UNIVERSITY OF HULL

Untangling molecular food webs of non-native invertebrates and their communities.

Marco Benucci

Department of Biological and Marine Sciences

University of Hull

A thesis submitted for the degree of

Doctor of Philosophy

January 2020

"*So it isn't the original building?*" I had asked my Japanese guide.
"*But yes, of course it is.*" he insisted, rather surprised at my question.
"*But it's burnt down?*"
"*Yes.*"
"*Twice.*"
"*Many times.*"
"*And rebuilt.*"
"*Of course. It is an important and historic building.*"
"*With completely new materials.*"
"*But of course. It was burnt down.*"
"*So how can it be the same building?*"
"*It is always the same building.*"
I had to admit to myself that this was in fact a perfectly rational point of view, it merely started from an unexpected premise.

Douglas Adams - "Last Chance to See" (1990)

Abstract

Invasive non-native species (INNS) are one of the main drivers of biodiversity loss globally, however to what extent INNS predators modify and utilise the invaded communities is still debated. This is particularly the case for arthropod INNS whose trophic interaction and predatory impact can be challenging to detect and describe. The application of DNA-based analysis, such as DNA metabarcoding, to the study of trophic interactions is often referred to as "MATI" (Molecular Analysis of Trophic Interactions). This approach has great potential in terms of increased sensitivity, higher resolution of prey identification and application to large-scale field studies, compared to previously established methods. This thesis focuses on describing trophic interactions in three non-native arthropod predators using this approach, with a key focus on intraguild predation (IGP), which has been shown to speed up invasion and facilitate establishment of invaders. In the first data chapter I focussed on the invasive amphipod, *Dikerogammarus villosus*, commonly known as "killer shrimp" to prove the concept that detection of prey DNA is possible in a controlled feeding experiment, and at a small field scale. From this proof of concept, I increased the scale of the field study by separately investigating the trophic interactions of three INNS: 1) *D. villosus* (Amphipoda: Gammaridae), 2) *Harmonia axyridis* (Coleoptera: Coccinellidae), and 3) the newly detected non-native *Crangonyx floridanus* (Amphipoda: Crangonyctidae). Data was collected for all species from UK sites across two seasons. The overarching goals were to investigate the broad trophic interactions of the target INNS across space and time, with a focus on detecting and understanding the importance of IGP in each target INNS. I predicted that high levels of IGP could be detected in all three target INNS. Firstly, I demonstrated that the method could detect prey species in both controlled and in field conditions, but detection success varied between prey taxa. I detected only low levels of IGP in *D. villosus* compared to a native amphipod, *G. zaddachi,* and found no evidence of IGP in *H. axyridis,* despite these species' reputations as important IG predators. I relate this to the availability of IG prey in the wider community. By contrast I detected high levels of reciprocal IGP between the newly detected *C. floridanus* and the established, non-native *C. pseudogracilis*. Interestingly, IGP was asymmetric, in favour of the new invader, which could

facilitate its establishment by eliminating competition. Together, these results demonstrate the applicability and also the challenges of DNA metabarcoding to molecular trophic interactions of INNS to understand the extent of their interactions in the invaded communities. I provide novel insight into the predatory dynamics of the three target species and their impact on the invaded communities.

Candidate Declaration

All the work submitted in this thesis is my own, except when otherwise stated. Several people have collaborated during this thesis. Drew Constable (DC), Katie Lee (KL), Alex Austin (AA), Lynsey Harper (LH), Rosetta Blackman (RB), Graham Sellers (GS), Lori Lawson-Handley (LLH), and Bernd Haenfling (BH).

Lori Lawson-Handley (LLH) and Bernd Haenfling (BH) have been involved with all the work.

Chapter 2: RB helped in the designing of the feeding arenas, KL helped on the fieldwork, RB and LH helped in the creation of the curated databases.

Chapter 3: KL helped on fieldwork, morphological indentification of kick samples and on DNA amplification. RB and LH helped for the creation of databases

Chapter 4: GS and AA helped on fieldwork

Chapter 5: DC helped in providing extra samples, KL helped on fieldwork, GS helped in tissue extraction. RB and LH helped for the creation of databases.

Marco Benucci, January 2020

Acknowledgements

First of all I would like to thanks my suprevisors Dr. Lori Lawson-Handley and Dr. Bernd Haenfling for your support, contibrutions and suggestions during the whole project. A particular thank goes to Lori for your incredible help and support during these years, during both the positive and the negative times. Your enthusiasm, encouragements and open mind to networking have been a driving force throughout the whole PhD. A special thanks to Helen Roy, Peter Brown and Rachel Farrow for their help and suggestions on the *Harmonia axyridis* chapter. Thanks to Drew Constable for the help given in the identification of *Crangonyx floridanus*.

A massive thanks to my ever-supporting family. Thanks for all your constant encouragement and kind words that you managed to transmit all the way from Italy. Thank you, I wouldn't have made it without you.

To my lab mates (past and present), thank you all. Rosetta Blackmann, Lynsey Harper, Graham Sellers, Alex Austin, Rob Donnelly, Jianlong Li, Cristina Di Muri, Helen Kimbell, Peter Shum, Nathan Griffith, and Rob Jaques; thank you all for sharing this time with me. Thanks for your help on fieldwork, for your recommendations in the lab and also for the unforgettable laughs we had. I found a group of friends inside this group of colleagues that made the time fly by and the heavier moments to feel lighter. Thanks to the whole EvoHull group, a place of discussion and improvement. The diverse knowledge contained in the group, and the routine sharing of those, made every lab chat a time of open discussion and debate.

Thanks to my partner Barbara, your constant help during this last year of writing this thesis has been enormous. You allowed me to achieve this submission with your encouragements and with the Sundays spent hiking. To all my D&D friends I found outside of the university, thank you for all the laughs and all the memories created.

Thank you all.

Table of Contents

Chapter 1 - Introduction

1.1 Ecological Communities.

Ecological communities are defined as “the organisms present within a space-time unit of any magnitude” (Palmer and White, 1994). Describing ecological communities, however, has also the additional complexity from the network of interactions among organisms and with the surrounding abiotic environment (Pilosof *et al.*, 2017). Thébault and Fontaine (2010) highlight how ecological communities and their corresponding networks are in a continuous dynamic equilibrium, whose stability is not only influenced by the species composition, but also by the types of interactions existing within the community (Thébault and Fontaine, 2010).

Although different indices can be used to describe and measure the stability of a network, greater connectance (i.e. the proportion of realized interactions among all possible ones (May, 1972)) is positively linked with robustness, resilience and resistance of a network (Dunne, Williams and Martinez, 2002; Allison, 2004). Dunne *et al.* (2002) highlight the importance of greater connectance by defining “power law” networks, which are networks more susceptible to lose key taxa and prone to drastic changes because they present very few taxa at the centre of a great number of links (Dunne, Williams and Martinez, 2002). In many cases the introduction of invasive non-native species (INNS) can lead to a similar situation with a reduction in biodiversity, simplification of the networks and an increase in the number of trophic links centred on the INNS (Galiana *et al.*, 2014).

1.2 INNS within communities.

Invasive non-native species (INNS) are one of the main drivers of biodiversity loss globally (Hulme, 2009). Due to the current trends in globalisation, wildlife trade and the movement of goods and people, the rate at which INNS are overcoming their natural ecological barriers is increasing (Westphal *et al.*, 2007); and this allows potential INNS to easily disperse outside of the native range (Olson, 2006; Keller *et al.*, 2011). Although only a small portion of the species

that are transported, actually successfully establish in the novel locations, the impacts that they can cause can be dramatic and can affect the health and functioning of an ecosystem, the economy of a country (e.g. through eradication and control plans) (Pimentel, Zuniga and Morrison, 2005), and even human health (e.g. the dermatitis caused by exposure to Giant Hogweed sap) (Nielsen *et al.*, 2005). Detecting newly introduced INNS and monitoring their spread is therefore a priority, although also challenging and more sensitive methods are needed to detect rare, elusive, or cryptic species.

Detecting species during the transport stage, represents the first possible barrier to prevent, or at least reduce, the chances for new species to accidentally be introduced. Conventional prevention methods relies on the current knowledge of distribution, of ecology and physiology of potential INNS in order to create “horizon scanning” models inclusive of habitat suitability, invasiveness and risk of invasion (Gallardo and Aldridge, 2013; Roy *et al.*, 2014). Although this approach is successful in prioritising and creating a list of high risk species, it can suffer from the disadvantage of being focused mostly on a restricted list of target species that needs expanding regularly in order to include changes in risk (Leung *et al.*, 2002), and on finding the right proxy for predicting impacts (Ricciardi and Cohen, 2006). In a similar way, detecting INNS already present in a territory with the aim of monitoring the distribution and rate of expansion also poses some challenges, because at the edge of the invasion range the density of specimens might be smaller than in the core of invasion (Jackson, Ruiz-Navarro and Britton, 2015). Successful examples of species monitoring uses wide networks relying on the engagement of trained volunteers and citizen scientists to collect information regarding expansion of INNS range (Hester and Cacho, 2017; Brown and Roy, 2018). Although with a higher land coverage and cost-effective monitoring than conventional methods, the citizen science approach can require some data to be validated by experts (Pocock *et al.*, 2014). Another important element, among others, in the study of INNS in a novel ecosystem is the study and understanding of the impacts that newly introduced species can have. Together with all the work done towards understanding invasion, invasive traits and predicting INNS dispersal (Roy *et al.*, 2014), the description and quantification of the impact of INNS in the invaded range has interested ecologists, conservationists and policy

makers to better understand the potential long-term consequences of INNS (Meyerson and Mooney, 2007; McGeoch *et al.*, 2010).

Assessing and quantifying the impacts of INNS has received a lot of attention in ecology, and there is evidence of widespread impact from the introduction of single INNS into a site (Snow and Witmer, 2010; Walsh, Carpenter and Vander Zanden, 2016). Although ecologists are able to detect impact and the changes, the challenge is to fully separate those changes caused by INNS from those changes caused by other global drivers of environmental change (Charles and Dukes, 2007). In addition to human induced pressures, the complexity and variability of ecosystems and their functioning also add another layer that needs to be taken into consideration when assessing INNS impacts (David *et al.*, 2017).

1.3 INNS within networks

Ecology is experiencing a radical change in the approach towards the study of INNS with, among others, a focus on understanding the mechanisms behind the success or failure of INNS invasion with a community and network perspective (Blüthgen, 2010; Roy and Lawson Handley, 2012). The inclusion of networks when investigating INNS in the invaded communities provides an important added information to the community; because rather than focusing only on the single components, the focus is expanded to include the linkages existing between each component (Thébault and Fontaine, 2010). As mentioned above, one of the main consequences attributed to INNS is the simplification of communities and the loss of biodiversity (Galiana *et al.*, 2014) and how INNS cause this biodiversity loss, still need to be further explored because different processes seem to be involved (Jeschke, 2014).

1.3.1 Niche availability and closely related species

The availability of niches in the receiving ecosystem is one of the different elements that seems to have an influence on the potential success of INNS, since it can provide them with access to resources (Sheppard *et al.*, 2018). The presence of closely related species seems to indicate that potential suitable niches for the INNS are available, and that there is potential for them to establish because of the similar niches that closely related species can occupy (Burns and Strauss, 2011; Violle *et al.*, 2011). However, because of this potential niche

similarity between a local and an invasive species, there is also an opportunity for the local closely related species to compete against the INNS for the shared niche and resources, thus limiting the INNS success (Violle *et al.*, 2011). Thuiller *et al*. (2010) expressed this concept under the Darwin's Naturalisation Conundrum, which describes how closely related species in the invaded site could indicate availability of resources suitable for INNS to establish but at the same time could also indicate a form of resistance to invasion due to the competition local species can have against the INNS (Thuiller *et al.*, 2010). Therefore to evaluate the potential invasion success and the potential niche INNS can occupy, it is needed to take into account the community and the networks that could facilitate or compete the INNS (David *et al.*, 2017).

1.3.2 Enemy release hypothesis (ERH)

The study of community networks and the interactions led to the development of the Enemy Release Hypothesis (ERH) (Torchin *et al.*, 2003; Liu and Stiling, 2006). The ERH tries to explain the success of INNS based on the lack of natural enemies (e.g. predators and/or parasites) that could hinder and limit the INNS population growth into the novel ecosystem (Torchin *et al.*, 2003; Colautti *et al.*, 2004). Examples such as the invasive grass *Brachypodium sylvaticum,* which appeared to thrive in the absence of two fungi that limit its population in the native range (Halbritter *et al.*, 2012), and the case of the invasive coccinellid *Harmonia axyridis* in the UK, which showed being parasitised to less extent by parasitic wasps and flies (Comont *et al.*, 2014); seem to provide support for the ERH explaining the success of INNS populations. Overall Prior *et al.* (2015) highlights that while ERH can explain some cases of invasion success, it does not apply to all species and systems (Prior *et al.*, 2015).

1.3.3 Intraguild predation (IGP)

Another process that can influence invasion success and of key importance in the current thesis, is intraguild predation. Intraguild predation (IGP) is defined as predation of an organism from the same guild that shares resources and is therefore also a competitor (Polis, Myers and Holt, 1989). Recent studies seems to highlight that IGP in natural conditions appear to be more important than initially expected; for example Wang *et al.* (2019) shows that even in non-invaded conditions the competition of species for shared resources can lead to an

increase in biodiversity and an improvement in ecosystem functions (Wang, Brose and Gravel, 2019). However, in invaded communities the IGP from INNS is often recorded to be stronger than the native species, and thus providing a benefit to the INNS during its settlement and range expansion (MacNeil, Platvoet and Dick, 2008; Katsanis *et al.*, 2013). As aforementioned, niche similarity and potential close relatedness between local species and INNS can also hinder invasion success through competition from the native species (Violle *et al.*, 2011); and there are cases, demonstrating this dichotomy, in which the result from IGP involving INNS led to a condition of cohabitation between the invader and the invaded organisms (Henkanaththegedara and Stockwell, 2014), or that led to a reduction in the INNS species (MacNeil, Elwood and Dick, 1999). Therefore in order to investigate the trophic interactions and better understand the impacts of INNS in the invaded system, it is necessary to include IGP for the potential key role this process can play.

1.3.4 Parasite/Parasitoid mediated invasion

In contrast to the Enemy Release Hypothesis, there are situations when parasites in the invaded range, either carried over with the INNS or naturally present, switch host towards the INNS or the organisms in the invaded range (Strauss, White and Boots, 2012). These processes have been described as Spillover and Spillback (Roy and Lawson Handley, 2012; Britton, 2013). Spillover happens when the INNS carries over a parasite that, in the invaded range, switches host and starts parasitising local organisms that are not adapted to it, as in the case of Crayfish Plague, carried over by the Signal Crayfish, and that has contributed to the reduction in the UK native White-Clawed Crayfish populations (Filipova *et al.*, 2013); or the Burmese Python that introduced parasitic pentastomes (Crustaceans) in the native US herpetofauna (Miller *et al.*, 2018).

Spillback on the other hand happens when a parasite in the invaded range switches host from native species to the INNS (Kelly *et al.*, 2009). There have been few cases reported of this, for example *Echinoparyphium recurvatum*, freshwater trematode, that infected the invasive *Dreissena polymorpha*; and because of the high abundance of this intermediate host, caused an increase in parasitism in the local wildfowl (the trematode final host) (Mastitsky and Veres, 2010). There is also the case in which a potential spillback is expected, as

Hartigan *et al.* (2011) who reported of two Myxosporea switching from endemic frogs to the invasive Cane Toad in Australia; process that could lead to a potential spillback into the endemic frogs and complete the spillback cycle (Hartigan *et al.*, 2011). Similar is the case described by Mlynarek (2015) in which the parasite preferred the invasive damselfly to the native damselfly (Mlynarek, 2015), potentially leading to the process of spillback into the native host.

1.4 DNA metabarcoding

High-Throughput Sequencing has enabled a significant technological advancement in eDNA research and application, moving from the single-species assays towards whole community, DNA metabarcoding (Taberlet *et al.*, 2012). The assays used in DNA metabarcoding are developed to target multiple species at the same time using conserved regions of the target gene that still allow for species identification. Depending on the target group the marker region selected can vary. For example, mitochondrial COI is mostly used arthropods (Zeale *et al.*, 2011; Leray *et al.*, 2013; Elbrecht and Leese, 2017); and the 12S region is being commonly used to describe Vertebrate and Fish communities (Evans *et al.*, 2015; Hänfling *et al.*, 2016; Li *et al.*, 2019). The development of DNA metabarcoding led to a complete rethinking of how ecologists can survey communities (Creer *et al.*, 2016), with a different exploration of the biological diversity and community composition (Deiner *et al.*, 2017).

Thanks to the advances in technology and instruments the number of samples that can be processed has also greatly increased, with a reduction in the unit costs and an increase in the sensitivity of detection of the whole process (Lawson Handley, 2015). In parallel to community-wide DNA monitoring (Olds *et al.*, 2016), DNA metabarcoding started to be applied in answering questions more related to community-network, such as investigation of direct predation (Krüger *et al.*, 2014), omnivory (De Barba *et al.*, 2014), herbivory (Kartzinel *et al.*, 2015), and parasitism (Kitson *et al.*, 2018).

1.4.1 environmental DNA

The application of molecular techniques to describe biological diversity was first applied to bacteria communities during the 1980s (Torsvik, 1980). From the early

2000s DNA barcoding started to be used as a method to identify individual species, including cryptic species, from their genetic material (Hebert *et al.*, 2003, 2004). These initial approaches paved the way to the development of methods that allowed detecting species by capturing the DNA they released into the environment they were living into, called environmental DNA (eDNA) (Ficetola *et al.*, 2008; Taberlet *et al.*, 2012), and thus understanding their distribution.

The first studies focusing on eDNA were mainly carried out in aquatic ecosystems, and focused on using species-specific assays to understand presence or absence of species within a site (Ficetola *et al.*, 2008; Dejean *et al.*, 2012). The first example of this molecular approach was for the detection of *Rana catesbeiana* in France (Ficetola *et al.*, 2008). eDNA detection is increasingly used for detecting rare and protected species, such as the Great Crested Newt monitoring programme in the UK (Biggs *et al.*, 2015); but also in detecting invasive species, such as Asian Carp (Jerde *et al.*, 2011) and the Burmese Python (Hunter *et al.*, 2015) in the USA.

1.5 Molecular Analysis of Trophic Interactions (MATI)

Understanding trophic interactions has interested ecology for long time (Layman *et al.*, 2015); however detecting species interactions has often been challenging, and several methods have been developed in an attempt to describe interactions in the wild (Sheppard and Harwood, 2005). The recent development and application of molecular approaches to study diet and host parasite interactions, known as “MATI”, through the use of DNA barcoding and DNA metabarcoding allowed for a reduction in the costs per unit of the analysis compared to previous methods (e.g. monoclonal antibodies, stable isotopes) and most importantly provided a resolution to species level (Sheppard and Harwood, 2005; Clare, 2014). Currently DNA barcoding/metabarcoding and stable isotopes analysis represent the main methods used to analyse trophic interactions (Traugott *et al.*, 2013). These two methods are currently integrated, as demonstrated in the study of rodents diet (Soininen *et al.*, 2014), because they can provide important and complementary information on species diet. The main one being that stable isotopes for example presents a longer resolution with the indication of the diet up to two months prior to the sampling (Sheppard and Harwood, 2005), while

DNA metabarcoding has the ability to capture the diet of an organisms within few hours to few days following predation (Kamenova *et al.*, 2018). However, one main element affecting stable isotopes is the potentially large effect that other environmental source of Nitrogen and Carbon (e.g. eutrophication in freshwater) can have on the Stable Isotopes signal (Traugott *et al.*, 2013). DNA metabarcoding also presents both methodological and analytical challenges (Deagle *et al.*, 2018); in particular in the case of predators and preys being closely related, the universal primers can lead to an overrepresentation of the predator DNA in the final results (Piñol *et al.*, 2014). Two major advantages in DNA metabarcoding however, is that it increases the identity resolution of the prey to species level and because of the universal assays does not require knowing the list of potential prey species *a priori*. For these reasons DNA metabarcoding is currently playing a key role in the study trophic interactions providing novel insight in the description of species niche breadth, diet and trophic networks even at large-scale (Clare *et al.*, 2019).

1.6 Aims and objectives

The aims of this thesis are to explore the trophic interactions of three generalist invasive arthropods present in the UK in order to understand: a) what are the trophic interactions of the target INNS in time and space; b) what is the rate of intraguild predation (IGP) of each species; c) what are the impacts the target INNS are causing to their respective communities. Each of the three species is introduced below, before describing the general aims of each chapter in more details.

1.7 Target species

Initially two target INNS had been selected in this project *Dikerogammarus villosus* (Sowinsky, 1984) commonly known as killer shrimp, and *Harmonia axyridis* (Pallas, 1773) commonly known as harlequin ladybird. The third non-native species involved in this project *Crangonyx floridanus* (Bousfield, 1963) was added at a later stage following its detection in one of the study sites selected during the sampling campaign.

Dikerogammarus villosus (Sowinsky, 1894)

Dikerogammarus villosus (Sowinsky, 1894), is an invasive freshwater amphipod (Crustacea) originally from the Ponto-Caspian region. Since the first half of the 1990s, *D. villosus* started to be recorded out of its native range and into European waterways (Rewicz *et al.*, 2015). Facilitated by the new connections in the eastern and central Europe canal systems (the Dnieper-Vistula basins, Danube-Rhein system and the Elbe-Oder basins) (Casellato, Visentin and La Piana, 2007; Bącela, Grabowski and Konopacka, 2008; Rewicz *et al.*, 2015), this invader has made its way across all European countries at a surprisingly fast rate reaching as far north as the Baltic countries (Šidagytė *et al.*, 2016), south in Italy (Casellato *et al.*, 2006), west in France (Bollache *et al.*, 2004), and since 2010 also in the UK (MacNeil *et al.*, 2010) (Fig. 1).

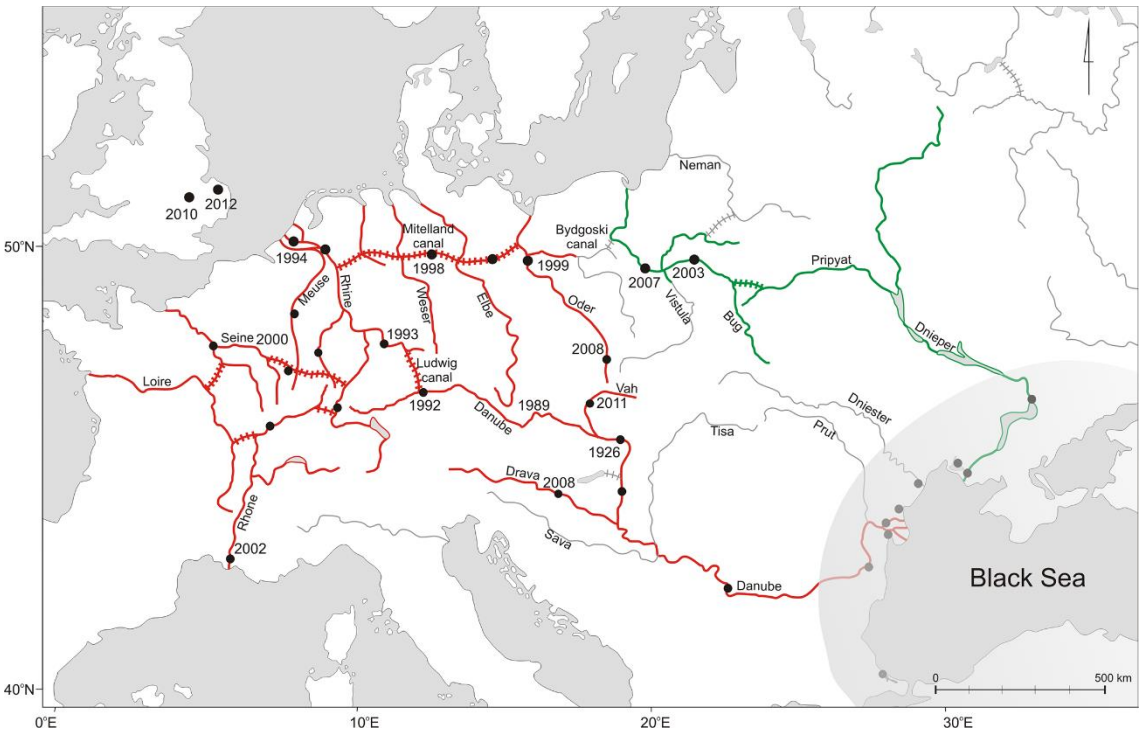


Figure 1. Map of *D. villosus* invasion routes across European river systems, modified from (Rewicz *et al.*, 2015). Reported are the dates of first records in each location. Green lines are the presumed Easter route, Red lines the presumed Western route.

Since its early records into the Oder River, *D. villosus* appeared to be causing extensive impacts on the freshwater ecosystems with reports highlighting

complete changes across all trophic levels of the communities after its arrival (Piscart *et al.*, 2009). The invasion of *D. villosus* has been strongly associated with a rapid exclusion or disappearance of other freshwater taxa (Dick and Platvoet, 2000; Lods-Crozet and Reymond, 2006; MacNeil and Briffa, 2019), and also with potential modifications on the movement of energy and nutrients cycling through the ecosystems (Dodd *et al.*, 2014). The impact from *D. villosus* has been linked to several biological traits (Bacela-Spychalska and Van Der Velde, 2013), in particular multivoltinism and the high number of eggs this species can produce (up to 200 for the larger individuals) (Pöckl, 2007; Dick *et al.*, 2013); by its larger body size than the native Gammarids (MacNeil, Platvoet and Dick, 2008), its strong environmental adaptability including heavy metals (Sebesvari, Ettwig and Emons, 2005), and ultimately a broad foraging behaviour including on other guild members (Dick, Alexander and MacNeil, 2012).

Similar to other invasive amphipods (Hänfling, Edwards and Gherardi, 2011), *D. villosus* shows traits associated with a strong predatory behaviour including stronger mouth parts morphology (Mayer *et al.*, 2008) and a higher Type II functional response in comparison to native amphipods (Dodd *et al.*, 2014) (Fig. 2).

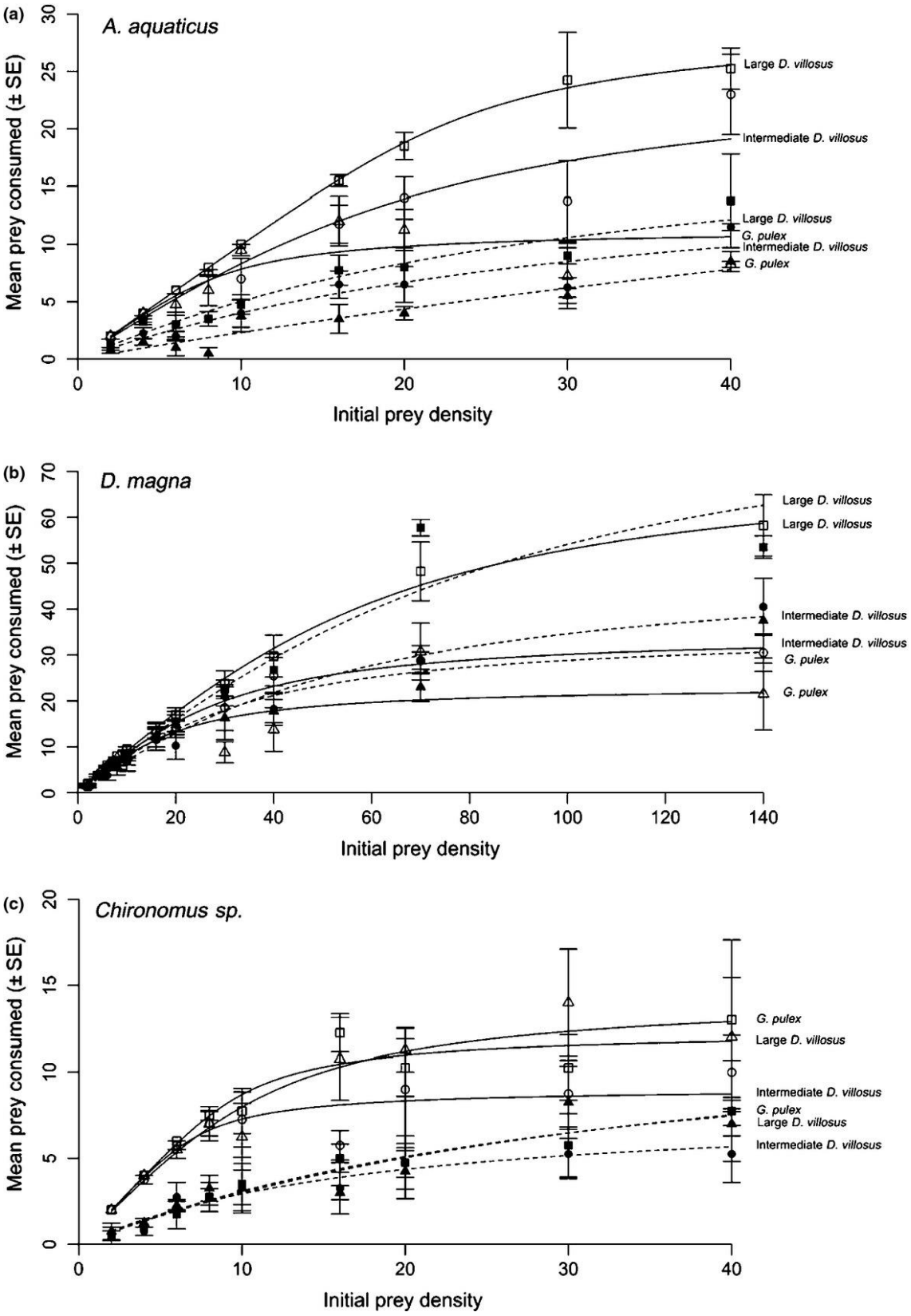


Figure 2. Modified from (Dodd *et al.*, 2014). Functional reposonses of non-native *D. villosus* in comparison with native *G. pulex* based on size ranges (large, intermediate) and on 3 different prey species: a) *A. aquaticus*, b) *D. magna*, c) *Chironomus sp.*

Mesocosm experiments have also demonstrated *D. villosus'* broad diet preferences and its ability to predate a wide variety of benthic invertebrates (Dick and Platvoet, 2000; Dick, Platvoet and Kelly, 2002; MacNeil and Platvoet, 2005), including competing amphipods (Dick and Platvoet, 2000; Kinzler and Maier, 2003; Kinzler *et al.*, 2008), and fish eggs (Taylor and Dunn, 2016). IGP is often assumed to be a key factor in in *D. villosus* invasion, however other non-predatory factors seem to be driving the displacement of native amphipods (Koester and Gergs, 2014; Koester, Bayer and Gergs, 2016; MacNeil and Briffa, 2019). Therefore, further describing the role of *D. villosus* as a predator of the wider invertebrate community can help in better understand the process of its invasion and its success.

Although not a focus of this thesis, *D. villosus* has recently been recorded outside of its native range to be associated with the infection from *Cucumispora dikerogammari*, a microsporidian which naturally occurs in *D. villosus* native range (Ovcharenko *et al.*, 2010). Although there are no records of *C. dikerogammari* infection in the UK yet (Bojko *et al.*, 2013), Bojko *et al.* (2015) reported the infection of *Dikerogammarus haemobaphes*, another Ponto-Caspian invader widely distributed across England, by *Cucumispora ornata* (Bojko *et al.*, 2015). This report increases the risk of potential threat from parasitic spillover into the freshwater Amphipod communities and a potential increase in the complexity of the interactions during its invasion.

Harmonia axyridis (Pallas, 1773),

Harmonia axyridis (Pallas, 1773), is an aphidophagous Coccinellid originally from Asia (Brown and Roy, 2018). *H. axyridis* is a natural predator of aphids and scale insects, which are known agricultural pests (Morales-Hojas, 2017), although it can feed also on honeydew, adelgids and larvae of other insects including other guild member species such as other coccinellids (Koch *et al.*, 2003; Pell *et al.*, 2008; Brown, Frost, *et al.*, 2011; Brown, Thomas, *et al.*, 2011; Roy *et al.*, 2016). Because its diet is largely composed of agricultural pests, *H. axyridis* has been widely transported as biological control agent in agricultural landscapes globally (Koch, 2003; Brown *et al.*, 2007). *H. axyridis* was first introduced for biocontrol in North America in 1916, but viable wild populations were only recorded in the USA following multiple releases during the 1980s (Koch, 2003; Brown, Thomas, *et al.*,

2011; Roy *et al.*, 2016). From the late 1960s introductions also took place in Eastern Europe, while starting from the 1980s and throughout all of the 1990s the same took place in Western Europe, North and South Africa (Brown *et al.*, 2007); and from the 2000s *H. axyridis* has been detected in South America (Grez *et al.*, 2016, 2017). The first detection of *H. axyridis* in the UK is dated back to 2004, and since then this specie has been the subject of a monitoring campaign showing its steady expansion over the years (Roy and Brown, 2015) (Fig. 2).

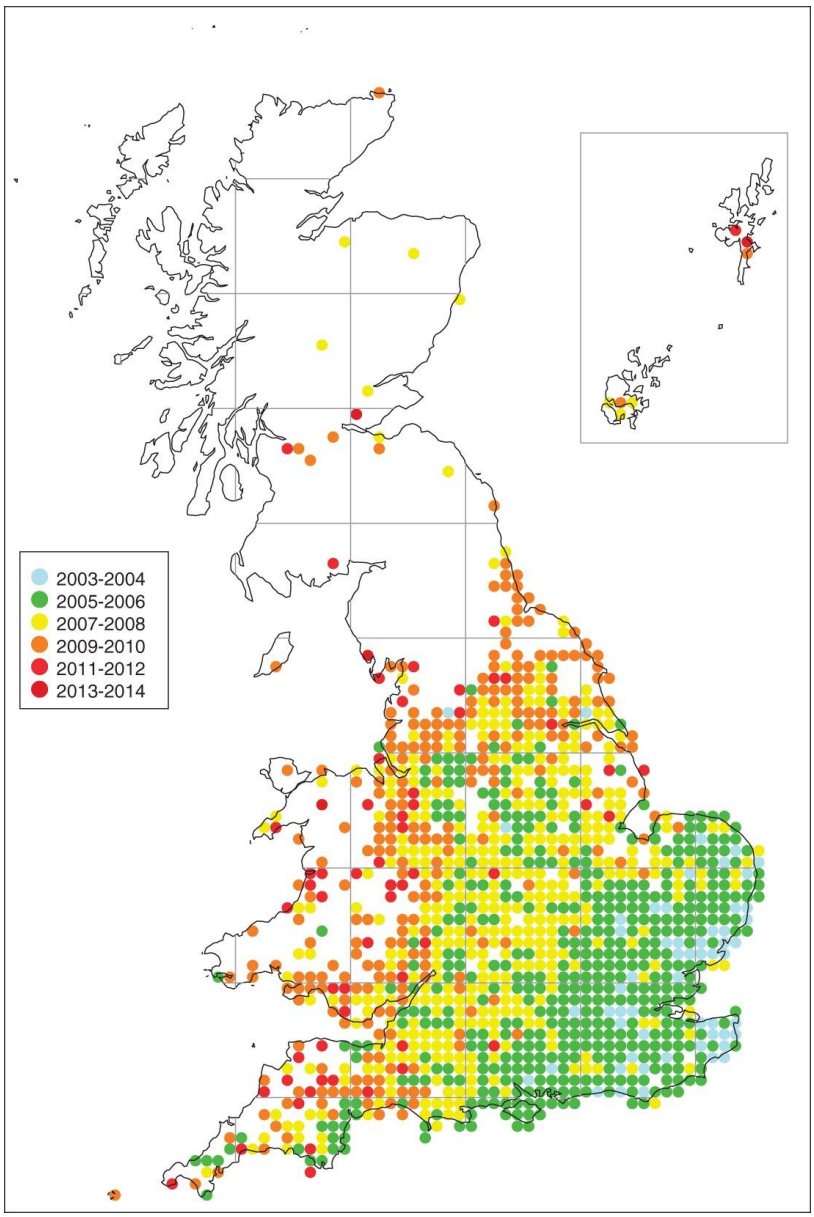


Figure 2. *Harmonia axyridis* occurrence in 10-km squares in Britain from 2004 to 2014 (Modified from (Roy and Brown, 2015). Where a square has been recorded in more than 1 year, occurrence in the earliest year is shown (blue, 2003–2004; green, 2005–2006; yellow, 2007–2008; orange, 2009–2010; red, 2011–2012; burgundy, 2013–2014).

The biology and life history traits of *H. axyridis* are key to its invasion success. Generally bivoltine in its native range, *H. axyridis* showed a high plasticity with up to three or four generations a year observed in invasive ranges, compared to many other UK native coccinellids, which are univoltine (Koch, 2003; Honek *et al.*, 2017). Plasticity and adaptability seem to be common traits characterising *H. axyridis*. The species is phenotypically highly variable, with more than 200 colour forms divided into four basic types that differ within and between form in the degree of melanisation, and exhibit plasticity linked to thermal tolerance (Michie *et al.*, 2010, 2011; Purse *et al.*, 2015). Although its diet is mainly composed by aphids and scale insects, *H. axyridis* can include in its diet a wider range of prey including mites, adelgids, barkflies and other coccinellids (Sloggett and Majerus, 2000; Sloggett, 2008; Brown and Roy, 2018). The diet is considered one of the main elements underlying the rapid success of *H. axyridis* invasions because it allowed the harlequin ladybird to easily exploit and adapt to the resources available in the new invaded range (Soares, Coderre and Schanderl, 2004). The strong predation of *H. axyridis* on aphids could have a massive impact on aphidophagous community in the invaded ecosystems (Majerus, Strawson and Roy, 2006); and since *H. axyridis* is now globally dispersed, there are several studies reporting this invasive ladybird switching diet to predate on other ladybirds (Michaud, 2002; Ware, Yguel and Majerus, 2009), on Coccidae, Lepidoptera (Koch, 2003), Lacewings, Psillidae and Psocidae among other taxa (Majerus, Strawson and Roy, 2006).

The IG interactions of *H. axyridis* against other UK native ladybirds is also considered a key driver of its invasion success and the following decline in native ladybirds populations (Roy and Brown, 2015). A number of recent studies have investigated the diet of *H. axyridis* using a targeted molecular approach, with an emphasis on detection of IGP, which Katsanis *et al.* (2015) reported to be more frequent at the fourth instar larval stage (Katsanis *et al.*, 2013). For example, using prey-specific PCR assays, evidence of IGP towards *Adalia decempunctata, A. bipunctata,* and *Episyrphus balteatus* has been detected in *H. axyridis* collected from several European countries at rates of 2.8-9.6% (Thomas *et al.*, 2013; Brown *et al.*, 2015; Rondoni *et al.*, 2015). Little is currently known about the more general diet of *H. axyridis*, specifically in field conditions and the relative

amount of IGP versus non-IG diet over seasons and across the invasive range. Overall, different processes seem to be involved in the success of *H. axyridis* invasion, including the lack of most of its natural enemies (Ceryngier *et al.*, 2018); although how much this is contributing to the spread of *H. axyridis* populations is still not fully explored.

Crangonyx floridanus (Bousfield, 1963)

Crangonyx floridanus (Bousfield, 1963) belongs to the Amphipod family Crangonyctidae, an exclusively freshwater family of which 80% of species are hypogean (Väinölä *et al.*, 2008). There are 47 known species of *Crangonyx*, of which 42 are native to North America (Zhang and Holsinger, 2003; Svavarsson and Kristjansson, 2006). In comparison, Eurasia only has five known native species, four of which occur in subterranean waters of Europe (Zhang and Holsinger, 2003; Svavarsson and Kristjansson, 2006).

C. floridanus is indigenous to eastern and east-central United States, including Florida and Louisiana, with non-native populations being found in western USA (Colorado, Oregon and California) (Toft, Cordell and Fields, 2002; Zhang and Holsinger, 2003), as well as Japan (Kanada *et al.*, 2007). Little is known about the population in the native range, and most information on *C. floridanus* comes from Japan, where it was first recorded in 1989 (Nagakubo *et al.*, 2011). *C. floridanus* was recently discovered in the UK (Mauvisseau *et al.*, 2019), and also during the course of the present study using molecular and morphological identification techniques. The timing of introduction into the UK, its colonisation pathway and current distribution are currently unclear, but it was identified in two locations (Windermere in Cumbria and Smestow Brook in the West Midlands) separated by 200 km; suggesting that it is a widespread, and established population (Mauvisseau *et al.*, 2019). Current knowledge regarding the impacts of *C. floridanus* are currently restricted to Japan, where this species has rapidly dispersed across the river network in the past decades, facilitated by its ability to utilise a wide variety of microhabitats, its high fertility rates and ability to withstand conditions of low oxygen (Nagakubo *et al.*, 2011). However, direct evidence of the impacts caused by the invasion by *Crangonyx floridanus* are still sparse (Tojo *et al.*, 2010).

The congeneric *Crangonyx pseudogracilis* (Bousfield, 1958), is also native to North American but has a wide non-native distribution across Europe. It was first

recorded in Europe, in England in 1936 (Crawford, 1937), then later in Ireland in 1975 (Holmes, 1975), and subsequently across Western Europe. Early reports pointed towards a low survival of *C. pseudogracilis* following introduction, mostly because of being predated by other, bigger, amphipod species, like *G. pulex* (MacNeil, Elwood and Dick, 1999; Van der Velde *et al.*, 2000). However, *C. pseudogracilis* can now be widely found throughout the UK and European freshwater systems. *C. pseudogracilis* has no known detrimental effects on native species, and some authors have suggested it may have positive ecological impacts including fulfilling the important trophic role of detritivore (MacNeil and Dick, 2014).

C. floridanus and *C. pseudogracilis* are morphologically highly similar, and although recent analysis have not fully resolved their phylogenetic relationship, they are still classified as separate species (Slothouber Galbreath *et al.*, 2010; Nagakubo *et al.*, 2011). During this thesis these two species were detected in cohabitation in a reservoir near Birmingham (Staffordshire, UK) and this opened the possibility to investigating also potential instances of IGP between *C. floridanus* and *C. pseudogracilis*.

1.8 Ethics

Dikerogammarus villosus specimens used in the feeding experiments presented in Chapter 2 were collected from Grafham Water (Cambridgeshire, UK TL 15333 67995). The prey species *Asellus aquaticus*, *Crangonyx pseudogracilis* were collected from Thwaite Gardens (Cottingham, UK TA 05510 32771), while *Daphnia magna* were collected from an ongoing culture already existing at the University of Hull. The feeding experiments were carried out in accordance with the University of Hull Ethics Committee approved protocol (ref: U108).

1.9 Rationale of the thesis

As mentioned above, understanding trophic interactions in INNS is a key element in understanding the impact these species can have on the invaded communities, however their study can be challenging especially in invertebrates communities. DNA metabarcoding in this regard is enabling an increase in our ability to monitor communities, species and their trophic interaction. The main goal of this thesis is to use DNA metabarcoding on gut contents and on communities samples to

detect and describe the trophic interactions of three INNS present in the UK, and to understand to what extent these species were using their respective communities.

Chapter 2: Detecting molecular trophic interactions of *Dikerogammarus villosus* in feeding experiments and field samples in the UK.

The aim of this chapter is to explore the use of DNA metabarcoding to detect prey DNA in predator gut contents. Initially the method is applied in controlled feeding experiments to verify for digestion rate in *D. villosus*. Following the feeding experiment, the same method was applied to a small-scale field study which included two sites in the UK invaded by the target species.

Chapter 3: DNA metabarcoding of gut contents of invasive *Dikerogammarus villosus* reveals low levels of intraguild predation compared to a native amphipod, *Gammarus zaddachi* in the UK.

This chapter is aimed at describing molecular trophic interactions of *D. villosus* using DNA metabarcoding on gut contents, to verify how much of the freshwater community this species is feeding on. In this study two invaded sites and a control non-invaded site were sampled across two seasons in 2017. The non invaded site was included as a control site, and for this reason the native amphipod *G. zaddachi* was sampled and its gut contents analysed. The communities associated with amphipod specimens were also collected and analysed.

Chapter 4: Seasonal and spatial patterns in molecular trophic interactions of *Harmonia axyridis* (Pallas, 1773), in the UK.

This chapter was aimed at detecting *H. axyridis* molecular trophic interactions in 12 sites split across two counties and across two seasons in the UK. In order to achieve this, I developed and tested the use of blocking primers in gut contents analysis. In this chapter I developed a set of blocking primers to reduce *H. axyridis* DNA during gut contents analysis. The blocking primers were tested both *in silico* and *in vitro*, before applying them to a large-scale field experiment. The field samples then were collected from six sites in Oxfordshire and six sites in

Yorkshire across two seasons. Similarly to Chapter 3, the arboreal communities were also collected and the bulk DNA from these samples were sequenced with the *H. axyridis* gut contents.

Chapter 5: Reciprocal intra-guild predation between newly detected *Crangonyx floridanus* and established *Crangonyx pseudogracilis* in a UK lake.

The final data chapter was aimed at detecting molecular trophic interactions and IGP in two cohabiting species of *Crangonyx* in one site. This study was developed over two seasons and involved first confirming the identification of the two *Crangonyx* species due to their crypticism using morphology, DNA barcoding and haplotype analysis. From there the gut contents analysis and community analysis were carried out to describe molecular trophic interactions and IGP in both species over both seasons.

Chapter 6: General Discussion

In this chapter I summarise the main results obtained in this thesis, and I frame the thesis into the wider research field of molecular trophic interactions and INNS. I delineate the main challenges in the methods that I have encountered, the main opportunities and ultimately, I highlight potential future research paths.

Chapter 2 - Detecting molecular trophic interactions of *Dikerogammarus villosus* in feeding experiments and field samples in the UK.

Abstract

Recent decades have witnessed a major shift in the way ecological communities are described and monitored using molecular methods, such as high-throughput sequencing (HTS). This technology is enabling ecologists to survey and monitor species at a larger scale and faster than before, and is allowing ecologists to investigate species diet and to detect direct interactions between species without eye-witnessing the events, field often referred to as molecular analysis of trophic interactions (MATI). However, inferring trophic interactions from DNA metabarcoding is complex because prey DNA detection can be influenced by the time from predation, the size of the prey, and amount of prey ingested; which are generally unknown in field conditions. Controlled laboratory feeding trials can thus facilitate the interpretation of MATI results, for example in comparing the detection rates of prey DNA from different sources (e.g. regurgitates, whole body or faeces), by testing whether longer or shorter DNA fragments can be better recovered, and by detecting the half-life of prey DNA at different digestion time from known predation events. In this study we performed a feeding experiment in controlled conditions as a proof of concept that we could detect prey DNA in *D. villosus* gut contents, followed by a small scale field trial with the same molecular method. Overall the aims were 1) to determine whether prey DNA could be detected in gut contents and to what extent, 2) to relate the gut contents data to the field conditions, and 3) to relate the gut contents data to the wider field community. Here we demonstrated the use of DNA metabarcoding to detect DNA belonging to single specimens of known prey species in controlled mesocosms, from gut contents of the invasive amphipod *D. villosus*. We confirmed that the DNA of single specimens of *Asellus*, *Daphnia* and *Crangonyx*, can be detected in the gut contents of an invasive consumer. In the small-scale field trial, we successfully detected trophic interactions also with instances of IGP against two other Amphipoda species. The rate of trophic interactions was similar to previous studies, and the rate of IGP was slightly higher than previously recorded in wild *D. villosus* individuals.

2.1 Introduction

Molecular ecology and molecular analysis of trophic interactions.

Recent decades have witnessed a major shift in the way ecological communities are described and monitored using molecular methods, such as high-throughput sequencing (HTS) (Creer *et al.*, 2016; Deiner *et al.*, 2017). This technology is enabling ecologists to survey and monitor species at a larger scale and faster than before. Another shift that HTS is driving forward is the ability to describe trophic interactions between species, and better understand how community networks are structured (Juen and Traugott, 2007; Evans *et al.*, 2016; Bohan *et al.*, 2017; Clare *et al.*, 2019). This rapidly evolving field is referred to as "molecular analysis of trophic interactions", or "MATI" (Clare, 2014).

Being able to investigate diet and to detect multiple direct interactions between species without eye-witnessing the events is enabling important new insights in ecology. Examples of molecular trophic interactions using HTS, in particular DNA metabarcoding, can be found now involving a variety of trophic systems including carnivorous plants (Littlefair *et al.*, 2018), species with external digestion like spiders (Wirta *et al.*, 2015), generalist and scavenging species (Siegenthaler *et al.*, 2018), and species like cnidarians and sponges (Leal *et al.*, 2014; Mariani *et al.*, 2019). The ability to infer direct interactions with a high taxonomic resolution obtained with DNA metabarcoding is increasing the information we currently possess about community networks and trophic interactions (Pompanon *et al.*, 2012; Clare, 2014). A field in particular that can strongly benefit from MATI is the impact assessment of invasive non-native species (hereafter INNS), and their integration within invaded ecological networks (Galiana *et al.*, 2014). MATI can be used, for example, to describe the diet of invasive predators and for understanding changes in the ecological networks of invaded communities following invasion (Shiels *et al.*, 2013; Thomas *et al.*, 2013). This is particularly the case in freshwater habitats, where trophic interactions are difficult to observe.

Freshwater ecosystems are particularly vulnerable to changes in species community structure and trophic networks (Moorhouse and Macdonald, 2015). This is linked to multiple stressors such as water abstraction, pollution, land reclamation, habitat degradation and the introduction of INNS (Geist, 2011). INNS

in particular can impact freshwater communities across multiple trophic levels, with top-down or bottom-up effects, or laterally along the same trophic level (Carvalheiro, Buckley and Memmott, 2010); and the introduction of single species have been demonstrated that can lead to changes that can ripple through the whole network and consequently impact broader ecosystem functioning (David *et al.*, 2017). Examples of this can be found spanning across INNS belonging to different kingdoms, from invasive plants, which can influence the biogeochemical processes in a reservoir (Ribaudo *et al.*, 2018), invasive herbivores and filter feeders that can greatly reduce the algae available to zooplanktonic species (Higgins and Zanden, 2010; Whitney, 2016), to consumers and top predators which can dramatically reduce the available herbivore species and lead to algal blooms (Dodd *et al.*, 2014; Walsh, Carpenter and Vander Zanden, 2016; Walsh, Lathrop and Vander Zanden, 2017). Freshwater amphipods are an important functional group in aquatic environments, playing multiple ecological roles (e.g. detritivory, predation, scavenging), and therefore influencing ecosystems across multiple trophic levels (Piscart, Roussel, *et al.*, 2011). For these reasons there are a number of examples of high-impact invasive amphipods that have been shown to drastically change the ecology and community of the sites they invade (Hänfling, Edwards and Gherardi, 2011; Paterson *et al.*, 2015), with native amphipods and other guild members that appear particularly affected (Piscart, Roussel, *et al.*, 2011).

One example of a high impact invasive amphipod is *Dikerogammarus villosus* (Sowinsky, 1894). Originally from the Ponto-Caspian region, this amphipod is now widespread in Europe and listed among the 100 world's worst invasive species because of its rate of spread and negative impact (Hulme, 2009). Although *D. villosus* populations have been poorly studied in the native range (Rewicz *et al.*, 2014), its invasion history has captured the attention of ecologists across continental Europe and North America, where it is feared that *D. villosus* might follow the same route of other Ponto-Caspian species across the Atlantic Ocean (Rewicz *et al.*, 2015). The first historic records of *D. villosus* outside of its native range are traced back to 1926 in the Danube, but it is since the opening of the Rhine-Danube canal in 1992 that *D. villosus* seems to have massively expanded its range westward in France (Bollache *et al.*, 2004), southward in Italy (Casellato *et al.*, 2006), eastward and northward in the Baltic seas (Bącela,

Grabowski and Konopacka, 2008; Šidagytė *et al.*, 2016); and it was first recorded in the UK in 2010 (MacNeil *et al.*, 2010).

D. villosus represents an ideal species to investigate the depth of impacts from invasive amphipods. It is described as a flexible omnivore (Platvoet *et al.*, 2009), although it is its predatory behaviour that caught the attention of ecologists, with studies associating the *D. villosus* invasion to the strong reduction of benthic fauna in the River Rhine (van Riel *et al.*, 2006). *D. villosus* is widely recognised also for its role as an important intraguild predator. Intraguild predation (IGP) is defined as the predation among competitor species over a shared resource, and it is a process that seems to be naturally occurring in ecology (Polis, Myers and Holt, 1989; van Riel *et al.*, 2007). IGP in freshwater systems appears to have a strong positive influence in shaping native invertebrate communities and their network, promoting biodiversity across different trophic levels by, among other effects, releasing lower trophic layers from predation pressure and by increasing available niches (MacNeil and Dick, 2014; Wang, Brose and Gravel, 2019). However, when INNS are directly involved as IG predators, IGP appears to be facilitating the invasion process and establishment of INNS in the invaded ecosystem (Hall, 2011). Evidence of predatory and IGP impact by *D. villosus* comes mostly from laboratory experiments in which direct predation could be observed against a wide variety of benthic invertebrate species, including other Amphipod species (Dick, Platvoet and Kelly, 2002; Kinzler *et al.*, 2008; Dick, Alexander and MacNeil, 2012), and also on fish eggs (Taylor and Dunn, 2016). Direct evidence of this predatory impact of *D. villosus* in field conditions are however more contradictory, with minor evidence of predation of other invertebrates by *D. villosus* (Koester and Gergs, 2014; Koester, Bayer and Gergs, 2016), or with evidence that other environmental factors have a greater or equal influence in shaping communities, rather than *D. villosus* invasion (e.g. physico-chemical parameters) (Koester *et al.*, 2018).

Considering the multiple ecological roles that amphipod species and *D. villosus* play in freshwaters, also as a flexible omnivore (Platvoet *et al.*, 2009), it becomes evident how the impact of this invasive amphipod on the broader community and on native amphipods has the potential to be perceived throughout the entire ecosystem (MacNeil and Platvoet, 2005). For example *D. villosus* has been reported to have a lower rate of leaf matter degradation in comparison to native

gammarid species, and in conjunction with the recorded reduction in amphipod diversity in invaded sites, shows the knock on effect that this invasive amphipod can have (MacNeil *et al.*, 2011; Piscart, Mermillod-Blondin, *et al.*, 2011; Little, Fronhofer and Altermatt, 2019).

Across the wider community, the impact of *D. villosus* on other amphipods is of particular interest (Dick and Platvoet, 2000), especially concerning native *Gammarus* species. Teasing apart the complexity of *D. villosus* effects on freshwater communities poses some challenges. For example Rewicz *et al.* (2014) highlights how omnivory in *D. villosus,* in conjunction with its body size, allows this species to access small refugia, either actively predating other amphipod species, or pushing them out of refugia and making them more vulnerable to other predators (Rewicz *et al.*, 2014). Laboratory mesocosm experiments provide evidence in support of both: Gammarid species subject to fear-induced displacement from the presence of *D. villosus* (MacNeil and Briffa, 2019) and in support of direct competition and IGP (MacNeil and Platvoet, 2005; MacNeil *et al.*, 2011). Therefore while field experiment appears to point at IGP of *D. villosus* as expected to have a strong detrimental impact that can be perceived across the entire ecosystem and its functioning (MacNeil *et al.*, 2011); the field data seems to express more caution and the support of the importance of IGP in *D. villosus* invasions has been equivocal, with some studies finding no evidence of IGP in the wild (Koester and Gergs, 2014). The lack of consensus over *D. villosus* as driver of change in benthic communities following its arrival (van Riel *et al.*, 2006; Koester *et al.*, 2018), highlights the knowledge gap still present in understanding the mechanisms of impact of *D. villosus*, and how its trophic interactions influences the invaded community. Ultimately detecting and describing these interactions could improve the understanding of the impacts from this invasive amphipod along its invasion range (Rewicz *et al.*, 2014).

Our current understanding about the role of *D. villosus*, from field conditions, within the invasion range has been inferred from stable isotopes (Koester and Gergs, 2014), and targeted PCR assays for specific prey species (Koester, Bayer and Gergs, 2016). Specifically, Koester and Gergs (2014) showed that *D. villosus* at the invasion front in Switzerland is occupying a similar trophic level as the native amphipods *G. pulex* and *G. fossarum*, with no indication that IGP was

happening. By contrast, the targeted PCR assays detected 12 different taxa in *D. villosus* gut contents, including a low rate (2.6%) of IGP against other Gammarids (6 individuals out of 226). While these studies provided important insight into *D. villosus* ecology, they require prior knowledge of potential prey species in order to design prey-specific PCR assays (Carreon-Martinez and Heath, 2010). DNA metabarcoding has potential for describing the wider *D. villosus* prey community and ultimately to understand the species' wider impact. However interpreting MATI data from metabarcoding is complex because prey DNA detection is influenced by the time from predation, the size of the prey, and amount of prey ingested, which are generally unknown in field conditions (Greenstone *et al.*, 2014). Controlled laboratory feeding trials can facilitate the interpretation of MATI results, for example in comparing the detection rates of prey DNA from different sources (e.g. regurgitates, whole body or faeces) and by targeting longer or shorter DNA fragments (Kamenova *et al.*, 2018), by detecting the half-life of prey DNA at different digestion time from known predation events (Harper *et al.*, 2005; Juen and Traugott, 2007; Rondoni *et al.*, 2018).

Despite all the progress made in understanding the ecological impacts of *D. villosus*; evidence of direct predation on the community and the rate of IGP on other amphipods still needs to be fully investigated (MacNeil, Platvoet and Dick, 2008; Koester and Gergs, 2014). Therefore here we performed controlled feeding trial experiments with *D. villosus* and three different prey species (*Asellus aquaticus, Daphnia magna, Crangonyx pseudogracilis)* followed by metabarcoding of gut contents, 1) to determine whether prey DNA could be detected in the guts via metabarcoding, and 2) to infer the digestion rates of three different prey items. We hypothesized that larger sized prey species could be detected over a longer range of time in comparison to the smaller sized species. We then performed a small scale field trial with the same molecular method, together with a survey of the wider community obtained by kick sampling and environmental DNA (eDNA) metabarcoding, 1) to determine whether prey DNA could be detected in wild conditions, and 2) to relate the gut contents data to the wider community.

2.2 Methods

a. Feeding trials

The feeding trials were based on methods from previous studies by Ingels *et al.* (2013), and Dodd *et al.* (2014), with modifications (Fig. 1).

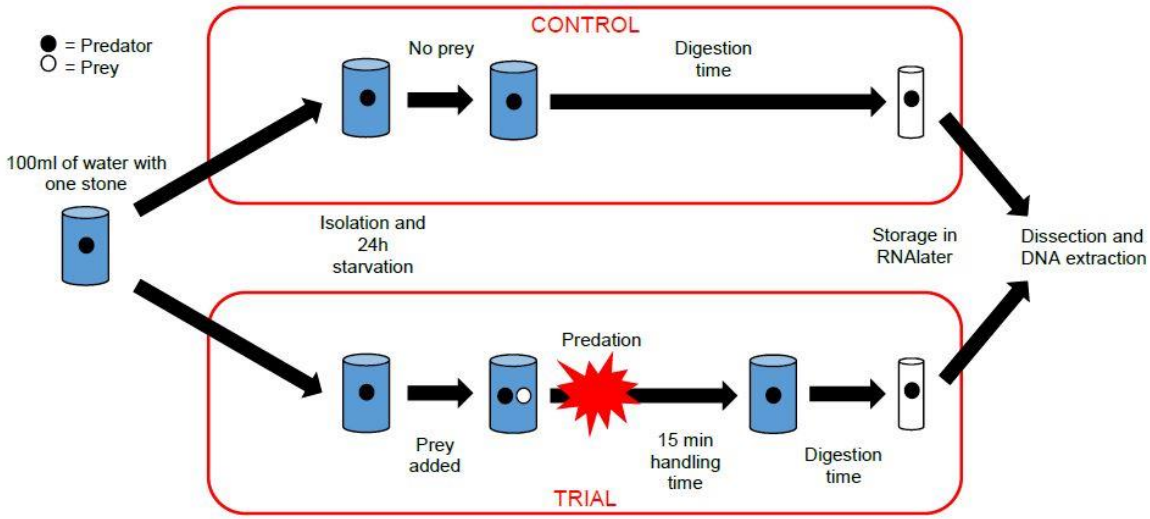


Figure 1. Workflow scheme of the experimental design for the feeding experiments involving *D. villosus*. Black circle=*D. villosus*, white circle=Prey taxa introduced.

The experiment consisted of 5 replicates for each of the three prey treatments (*Asellus aquaticus, Crangonyx pseudogracilis* and *Daphnia magna*) and six digestion time intervals (0h, 4h, 8h, 12h, 24h and 36h following (Juen and Traugott, 2005)), and 1 control for each digestion time per prey (N total = 108). All predators were isolated in the arenas and starved for 24h, before prey specimens were introduced, in order to standardise levels of hunger across the different arenas (Dodd *et al.*, 2014). The control trials were handled in the exact same way, including starvation period, with the only difference that no prey was added and their digestion times were counted starting directly after the 24h starvation period had passed (Fig. 1). The time 0h included 15 minutes of handling time for the predators to consume and start digesting the prey (Fig. 1). All taxa involved in the trials were collected in spring 2016 and maintained in separate oxygenated tanks for a few months to acclimatise before the trials.

Asellus aquaticus and *C. pseudogracilis* were collected in Thwaite Gardens, Cottingham, UK (lat/long: 53.780623 N, 0.40031433 W), while *D. villosus* were collected from Grafham Water (lat/long: 52.298037 N, 0.31814828 W). *D. magna* was taken from an existing culture in the University of Hull. During acclimatisation the taxa were fed with leaves of *Aesculus hippocastanum*, with the exception of *D. magna* which were fed with *Chlamydomonas spp.* All tanks and arenas for the trials were maintained at the same conditions of room temperature and photoperiod (Temp. = 18-20°C, and photoperiod of 14h Light:10h Dark). The arenas were set up to host one predator and one prey each. We used a 1:1 predator:prey to avoid multiple predation events on different prey individuals at different times (Ingels *et al.*, 2013; Dodd *et al.*, 2014). Arenas contained 100ml of water taken from the main predator tank, with a single pebble (See Appendix 2.1), both the arenas and the pebbles were sterilised with 10% v/v commercial bleach and dried for 3 days before being used. Once the prey individuals were introduced, all arenas were checked every 30 mins during the day. If predation did not occur, preys were removed overnight and re-introduced in the morning. All *D. villosus* specimens that successfully attacked the prey and that were alive at the end of the trials, were frozen for few minutes and then immersed in RNAlater for long term storage.

b. Field trials

For the field trials, *D. villosus* were collected from field sites in Grafham Water (Cambridgeshire, UK) (lat/long: 52.298037 N, 0.31814828 W), and Wroxham Broad (Norfolk, UK) (lat/long: 52.699442 N, 1.4200367 E) in May 2017 (Fig. 2).

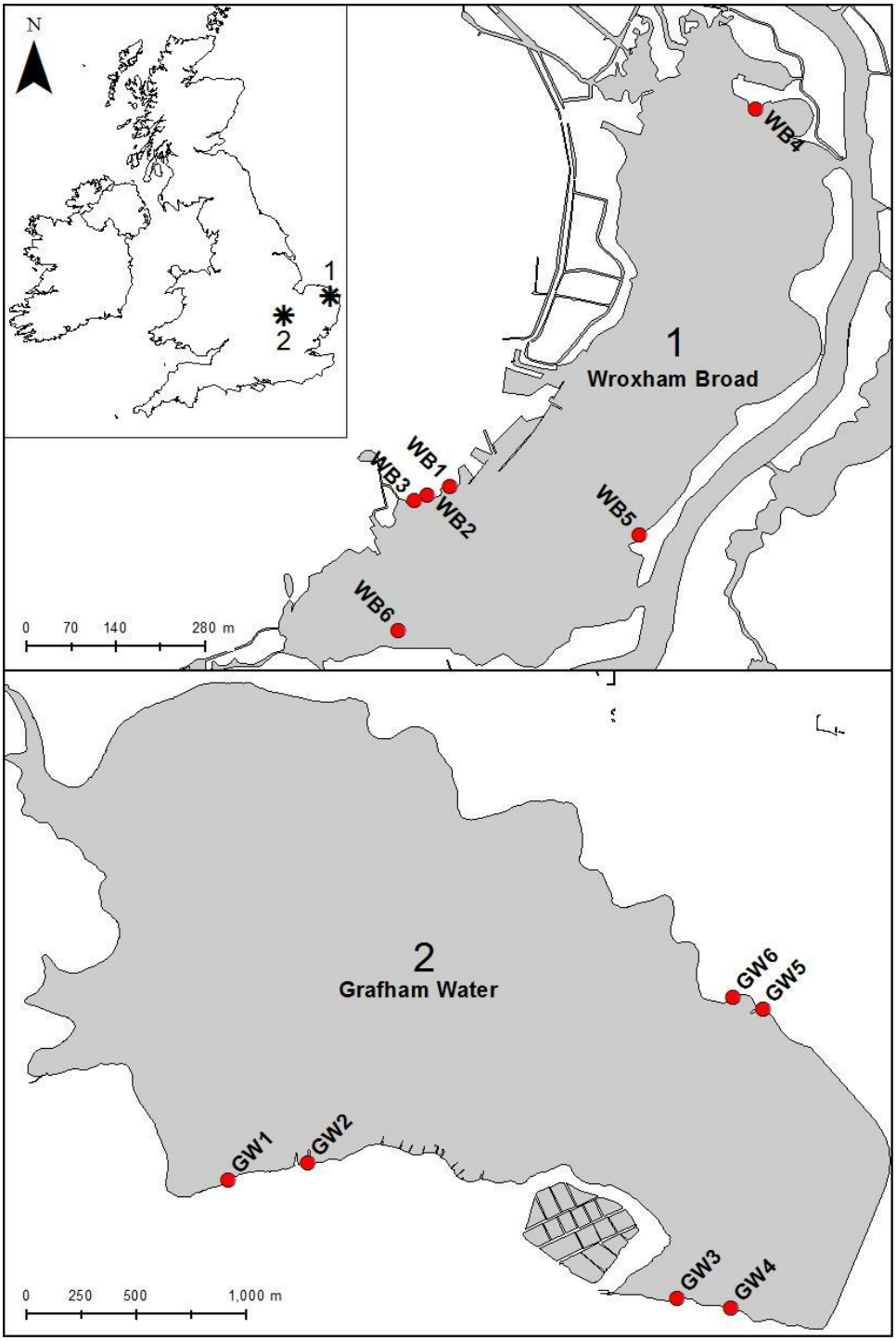


Figure 2. Sampling sites used for the small-scale field study. 1) Wroxham Broad (Norfolk, UK) and, 2) Grafham Water (Cambridgeshire, UK).

Six sampling locations were selected in each lake. Samples were collected in the order: i) water collection for eDNA analysis, ii) kick-sampling for community identification, iii) and collection of amphipod specimens for gut content analysis. At each site we collected 2 L of water for eDNA analysis (N=12). Water samples were collected using a Phil Sampler (Hydro Technologies Inc.) and Pyrex Media

bottles (500 mL), which were sterilised between each lake using 10% v/v commercial bleach solution and 5% v/v Lipsol solution before being rinsed with purified water from the University of Hull. Each Pyrex bottle was then rinsed twice with lake water at the sampling location to remove any possible remaining bleach or detergent. Each 2 L water sample collected was the sum of four 500 mL subsamples from each site. Two 2 L bottles containing milliQ water were taken into the field as sampling blanks (one bottle per lake). All water samples were stored on ice and filtered within 24 h of collection. Filtration took place in a dedicated eDNA laboratory at the University of Hull using 0.45 µm cellulose nitrate membrane open filters (Whatman, GE Healthcare) and sterilised Nalgene units (ThermoFisher Scientific Inc., UK) attached to a vacuum pump. Because of particulates and sediments diluted in the water, the samples from both lakes needed two filters to be processed. The filters belonging to the same sample were placed in the same petri dish with the DNA side of the two filters facing each other. Each petri dish was sealed with parafilm and stored at -20ºC until DNA extraction. Filters belonging to the same sample were also extracted together (more details on DNA extraction in section 2.2.c). All filtration equipment (Nalgene filter units and tweezers) were sterilised in between each sample with 10 min immersion in 10% v/v bleach and 5 min immersion in 5% v/v lipsol before being rinsed with purified water and let to dry. The bench surfaces where filtration took place were sterilised with 10% v/v bleach in between each sample.

Following the eDNA sampling, standardised 3-minute kick-samples were collected from each sampling location (N=12). From these kick-samples, a minimum of 50 *D. villosus* per lake per sampling event were picked out for gut contents analysis and immediately stored in sterile 1.5 mL screw-cap tubes with 1 mL of RNAlater. The amphipods in RNAlater were then frozen at -20°C upon return to the laboratory. In total 117 amphipods (of which 115 were *D. villosus*, and 2 were *Gammarus tigrinus*) were collected for gut contents analysis. The 12 kick-samples were placed in Whirl Pak plastic bags (Nasco, USA) and preserved in 100% ethanol. Kick-samples were identified under microscope to the lowest taxonomic level possible back at the University of Hull. In the case of specimens that were damaged, or species identification was not possible, then genus and in few cases family level identification were used (See Appendix 2.5 for complete table).

In this field campaign 50 ml of sediment samples were also collected at each sampling site (N=12), using individual sterile 50 ml Centrifuge tubes, to test the ability to describe the benthic community from sediment.

c. DNA extraction

The same DNA extraction protocol was used for the gut contents samples from both the feeding trials and wild *D. villosus* specimens. Whole digestive tracts were removed from individual *D. villosus* under a stereomicroscope using single-use scalpel blades and petri dishes. Scalpel blades and forceps were sterilised by immersion in 10% v/v commercial bleach for few minutes and rinsed with milliQ water before being used. Scalpel blades were then discarded after each specimen. All other materials (e.g. petri dishes) and work surfaces were wiped with 10% v/v commercial bleach before and after each dissection to prevent cross-contamination. Each specimen was also rinsed with milliQ water and briefly dried with blue roll prior to dissection. The dissected material was transferred immediately into sterile 1.5 mL Eppendorf tubes stored on ice, and DNA extracted within 2 h of dissection. DNA from gut contents was extracted following the Mu-DNA tissue extraction protocol as described by Sellers *et al.* (2018), with the addition of a grinding step with individual sterilised pestles to allow a better digestion by the Proteinase-K (Sellers *et al.*, 2018). The grinding was carried out after the addition of the tissue lysis solution, and directly into the 1.5 mL tubes used after the dissection. The individual plastic pestles used to grind the material were sterilised with 10% v/v commercial bleach, stored in 100% ethanol and exposed to UV light for 3 h. All tissue samples were digested on a Thermo mixer (ThermoFisher Scientific Inc., UK) at 56°C and 700 rpm for 4 h, until all material was digested. For the eDNA samples, DNA was extracted from the membrane filters using MoBio Power Water kit (Qiagen, UK) following manufacturer's protocol. DNA extracted from the guts and DNA from the filters were both eluted in 200 µl final volume. Field blanks were extracted in parallel to the field samples using 1 filter per field blank. In addition to the field blanks, no extraction blanks were included in this study.

The sediment samples were processed by resuspending the sample in 200 ml of purified water using a sterilised graduate cylinder. To ensure homogenous resuspension the water and sediment solution were inverted 10 times before

allowing the larger particulates to settle for 30 sec. The ~200 ml supernatant was poured into sterile 2 L bottles, identical to the ones used for the water samples. This resuspension was repeated twice for a total of ~400 ml of supernatant. These 400 ml samples were pre-filtered using 20 µm filters as described to process the water samples to further remove the fine sediment, and then were filtered with 0.45 µm as described for the filtration of water samples.

d. Library preparation

Two separate sequencing libraries were generated. The first library contained only the feeding trials against *A. aquaticus* and *D. magna*. These samples were amplified in 4 PCR replicates to verify if technical replicates were consistent across the different biological replicates. The second library contained the feeding trials against *C. pseudogracilis* and the wild samples collected in May, which were all amplified with only 1 PCR replicate, based on the consistent amplification between PCR replicates observed in the biological replicates of the first library. Each 96 well plate contained a minimum of two *Osmia bicornis* (Linnaeus, 1758) genomic DNA positive controls (N = 4 first library, and N = 5 second library), and a minimum of two PCR negative controls (2 µl of molecular grade water) per plate (N = 13 first library, and N = 7 second library). The total number of samples was 722, with 320 sequenced in the first library and 402 in the second. PCRs targeted a 313 bp region of COI using metazoan universal primers mtCOIintF - jgHCO2198 (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013). The amplicon primers were modified to enable a nested-barcode approach for library preparation by adding 20 different barcodes, and 8 different heterogeneity spacers (N = 96 primer combinations) (Kitson *et al.*, 2018) (See Appendix 2.2 for the information on the Sequencing primers). Because the number of samples was greater than the number of tags that can be introduced in the initial PCR, all samples were split into separate plates that were individually tagged in the second PCR. Plates were processed separately to minimise cross-contamination. Library preparation followed a two-step protocol, which included an initial PCR to amplify the COI amplicon and to tag each sample with one the 96 primers combinations. The reagents used for this first PCR were 0.4 µM (1 µl) of each Primer, 1x (12.5 µl) MyFi™ Mix *Taq* polymerase (Bioline, UK), 8.5 µl molecular grade water and 2 µl of DNA template in a 25 µl reaction volume. PCR conditions were 95°C for 3

mins, followed by 40 cycles of 95°C for 15 sec, 53°C for 30 sec, 72°C for 30 sec, and a final extension step of 72°C for 10 mins.

Products from the first PCR were individually purified with Mag-Bind RxnPure Plus (Omega Biotek, US) on a 96 well magnetic stand to remove primer dimers and to isolate the target fragments. The clean-up protocol was based on the size-selection protocol described by Quail, *et al.* (2009) with a few modifications, as described below (Quail, Swerdlow and Turner, 2009). The initial ratio was changed to 0.5x of volume of magnetic bead per 20 µl PCR product, and samples were allowed to stand for 15 min before transferring the supernatants to new wells. The second ratio was changed to 0.12x of magnetic beads per initial volume of PCR products, and samples were then allowed to stand for 5 mins to allow target fragments to bind to the beads. The supernatants were then discarded, and the beads were washed twice with 200 µl of 80% ethanol. The DNA bound to the beads was ultimately eluted in 15 µl of 10 mM Tris-HCl (pH 8). After visualisation on 2% agarose gels, PCR products from the same 96 well plate were pooled. The individual plates were then uniquely tagged with the Illumina MiSeq adapter primers in a second PCR, which followed the protocol and reaction volumes as described in the “Amplicon, Clean-Up and Index, 2013” (Illumina technologies, online) using MyFi™ Mix *Taq* polymerase (Bioline, UK). Following the second PCR and gel visualisation, the PCR products were cleaned again with magnetic beads as described above. Products were checked on agarose gels after each step.

After the clean-up of the second round PCR products, the individual plates now individually tagged were quantified for dsDNA with a Qubit fluorometer (ThermoFisher Scientific Inc., UK) and pooled together in equimolar amounts, thus taking into account the number of samples in each plate. The pooled libraries were then quantified with qPCR using the NEBNext Library Quant Kit (New England BioLabs Ltd., UK) and the fragment size was further checked on a TapeStation Automated Electrophoresis (Agilent Technologies Inc., US) using a High Sensitivity D1000 Screen Tape kit. The final libraries were denatured and diluted following Illumina “MiSeq System Denature and Dilute Libraries Guide”. Both libraries were sequenced on an Illumina MiSeq using a V2 kit at 250 cycles per reads, loading 15 pM library with 10% PhiX.

e. Bioinformatics

Illumina raw sequences (GenBank SRA: PRJNA434643) were processed using the custom pipeline metaBEAT v0.97.10 (https://github.com/HullUni-bioinformatics/metaBEAT). This includes trimming raw Illumina sequences with Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014) using a Phred score of 30 or higher using a 5-base sliding window. The sequences were merged with FLASH v1.2.11 (Magoč and Salzberg, 2011), checked for chimeras and clustered at 97% similarity with VSEARCH v1.1.0 (Rognes *et al.*, 2016). The centroids of the clusters that contained more than 2 sequences were taxonomically assigned using BLASTn v2.2.28+ (Altschul *et al.*, 1990) and the Lowest Common Ancestor (LCA) approach using Taxtastic v0.8.5 (https://github.com/fhcrc/taxtastic). Sequences were assigned with 97% identity match, and a minimum of 80% alignment. The taxonomic assignment was run against databases of macroinvertebrate COI sequences mined from GenBank and EMBL, and curated using SATIVA v0.9 (Kozlov *et al.*, 2016) to highlight errors in the mined records. Sequences that remained unassigned to the reference database were assigned against the NCBI nt database (updated at the end of August 2018), with the same BLASTn and LCA approach. The final output was a table of DNA reads in each sample collapsed by taxonomy. (Detailed information on the curated databases are available in the Appendix 2.3).

f. Statistical analysis

Downstream analyses were performed in R v.3.5.1 (R Core Team, 2018). The bioinformatic output, containing read counts collapsed by taxonomy, was initially quality controlled to remove low coverage samples. The sediment samples did not generate enough sequencing depth (< 200 reads per sample), so they were excluded from downstream analysis. The sequencing data from the remaining eDNA and gut contents samples were quality controlled and samples that contained less than 1000 reads (first library) and 500 reads (second library) were excluded from analysis.

A lower read count threshold was used for the second library due to more samples (hence lower coverage per sample) and higher standard error of the mean read depth. Low levels of contamination were detected in the positive control samples in both sequencing runs, so DNA reads in each sample that were

lower than 0.1% and 0.05%, respectively for each library, were removed. Following these QC steps, all retained read counts were used to create a presence/absence table. The feeding trials presence/absence data were used to calculate the proportion of positive consumers to prey DNA. Presence/absence data from the wild gut contents were used to calculate interaction strengths between predator and prey, defined as the number of predators that were positive for prey species DNA. Predator-prey interactions were visualised using the R-package `bipartite` v2.11 (Dormann, Gruber and Fründ, 2008). Kick sample, gut contents and eDNA data were compared using barplots constructed in ggplot2 (Wickham, 2011) to investigate the overlap between gut contents and the wider community. To ensure reproducibility, all scripts have been deposited (https://github.com/mbenucci/Dv_feeding_trials).

2.3 Results

The first library generated 14,239,823 reads of which 87.36% passed the filter (12,471,227 reads), with 91.47% of bases ≥Q30 and error rate of 1.52%. Mean read depth resulted 19076.56 (±807.34) reads. The second library was similar, with 15,538,383 total reads generated of which 90.33% passed the filter (14,008,944 reads), with 92.12% of bases ≥Q30 and an error rate of 1.70%. Mean read depth was 21788.45 (±1548.57) reads.

Feeding trials

A total of 87 feeding trials were successfully completed (i.e. the predator consumed prey and survived to the end of the experiment) and all of the corresponding samples were sequenced. Of these samples, 24 were from the *A. aquaticus* trials (N trials = 18, N controls = 6), 30 from the *D. magna* trials (N trials = 27, N controls = 3), and 33 from the *C. pseudogracilis* trials (N trials = 27, N controls = 6).

All three prey species were detected by metabarcoding of *D. villosus* guts, however there were important differences in detection rates and times between them. *A. aquaticus* was detected up to 36 h after predation, while *D. magna* and *C. pseudogracilis* were detected up to 8 h and 4 h after a predation event respectively (Fig. 3). The proportion of the prey to predator reads was low. Mean prey read proportions (relative to *D. villosus* in each case) were 7.55% (±1.25)

for *A. aquaticus,* 0.0722% (±0.055) for *C. pseudogracilis* and 0.014% (±0.0076) for *D. magna. Asellus aquaticus* was detected from time 0 h through till 36 h, although very low read counts were observed in all three replicates at 0h and in 2 out of 3 replicates at 36h. Peak read counts were observed at 36 h for trial replicate 1, between 8 and 12 h after digestion for trial replicate 2, and at 4 and 24 h for trial replicate 4. There were also differences in prey read count between PCR replicates of the same trial (Fig. 3). *D. magna* was only detected in one trial replicate (number 2) at 4 h, while *C. pseudogracilis* was detected in two trial replicates at 4 h and one trial replicate at 8 h only (Fig. 3).

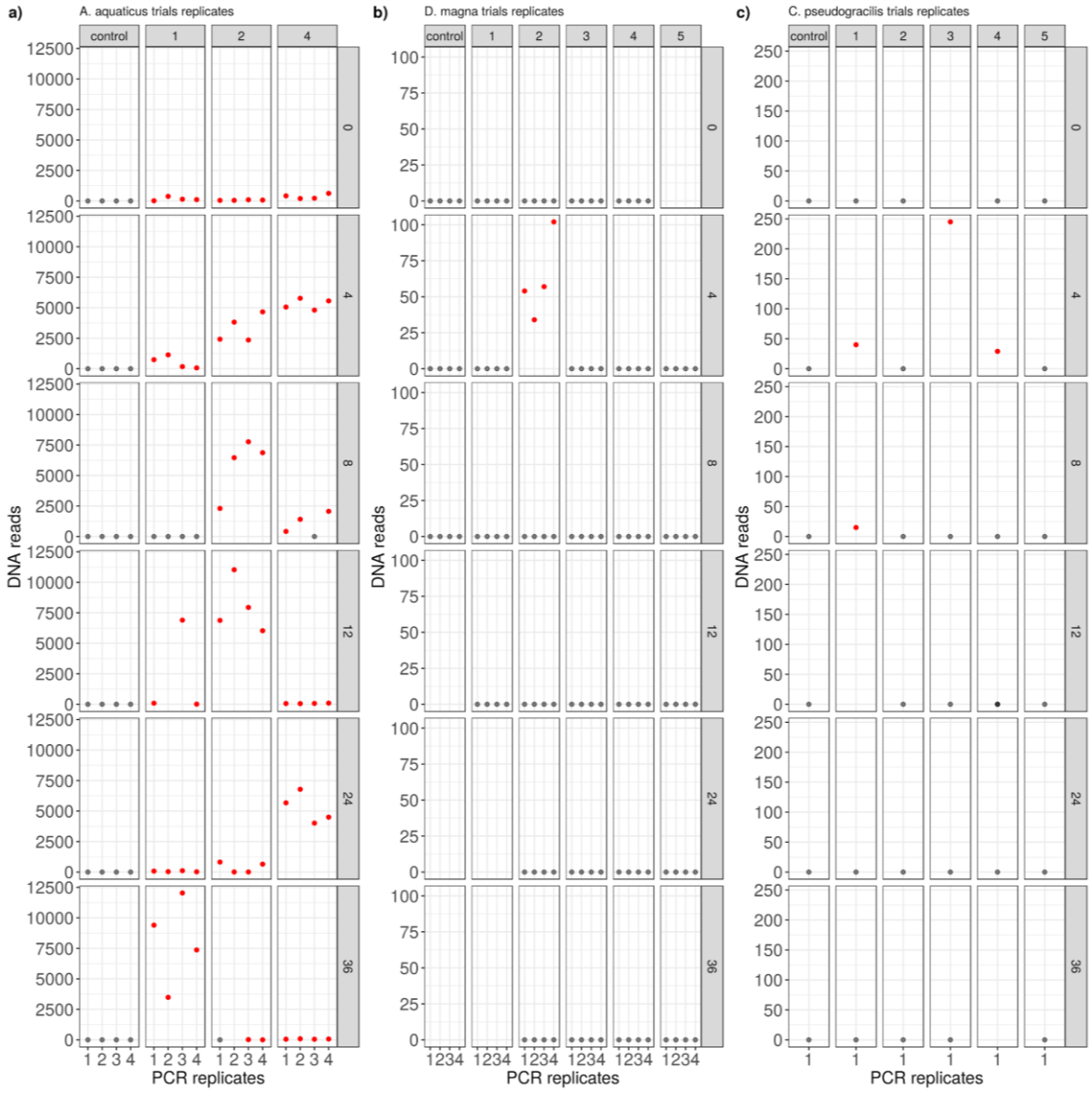


Figure 3. Read count of prey species from feeding trials, divided by digestion time, PCR replicates, and trials replicates. Samples with DNA reads greater than

0 are shown in red. Samples with DNA reads equal to 0 are in grey. Empty boxes correspond to samples that were not sequenced.

A. aquaticus DNA was detected in 100% of the consumers at 0 h + 15 mins, and at 4 h after predation. The detection dips at 67% around 8 h, however we then continued to detect *A. aquaticus* DNA in 100% of the consumers' guts at 12 h, 24 h and 36 h without changes. *D. magna* instead was only detected at 4 h in 20% of the *D. villosus* guts; while *C. pseudogracilis* DNA was detected at 4 h and 8h, in respectively 60% and 20% of *D. villosus* guts (Tab. 1). All Control replicates of the feeding trials were negative for prey DNA.

Table 1. Percentage detection of the feeding trials preys in the *D. villosus* gut contents.

	A. aquaticus		D. magna		C. pseudogracilis	
Times	N replicates	% detection	N replicates	% detection	N replicates	% detection
0h	3	100	5	0	5	0
4h	3	100	5	20	5	60
8h	3	66.7	5	0	5	20
12h	3	100	5	0	5	0
24h	3	100	5	0	5	0
36h	3	100	5	0	5	0

Detection of prey DNA in wild D. villosus.

We detected DNA from potential prey species in 19/117 (16%) *D. villosus* (N Grafham Water = 6/50, N Wroxham Broad = 13/67) (Tab. 2).

Table 2. Summary of detection (presence/absence) of prey species DNA in wild *D. villosus* gut contents.

Sample type	gut contents	
Predator ID	*D. villosus*	
Lake	GW	WB

N specimens sequenced	50	67
N specimens passing QC	50	65
N specimens with detected interactions	6	13
Detection rate	12%	20%
Total N interactions	6	18
Bothrioneurum vejdovskyanum	0	1
Chironomus luridus	1	0
Chydorus brevilabris	0	1
Cricotopus	0	1
Cricotopus laricomalis	2	0
Cyclopidae	0	4
Daphnia	0	1
Dreissena polymorpha	0	1
Gammarus tigrinus	3	6
Gammarus zaddachi	0	1
Polyphemus pediculus	0	2

From these 19 wild *D. villosus* we detected 11 prey species belonging to 6 orders: 3 species belonging to Cladocera, 1 to Copepoda, 2 to Amphipoda, 3 to Diptera, 1 Mollusca, and 1 Oligochaeta (Fig. 4 and Tab. 2). The Amphipoda prey detected were *Gammarus tigrinus* (N interactions = 3 in Grafham Water, and N interactions = 6 in Wroxham Broad) and *G. zaddachi* (N = 1 in Wroxham Broad).

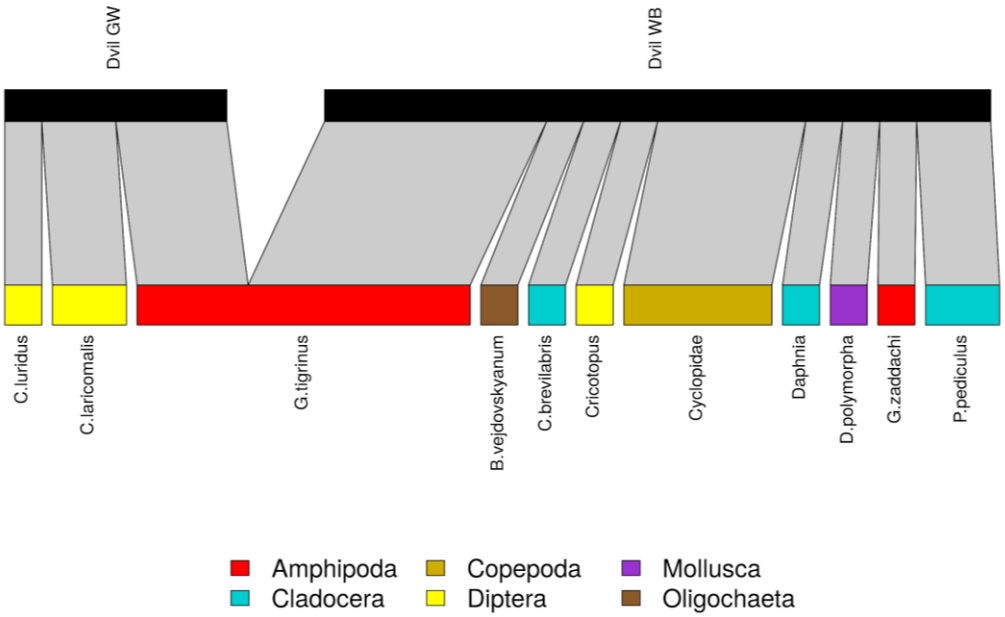


Figure 4. Bipartite network of wild *D. villosus* obtained from DNA metabarcoding of gut contents.

Integration of community information and gut contents

We identified 96 taxa in total from the kick and eDNA samples (Grafham Water: 44 taxa from the eDNA samples, 23 from the kick samples; Wroxham Broad: 26 from the eDNA samples, 22 from the kick samples) (See Appendix 2.5 for complete table).

In Grafham Water *Cricotopus laricomalis* and *Gammarus tigrinus* were detected in both the gut contents and the community samples (eDNA and kick samples), while in Wroxham Broad we detected Cyclopidae, Daphnia sp., and *Dreissena polymorpha* DNA in both gut contents and the community samples (Tab. 3). In Grafham Water prey DNA in *D. villosus* gut contents was composed for about 25% by *G. tigrinus* DNA, and ~75% Diptera DNA, specifically *Chironomus luridus* and *Cricotopus laricomalis* (Fig. 5). The community obtained by combining all the eDNA samples is composed of ~50% Cladocerans and Copepod DNA; with Annelida, Diptera and Nematoda DNA making nearly 30% of the eDNA community. The kick samples from Grafham Water are dominated (~50%) by Diptera (mostly identified as Chironomidae), with Amphipods (mostly *D. villosus* and few *G. tigrinus* specimens) comprising ~ 10% of the community (Fig. 5).

In Wroxham Broad, gut contents contained a high proportion (~80%) of Amphipod DNA (mostly composed of *G. tigrinus* and to a lesser extent *G. zaddachi*), with the remaining DNA belonging to Diptera (*Cricotopus sp.*) DNA (~15%), plus Copepoda, Cladocera (Daphnia, *Chydorus brevilabris*, and *Polyphemus pediculus*), Annelida (*B. vejdovskanium*) and Mollusca (*D. polymorpha*) combined accounting for ~5%. Wroxham eDNA community consisted of ~50% Nematoda, with Cladocerans, Copepoda and Rotifera DNA making ~40% of the community. The remaining 10% of the eDNA community consisted of Amphipoda, Diptera, Mollusca, and Ostracoda DNA. The Wroxham kick samples were dominated (~65%) by Amphipoda, all of which were *D. villosus*, followed by Hemiptera (~20%), Diptera (~10%), Mollusca (<5%) and very few Ephemeroptera (Fig. 5).

Table 3. Species overlap between gut contents and community (eDNA plus kick samples). The data represents the sum of detections in gut contents over the number of detections in the community. Highlighted in white are the shared taxa.

	shared guts/eDNA		shared guts/kick	
Lake	GW	WB	GW	WB
Cricotopus laricomalis	2/3	0/0	2/0	0/0
Cyclopidae	0/2	4/3	0/0	4/0
Daphnia	0/4	1/1	0/0	1/0
Dreissena polymorpha	0/1	1/1	0/0	1/5
Gammarus tigrinus	3/0	6/0	3/6	6/0

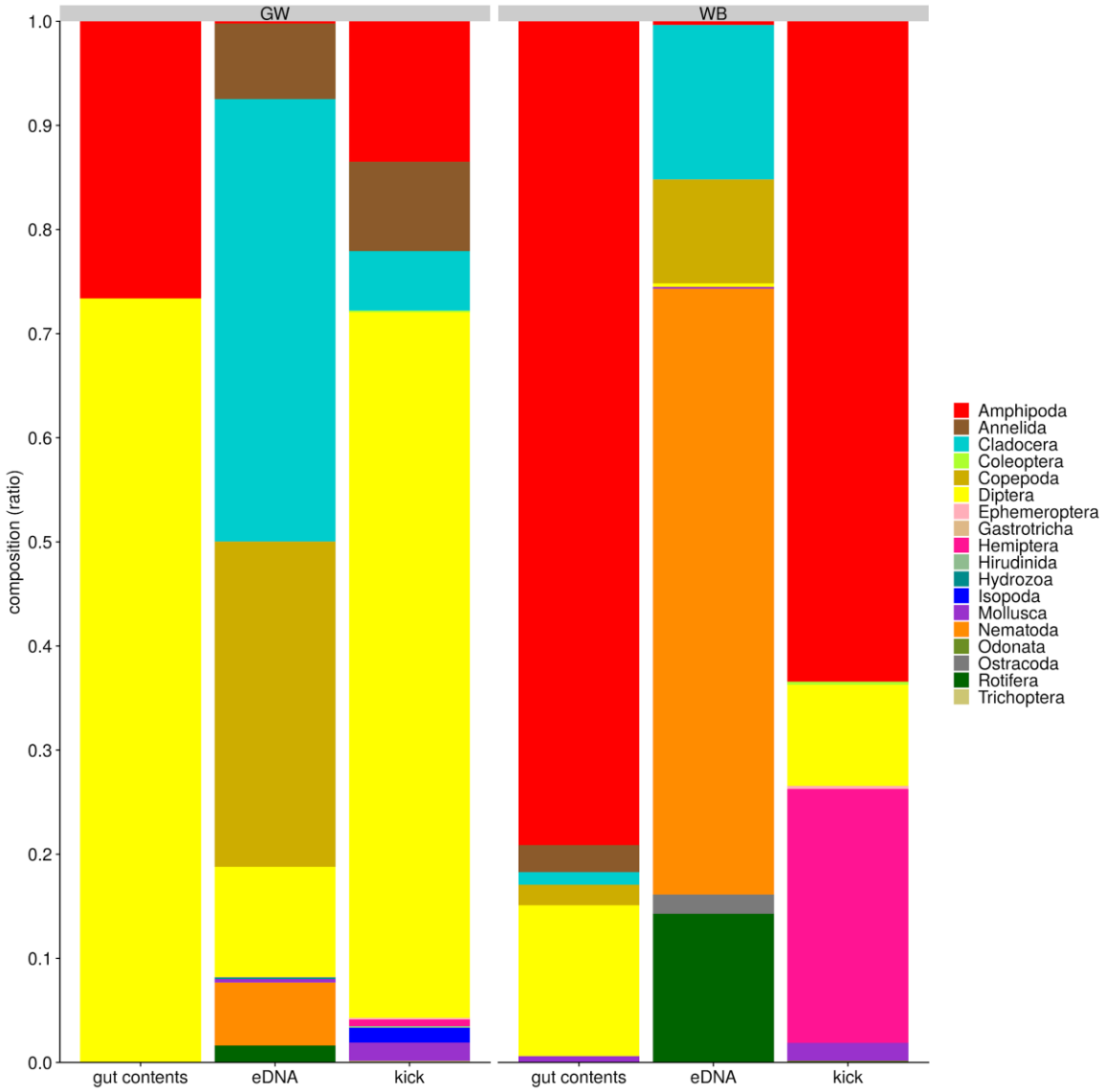


Figure 5. Barplot of Grafham Water (GW) and Wroxham Broad (WB) species composition obtained from gut contents (DNA read counts), eDNA (DNA read counts) and kick-samples compositions (count data). Gut contents data include prey DNA only (i.e. *D. villosus* DNA is excluded), so Amphipoda in gut contents indicated intra-guild predation of *Gammarus zaddachi* and *G. tigrinus*.

2.4 Discussion

We successfully detected DNA from 3 known prey species in gut contents of 22 (25.2%) of 87 *D. villosus* from the controlled mesocosms, and at different digestion times from a known predation event. However, only one prey species, *Asellus aquaticus,* could be detected throughout the experiment in multiple replicates. The other prey items, *Daphnia magna* and *Crangonyx pseudogracilis,*

were detected in just a single or three trial replicates respectively. We then applied the same methodology in a small field trial using gut contents from 117 wild *D. villosus* from two lakes, and compared gut contents with data on the wider community obtained from 12 eDNA samples, and 12 kick-samples. Eleven different prey species were detected in 19 (16%) of the 117 *D. villosus.* Nine of these 19 *D. villosus* were positive for *Gammarus tigrinus* or *G. zaddachi,* which are intraguild preys.

Sediment samples applied to the wider community

The failed attempt to describe the benthic community using DNA from sediment highlights important limitations in the methodology and in the ecological interpretation. The presence of inhibitors in the DNA extract might be behind the failed sequencing of the samples in our study (Turner *et al.*, 2015), but it cannot be excluded that the chemical composition of the sediment collected didn't allow for the appropriate release of the DNA during resuspension and extraction (Fisher *et al.*, 2017) without manipulating the pH during the resuspension.

In addition to this the ecological interpretation also needed important considerations linked to the rate of sedimentation of each study site. Although the 50 ml of sediment collected in this study could be considered surface sediment, there is the risk of describing and comparing communities from different temporal scales, thus introducing a bias by including a community that might not reflect the current conditions (e.g. Bennion *et al.*, 2011, Dong *et al.*, 2016).

Feeding experiments

The results from the controlled mesocosms confirm our expectations that it is possible to detect prey DNA in *D. villosus* gut contents with DNA metabarcoding. However, detection depends on which taxa is being consumed, and the period in which DNA can be detected appears to vary greatly. *A. aquaticus* was detected consistently, up to 36h post feeding, whereas the probability of detecting smaller prey, *D. magna* and *C. pseudogracilis*, was very low. We detected DNA from *D. magna* in ⅕ trial replicates 4 hours after predation, and *C. pseudogracilis* in ⅗ trial replicates 4 hours after predation, and ⅕ replicates 8 hours after predation. In contrast with other feeding trials studies (King *et al.*, 2008), we decided to use a broad target assay (DNA metabarcoding) on dissected gut contents rather than

on whole specimens. Dissecting whole digestive tracts is a labour and time intensive process, but we believe this is beneficial for increasing the prey:predator DNA ratio. We managed to obtain good DNA detection in *A. aquaticus* feeding trials, with an average of 7.5% of prey:predator DNA. By contrast the two smaller prey species were detected on average at less than 1% (*D. magna* 0.07%; *C. pseudogracilis* 0.014%). Of these smaller prey species, *D. magna* was the one more difficult to detect as only 1 predator was positive *D. magna* DNA. This result highlights one of the main challenges in molecular analysis of trophic interactions, that the amount of food ingested (e.g. either in the form of larger/smaller items or greater/fewer number of smaller items) can influence the DNA recovery. This was also demonstrated by Weber and Lundgren (2009) who detected an increasing qPCR signal as they fed *Coleomegilla maculata* (Coleoptera: Coccinellidae) with increasing number of Potato Beetle eggs (Weber and Lundgren, 2009).

Detection of prey DNA in wild D. villosus

We successfully detected 11 prey species in the gut contents of wild *D. villosus* across two separate lakes. The number of *D. villosus* with prey DNA was quite low, with 16% of the total number of specimens (N total =117) positive for prey DNA. This was somewhat surprising given the reported generalist diet of *D. villosus* (Dick, Platvoet and Kelly, 2002; van Riel *et al.*, 2006; Platvoet *et al.*, 2009). Similar values of detection however have been reported by Koester *et al.* (2016), who detected prey DNA in around 15.5% of the total sample size in wild populations of *D. villosus* from Lake Constance and the River Rhine (Koester, Bayer and Gergs, 2016). Although detection rates were low, it is worth noting that DNA from other Gammaridae (*G. zaddachi* and *G. tigrinus*) was detected in *D. villosus* gut contents, indicating intraguild predation (IGP). The rates of IGP were approximately 5.8% in Grafham Water (N specimens = 52), and 10.8% in Wroxham Broad (N specimens = 65). The values obtained in this study represent a limited temporal transect, however these levels appear slightly higher than the ~3.9% of IGP reported by previous studies (Koester, 2016). *G. tigrinus* was detected in the gut contents in both Grafham and Wroxham, whereas *G. zaddachi* was only detected in Wroxham. Nine other prey species were detected in addition to the two *Gammarus* species. This included: 3 Diptera species (*Cricotopus sp.*, *C. laricomalis*, *Chironomus luridus*), 3 Cladocera species (*Daphnia sp.*,

Polyphemus pediculus, *Chydorus brevilabris*), 1 Mollusca (*Dreissena polymorpha*), 1 Copepoda (Cyclopidae), and 1 Oligochaeta (*Bothrioneurum vejdovskyanum*). Despite the low detection rates of *D. magna* in the feeding trials, this prey species was detected in the gut contents of 1 (5.2% of the positive gut contents) wild *D. villosus*.

Overlap between gut contents and the wider community

Five of the eleven taxa detected in the gut contents were also detected in the the wider community (i.e. eDNA and kick samples combined): *Cricotopus laricomalis* (Diptera) and *G. tigrinus* (Amphipoda) in Grafham Water; and Cyclopoidae, *Daphnia sp.* (Cladocera) and *D. polymorpha* in Wroxham Broad. Three taxa were detected in both eDNA and kick samples; Chironomidae and *D. villosus* in Grafham Water, and *Dreissena polymorpha* and *D. villosus* in Wroxham Broad. This limited number of species can be associated with species for which lower resolution in identification could be obtained (e.g. Chironomidae that could be identified by microscopy only to family level), and species that were detected in high abundance (e.g. *D. villosus* and *D. polymorpha* in both GW and WB). The remaining of the community were detected in either method (eDNA or kick sample) only. Overall eDNA and kick samples seems to target different species from the same community, with eDNA that in this small scale study detected mainly microinverterbrates species, like Cladocera, Copepoda, Oligochaeta, Rotifera, and Nematoda; while the kick samples detected mainly macroinvertebrates species, like Mollusca, Amphipoda (*D. villosus* and *G. tigrinus*), Diptera.

Another element contributing to the low overlap in terms of species was the low resolution of identification in some taxa from the kick samples (e.g. Chironomidae, Oligochaeta) that were identified and recorded only to family and order levels; in the eDNA samples instead the same taxa could be identified to much lower resolution often achieving genus and species level (e.g. *Cricotopus laricomalis*, *Chironomus nuditarsis* which are both Chironomidae). This difference caused by challenges in the morphological identification from kick samples highlights the importance of integrating molecular and morphological sampling and identification methods to be able to capture a broader community than the individual methods alone. The integration between community information and

gut contents information demonstrates, for example, that the lack of interactions (e.g. low rate of IGP between *D. villosus* and *G. tigrinus* in GW) could be driven also by a low abundance of potential guild member species, hence a reduced chance of encounter between the two IG competitors.

The small overlap between gut contents and communities is consistent with previous studies on generalist and opportunist species which can feed on the wider community by scavenging or direct predation (Siegenthaler *et al.*, 2018). *D. villosus* is a facultative omnivore with cannibalistic behaviour, and a detritivore (Dodd *et al.*, 2014; Little, Fronhofer and Altermatt, 2019).

2.5 Conclusions

Here we demonstrated the use of DNA metabarcoding to detect DNA from single specimens of known prey species in controlled mesocosm be the invasive amphipod *D. villosus*. We confirmed that the DNA of single specimens from *Asellus*, *Daphnia* and *Crangonyx*, can be detected in the gut contents of an invasive consumer. However, consistent detections were only obtained with the larger prey species, *Asellus*. We applied the same methods to investigate the trophic interactions in wild specimens of *D. villosus* and successfully detected trophic interactions in two study lakes. Interactions involved mostly other Amphipods (*Gammarus spp)*, indicating IGP, as well as Diptera, Cladocera, Copepods, Molluscs and Oligochaetes. The rate of trophic interactions was similar to previous studies, and the rate of IGP was slightly higher than previously recorded in wild *D. villosus* individuals. Further work is needed to determine whether the rates of IGP obtained here for *D. villosus* are comparable to, or greater than that in native Amphipods.

Chapter 3 - DNA metabarcoding of gut contents of invasive *Dikerogammarus villosus* reveals low levels of intraguild predation compared to a native amphipod, *Gammarus zaddachi* in the UK.

Abstract

Invasive Non-Native species (INNS) are one of the main causes of biodiversity loss globally. Freshwater habitats are particularly vulnerable to invasions, with evidence that even the introduction of a single species can deeply alter the trophic interactions of a community. This can have deep ramifications for the community stability when the introduced species also predates on other guild members, interaction called intraguild predation (IGP), interaction that can speed up the invasion process and the establishment INNS into the novel habitat. There is therefore considerable interest in understanding how INNS influence the stability of ecological networks, but detecting trophic interactions is notoriously challenging in field conditions. Given this challenge, there has been considerable interest in recent years in using molecular methods to detect trophic interactions, an approach sometimes referred to as “MATI” (Molecular Analysis of Trophic Interactions). The objectives in this study were to 1) carry out gut contents molecular analysis of direct trophic interactions of *D. villosus* and native *G. zaddachi* to investigate breadth of trophic interactions, including the proportion of IGP interactions of the invasive amphipod compared to a native amphipod, and 2) to use both eDNA and kick sampling of invertebrate communities in order to describe the available prey resources, and understand the composition of invaded communities. Here we demonstrated the use of broad-spectrum molecular methods, DNA metabarcoding, in conjunction with thorough community information, to detect direct trophic interactions in two species of amphipods, an invasive and a native species in the UK across two seasons. We detected different levels of trophic interactions, which appears in agreement with the ecology and omnivory expected from these amphipods. However, our data show that, despite similarities in the diet breadth of the target amphipods, their way of using the communities available to them had important differences. The importance of IGP in both invaded and non-invaded communities is still not fully explored, and we might suspect, in agreement with current literature, that this interaction plays an important role in shaping species communities.

3.1 Introduction

Invasive Non-Native species (hereafter INNS) are listed as one of the main causes of biodiversity loss globally (Secretariat of the Convention on Biological Diversity, 2010). Freshwater habitats are particularly vulnerable to invasions (Sala *et al.*, 2000), with evidence of INNS having repercussions across whole ecosystems, impacting vital ecosystem services such as nutrient cycling and water quality (Dudgeon *et al.*, 2006; Walsh, Carpenter and Vander Zanden, 2016), causing physical damage to structures via biofouling (Sousa, Pilotto and Aldridge, 2011), introducing novel parasites (Bojko *et al.*, 2015), changing community composition (Gergs and Rothhaupt, 2014), and disrupting existing and introducing novel trophic interactions (Bašić *et al.*, 2019).

The introduction of a single species can deeply alter the trophic interactions of a community, with knock on effects throughout the whole ecological network (Higgins and Zanden, 2010; Jackson *et al.*, 2017). This can be particularly important if the introduced species is also an intraguild predator. Intraguild predation (IGP), i.e. predation of a competitor species that uses similar resources (Polis, Myers and Holt, 1989; Aebi *et al.*, 2011), plays a major role in the functioning of an ecosystem, shaping and promoting stability and resilience of communities against disturbance (Schneider *et al.*, 2016; Wang, Brose and Gravel, 2019). Invaders that consume native competitors have an advantage if IGP is not reciprocal, and increasing levels of IGP can potentially increase invasion speed (MacNeil and Platvoet, 2005; Hall, 2011). Other interactions, such as mutualism between multiple INNS, can lead to synergistic impacts and facilitate further invasions, a situation referred to as “invasional meltdown” (Green *et al.*, 2011; Gallardo and Aldridge, 2015). Depending on the species, the modifications of INNS on ecological networks from the afore mentioned interactions can follow a “bottom-up” process, with changes in nutrient loading (Kuiper *et al.*, 2015), a “top-down” process, with changes driven by release of grazing on primary producers from higher trophic levels (Walsh, Carpenter and Vander Zanden, 2016), or laterally, via IGP, across the same trophic level (David *et al.*, 2017). Recovery from such impacts on ecological networks can be slow (Geist and Hawkins, 2016), requires considerable management efforts and might not be fully reversible to the original state (Suding, Gross and Houseman, 2004).

There is therefore considerable interest in understanding how INNS influence the stability of ecological networks (David *et al.*, 2017), but detecting trophic interactions is notoriously challenging in field conditions without witnessing the event or disturbing the system (Aebi *et al.*, 2011).

Given this challenge, there has been considerable interest in recent years in using molecular methods to detect trophic interactions, an approach sometimes referred to as “MATI” (Molecular Analysis of Trophic Interactions) (Symondson, 2002; King *et al.*, 2008; Clare, 2014; Symondson and Harwood, 2014). Most of the current knowledge on trophic interactions in invertebrates comes from monoclonal antibodies, stable isotopes and, more recently, from single-species molecular assays (Harwood *et al.*, 2007; Thomas *et al.*, 2013; Koester and Gergs, 2014; Frossard and Fontvieille, 2018). These methods have provided important information regarding trophic positions and direct interactions, however, as also highlighted by Soininen *et al.* (2014), there are still opportunities for applying these methods and for integrating them in diet analysis since they produce different and complementary information on dietary analysis (Soininen *et al.*, 2014). Stable isotopes, for example, can produce dietary information representing a 2-months period, however it can be subject to confounding signals in migrating species that use a multiple areas for gathering resources, as in the case of Norwegian and Finnish rodents (Soininen *et al.*, 2014). The molecular assays, like single-species assays and DNA metabarcoding, on the other hand can describe a very recent interactions, but can provide a finer taxonomic resolution and a better understanding of the degradation of DNA following predation (Kamenova *et al.*, 2018).

Single-species molecular assays have increased our understanding of known predator-prey interactions; however, they are limited to investigating known or hypothesized interactions, and require the development of one-assay per target or potential target prey (Koester, Claßen and Gergs, 2013), making the process time- and resource-intensive. DNA metabarcoding and High-Throughput Sequencing (HTS) are emerging as powerful methods that can allow the detection of both known and uncharacterised interactions (Lawson Handley, 2015; Buglione *et al.*, 2018), across trophic levels (Kartzinel *et al.*, 2015; Jakubavičiūtė *et al.*, 2017; Siegenthaler *et al.*, 2018). This is demonstrated by their employment for studying interactions within and among predators (Clare *et*

al., 2009; Lyngdoh *et al.*, 2014), such as in the case of generalist bats for which the cryptic and highly diverse flying arthropods they feed on could be described using DNA metabarcoding on guano (Clare *et al.*, 2009), herbivores (Kartzinel *et al.*, 2015), parasites (Kitson *et al.*, 2018), arthropods (Kaunisto *et al.*, 2017; Sint *et al.*, 2019) as in the description of pioneer communities interactions in alpine habitats following glacier's retreat (Sint *et al.*, 2019), and plants (Littlefair *et al.*, 2018).

DNA metabarcoding, as with other methods, has its own challenges and limitations associated with method development, use and analysis (Deagle *et al.*, 2018; Nielsen *et al.*, 2018). Universal primers, for example, are an important element to describe the broad diversity of species, but not without introducing important challenges in describing invertebrate-invertebrate interactions (Piñol, Senar and Symondson, 2018). Universal primers used in DNA metabarcoding of invertebrates can often include degenerated bases in their sequences (e.g. Leray *et al.*, 2013), and while having degenerated bases allow for the amplification of the broad range of closely and distant species; on the other hand it comes at the cost of amplifying DNA also from the predator, which ends up dominating the number of reads in each sample (Pinol *et al.*, 2014; Piñol, Senar and Symondson, 2018). The use of universal primers with the aim of targeting a broad number of species also highlights the limit of DNA metabarcoding in not being able yet to detect cannibalism in invertebrates communities, which instead still can be achieved through immuno assays as Zilnik and Hagler (2013) demonstrated on two species of Coleoptera (Zilnik and Hagler, 2013). While the detection of cannibalism still remains inaccessible with DNA-based methods, there is a small number of studies that have successfully limited predator DNA amplification in molecular trophic interaction analysis, for example Vestheim and Jarman (2008) developed blocking primers and successfully removed Arctic Krill DNA to investigate its diet over two seasons (Vestheim and Jarman, 2008). There is a growing body of studies that managed to demonstrate the applicability of DNA metabarcoding analysis in detecting and describing diet, niche breadth and partitioning (Razgour *et al.*, 2011; Lyngdoh *et al.*, 2014; Littlefair *et al.*, 2018), and to describe trophic interactions in great detail (Evans *et al.*, 2016).

Dikerogammarus villosus (Sowinsky, 1894)

Dikerogammarus villosus (Sowinsky, 1894) (Amphipoda: Gammaridae) is an example of a fast dispersing INNS with widespread impacts (Rewicz *et al.*, 2014), and is considered one of the 100 worst invasive species in Europe (Hulme, 2009). This invasive amphipod, from the Ponto-Caspian region, has a long and well documented history of expanding its range across Europe (Bacela-Spychalska and Van Der Velde, 2013; Rewicz *et al.*, 2015; Borza *et al.*, 2017), appearing as early as 1926 in the Danube (Nesemann and Pöckl, 1995). From this early record, *D. villosus* greatly expanded throughout Europe, reaching southwards to Italy (Casellato *et al.*, 2006; Tricarico *et al.*, 2010), westwards across France (Bollache *et al.*, 2004), and northwards to the Baltic and the UK (Bącela, Grabowski and Konopacka, 2008; MacNeil *et al.*, 2010). It is also expected to follow other Ponto-Caspian species across the Atlantic Ocean to North America (Rewicz *et al.*, 2015). Due to its fast range expansion, *D. villosus* has attracted a lot of research interest, with widespread (but not universal) agreement about its negative impacts, which can include reduction of leaf matter processing (MacNeil *et al.*, 2011; Kenna *et al.*, 2016; Little and Altermatt, 2018), generalist predation as recorded both in lab (Dick and Platvoet, 2000; Jackson *et al.*, 2017), and field conditions (Hellmann *et al.*, 2016; Kobak *et al.*, 2016); and causing changes in community composition (Gergs and Rothhaupt, 2014). *D. villosus* is considered an ecosystem engineer, capable of modifying freshwater habitats across all trophic levels (MacNeil and Platvoet, 2005; MacNeil and Briffa, 2019). Despite being an omnivore (a trait shared across other invasive amphipods (Hänfling, Edwards and Gherardi, 2011)), *D. villosus* shows traits associated with a strong predatory behaviour, including mouth parts morphology (Mayer *et al.*, 2008) and a higher Type II functional response in comparison to native amphipods (Dodd *et al.*, 2014). These morphological and functional traits appear to explain its ability to destabilise and cause the local extinction of other invertebrates (Bacela-Spychalska and Van Der Velde, 2013; Dodd *et al.*, 2014). Mesocosm experiments have demonstrated *D. villosus'* ability to predate a wide variety of benthic invertebrates, including planktivorous Cladocera, detritivorous Isopoda, predators such as Hemiptera and Odonata (Dick and Platvoet, 2000; Dick, Platvoet and Kelly, 2002; MacNeil and Platvoet, 2005), and fish eggs (Taylor and Dunn, 2016). Laboratory studies have also demonstrated strong intraguild predation of competing amphipods (such as *Gammarus pulex, G. roeselii, G. duebeni and G. tigrinus* and *Echinogammarus ischnus and E. berilloni,* (Dick and

Platvoet, 2000; Kinzler and Maier, 2003; Kinzler *et al.*, 2008)) and other macroinvertebrates by *D. villosus*, for which IGP is often assumed to be a key factor in the displacement of intraguild competitors in field conditions as well (Dick and Platvoet, 2000; Kinzler *et al.*, 2008).

While field studies have further demonstrated a link between the arrival of *D.villosus* and the decline of benthic invertebrates in both lakes (e.g. Lake Constance (Gergs and Rothhaupt, 2014)), and rivers (e.g. River Rhine (Hellmann *et al.*, 2016)), evidence of impact in the field has not been universal, with other studies suggesting a combination of environmental factors (e.g. physico-chemical parameters), rather than *D. villosus,* structure benthic communities such as that of the River Rhine (Koester *et al.*, 2018). Of particular note, two field studies used a combination of stable isotopes and a gammarid specific molecular assay to specifically test whether IGP by *D. villosus* has contributed to displacement of native amphipods at the invasion front on the River Untere Lorze in Switzerland (Koester and Gergs, 2014) and on the River Rhine in Germany (Koester, Bayer and Gergs, 2016). No differences were found between stable isotopes of *D. villosus* and native gammarids, and no native gammarids were detected in *D. villosus* gut contents on the Untere Lorze (Koester and Gergs, 2014). Similarly, on the River Rhine, only 1% of *D. villosus* guts were positive for intraguild prey, and stable isotope values were comparable to those of primary consumers (Koester, Bayer and Gergs, 2016). These combined results suggest that factors other than IGP are driving the displacement of native amphipods (Koester and Gergs, 2014; Koester, Bayer and Gergs, 2016). However, the role of *D. villosus* as a predator of the wider invertebrate community is poorly understood, since studies have so far focussed on a few target prey taxa.

In this study, we carried out DNA metabarcoding of gut contents to investigate the direct trophic interactions of *D. villosus* compared to a native amphipod, *Gammarus zaddachi* (Sexton, 1912) from a non-invaded site. A direct comparison of *D. villosus* with native amphipods was not possible at invaded sites as very few native amphipods were recovered from kick samples. We collected environmental DNA (eDNA), i.e. DNA from organisms that can be collected directly from the environment (Taberlet *et al.*, 2012), in parallel to standardised kick-samples to describe the macroinvertebrate communities associated with

both species. Specifically, our objectives were to 1) carry out gut contents molecular analysis of direct trophic interactions of *D. villosus* and native *G. zaddachi* to investigate breadth of trophic interactions, including the proportion of IGP interactions of the invasive amphipod compared to a native amphipod, and 2) to use both eDNA and kick sampling of invertebrate communities in order to describe the available prey resources, and understand the composition of invaded communities. Given the previously reported impacts of *D. villosus* we hypothesised that diet breadth of the invasive amphipod would be wider, and that it would include more IGP interactions than in native amphipods; further, we hypothesised that the community composition would be less diverse and more similar across the invaded sites, in comparison to the control non-invaded sites, and that the invasive amphipod would have been a driver in shaping the communities.

3.2 Methods

a. Sampling strategy

The sampling took place on two days in May and October 2017. Three sites were included in the study: Grafham Water (Cambridgeshire, UK, TL 15333 67995), Wroxham Broad (Norfolk, UK, TG 31064 16710) and Rockland Broad (Norfolk, UK, TG 33238 05158) (Fig. 1), and six locations were selected in each of the sites. Grafham Water (labelled GW) and Wroxham Broad (labelled WB) had recorded populations of *D. villosus* (labelled Dv) respectively since 2010 and 2012. We therefore sampled native *G. zaddachi* (labelled Gz) from Rockland Broad (labelled RB), which has not yet been invaded by *D. villosus,* and is in the same catchment of Wroxham Broad (Fig.1).

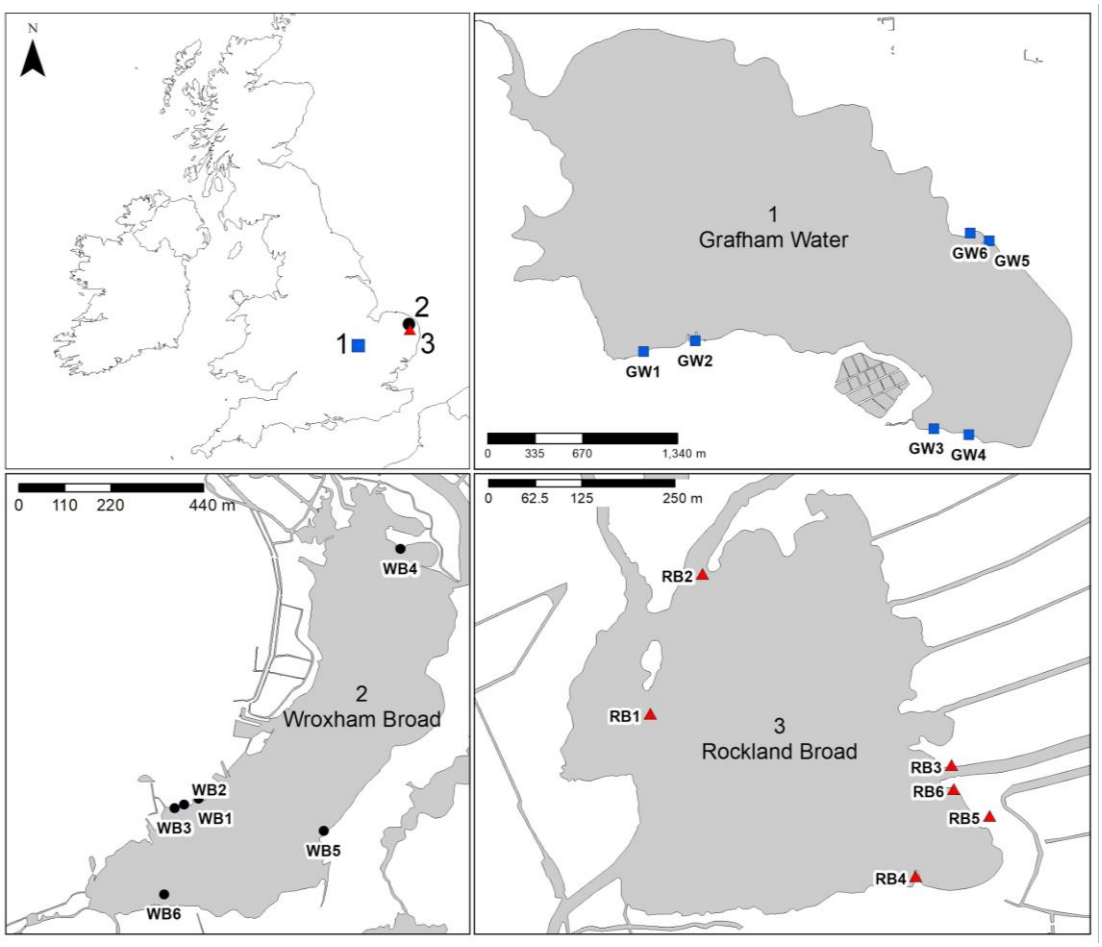


Figure 1. Map of the 3 study lakes with the sampling locations. Grafham Water (GW) and Wroxham Broad (WB) with *D. villosus* (Dv), Rockland Broad (RB) was used as a non-invaded control site to collect native *G. zaddachi* (Gz).

All samples were collected using the following methods in the order: i) water collection for eDNA analysis, ii) kick-sampling for community identification, iii) and collection of amphipod specimens for gut content analysis.

At each site we collected 2 L of water for eDNA analysis in May and October 2017 (N=36). Water samples were collected using a Phil Sampler (Hydro Technologies Inc.) and Pyrex Media bottles (500 mL), which were sterilised in between each lake using 10% v/v commercial bleach solution and 5% v/v Lipsol solution before being rinsed with purified water. Before sampling, each Pyrex bottle was rinsed twice using lake water at the sampling location to remove any possible remaining contamination. An extra 2 L bottle, as blank sample, per lake per season was carried on field (N=3 in May and N=3 in October). These blank samples were filled with milliQ water in the contained facility at the University of Hull and were

treated in parallel to the other samples. Samples were stored on ice and filtered within 24 h from collection. Filtration took place in a dedicated eDNA laboratory at the University of Hull using 0.45 µm cellulose nitrate membrane open filters (Whatman, GE Healthcare) and sterilised Nalgene units (Thermo Fischer Scientific, UK) attached to a vacuum pump. Each water sample required the use of 2 filters which were processed on the same unit. Filters from the same sample were placed dry in sterile petri dishes, sealed with parafilm and stored at -20 ^{o}C until DNA extraction.

Following the eDNA sampling, standardised 3-minute kick-samples were collected from each sampling location (N=36). From these kick-samples, 50 amphipod specimens per lake per sampling event (N=300) were manually picked out for gut contents analysis and immediately stored in sterile 1.5 mL screw-cap tubes with 1 mL of RNAlater. The amphipods in RNAlater were then frozen at -20 ^{o}C upon return to the laboratory. In total 324 amphipods were collected for gut contents analysis (N=175 in May, N=149 in October), of these 11 amphipods were mis-identified during collection and excluded from the analysis; specifically the species were *Corophium multisetosum* (2), *Crangonyx pseudogracilis* (7), and *Gammarus tigrinus* (2). The kick-samples with the communities were placed in Whirl Pak plastic bags (Nasco, USA) and preserved in 100% Ethanol. During the October sampling three environmental parameters (pH, Temperature, and Conductivity) were also collected at each site. The kick-samples collected were identified to the lowest taxonomic level possible. In the case of specimens that were damaged, or species identification was not possible, then the genus and in few cases family level identification were used.

b. Sample preparation

Guts were removed from *D. villosus* or *G. zaddachi* under a stereomicroscope using single-use scalpel blades and petri dishes. Scalpel blades and forceps were sterilised by immersion in 10% v/v commercial bleach for few minutes and rinsed with milliQ water before being used. Scalpel blades were then discarded after each specimen. All other materials (e.g. petri dishes) and work surfaces were wiped with 10% v/v commercial bleach before and after each dissection to further prevent cross-contamination. Each specimen was also rinsed with milliQ water

and briefly dried with blue roll prior to dissection. The dissected material was transferred immediately into sterile 1.5 mL Eppendorf tubes stored on ice and processed for DNA extraction within 2 h after dissection.

DNA extractions followed two separate protocols based on the sample material. The membrane filters for the eDNA samples were processed using MoBio Power Water kit (Qiagen, UK) following manufacturer's protocol. DNA from gut contents was extracted following the Mu-DNA tissue extraction protocol as described by Sellers *et al.* (2018), with the addition of a grinding step with individual sterilised pestels to allow a better digestion by the Proteinase-K (Sellers *et al.*, 2018). The grinding was carried out after the addition of the tissue lysis solution, and directly into the 1.5 mL tubes used for the dissection. The individual plastic pestles used to grind the material were sterilised with 10% v/v commercial bleach, stored in 100% ethanol and exposed to UV light for 3 h. All tissue samples were digested on a Thermo mixer (Fisher) at 56 ^{o}C and 700 rpm for 4 h, until all material was digested.

Two separate sequencing libraries were generated: the first library containing the samples collected in May, the second library containing the samples collected in October. Each 96 well plate contained a minimum of two *Osmia bicornis* (Linnaeus, 1758) genomic DNA positive controls (N = 4 first library, and N = 7 second library), and a minimum of two PCR negative controls (2 µl of molecular grade water were used instead of the template DNA) per plate (N = 6 first library, and N = 10 second library). PCRs targeted a 313 bp region of COI using metazoan universal primers mtCOIintF - jgHCO2198 (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013). The primers were modified to enable a nested-barcode approach for library preparation by adding 20 different barcodes, and 8 different heterogeneity spacers (N = 96 primer combinations) (Kitson *et al.*, 2018) (See Appendix 3.1 for further details). Library preparation followed a two-step protocol, which included an initial PCR with the 96 primers combinations, using 0.4 µM of each Primer, 1x MyFi™ Mix *Taq* polymerase (Bioline, UK), and 2 µl of DNA template in a 25 µl reaction volume. PCR conditions were 95 ^{o}C for 3 mins, followed by 40 cycles of 95 ^{o}C for 15 sec, 53 ^{o}C for 30 sec, 72 ^{o}C for 30 sec, and a final extension step of 72 ^{o}C for 10 mins.

Products from the first PCR were individually purified with Mag-Bind RxnPure Plus (Omega Biotek, US) on a 96 well magnetic stand to remove primer dimers and to isolate the target fragments. The clean-up protocol was modified from the size-selection protocol described by Quail, *et al.* (2009) as described below. The initial ratio was changed to 0.5x of magnetic bead per 20 µl PCR product and allowed to stand for 15 min before transferring the supernatants to new wells. The second ratio was changed to 0.12x of magnetic beads per initial volume of PCR products and allowed to stand for 5 mins to allow target fragments to bind to the beads. The supernatants were then discarded, and the beads washed twice with 200 µl of 80% ethanol. The DNA bound to the beads was ultimately eluted in 15 µl of 10mM Tris-HCl (pH 8) (Quail, Swerdlow and Turner, 2009). After visualisation on 2% agarose gels to confirm successful clean-up, the samples products from the same 96 well plate were pooled together. The individual plates were then uniquely tagged with the Illumina MiSeq adapter primers in a second PCR, which followed the protocol and reaction volumes as described in the "Amplicon, Clean-Up and Index, 2013" (Illumina technologies, online) using MyFi™ Mix *Taq* polymerase (Bioline, UK). Following the second PCR and gel visualisation, the PCR products were cleaned again with magnetic beads as described above and visualised on gel again to confirm the success of the clean-up step.

After the clean-up of the second round PCR products, the individual plates were quantified for dsDNA with a Qubit fluorometer (ThermoFisher Scientific Inc., UK) and pooled together in equimolar amounts, taking into account the number of samples in each plate. The pooled libraries were then quantified with qPCR using the NEBNext Library Quant Kit (New England BioLabs Ltd., UK) and the fragment size was further checked on a TapeStation Automated Electrophoresis (Agilent Technologies Inc., US) using a High Sensitivity D1000 Screen Tape kit. The final libraries were denatured and diluted following Illumina "MiSeq System Denature and Dilute Libraries Guide". Both libraries were sequenced on an Illumina MiSeq using a V2 kit at 250 cycles per reads, loading 15 pM libraries with 10% PhiX.

c. Bioinformatics

Illumina raw sequences (NCBI SRA: PRJNA575167) were processed using the custom pipeline metaBEAT v0.97.10 (https://github.com/HullUni-bioinformatics/metaBEAT). This includes trimming raw Illumina sequences with Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014) using a Phred score of 30 or higher using a 5-base sliding window. Following, the sequences were merged with FLASH v1.2.11 (Magoč and Salzberg, 2011), checked for chimeras and cluster at 97% similarity with VSEARCH v1.1.0 (Rognes *et al.*, 2016). The centroids of the clusters that contained more than 2 sequences were taxonomically assigned using BLASTn v2.2.28+ (Altschul *et al.*, 1990) and the Lowest Common Ancestor (LCA) approach using Taxtastic v0.8.5 (https://github.com/fhcrc/taxtastic). Sequences were assigned with 97% identity match, and a minimum of 80% alignment of the target sequence against the reference sequence. The taxonomic assignment was run against curated databases of macroinvertebrate COI sequences mined from GenBank and EMBL, and curated using SATIVA v0.9 (Kozlov *et al.*, 2016) to highlight errors in the mined records (See Appendix 3.2 for details on the creation of the databases). Sequences that remained unassigned to the reference database were assigned against the NCBI nt database (updated at the end of August 2018), with the same BLASTn and LCA approach. The final output was a table of DNA reads in each sample collapsed by taxonomy.

d. Statistical analysis

Downstream analysis was performed in R v.3.5.1 (R Core Team, 2018). The bioinformatic output containing read counts collapsed by taxonomic ID, were initially quality controlled and filtered to remove samples containing less than 500 reads. Next, we checked for cross contamination present in the positive samples which were expected to contain 100% DNA of *O. bicornis* (Hymenoptera). Low levels of contamination were detected in the positive samples from the first sequencing run, specifically 7 (0.046942%) reads belonging to *D. villosus* and 8 (0.020173%) reads belonging to *Gammarus zaddachi*. Based on these values we decided to apply a 0.03% filter threshold, which we acknowledge might not remove all contamination, but with a higher threshold we were risking of losing potential rare detections. The second run presented higher levels of contamination from *D. villosus* and *Crangonyx floridanus*. In particular, 3 of the 7

positive controls had contamination levels between 600 and 2000 reads, which represented between 40% and 70% of their respective samples. We could not account for this high contamination which came from *D. villosus*, and we had to exclude those samples from analysis. The remaining 4 positive samples had a lower level of contamination from *D. villosus*, Diptera and Cyclopidae. The total number of contaminating reads in this case were 37 (0.05%), 14 (0.28%), 28 (1%) and 4 (0.47%). Again, acknowledging that we could not remove all contamination, we decided for the second sequencing run to apply a threshold of 0.5%. Following these QC steps, all retained read counts were used to create a presence/absence data set (eDNA and gut contents samples).

For the gut contents analysis, the interaction strengths between consumer and potential prey species were quantified based on the total number of links detected in each consumer species at each site and each sampling time. The links were obtained from the presence/absence data of prey DNA in consumer gut contents, and were visualised using the R-package 'bipartite' v2.11 (Dormann, Gruber and Fründ, 2008). Niche breadth and niche overlap of the target amphipod taxa were calculated using the standardised Levin's and Shannon indices (Krebs and Others, 1989; Lyngdoh *et al.*, 2014; McClenaghan *et al.*, 2015), and the Pianka index for similarity from the 'spaa' R-package (Zhang, 2016).
The number of sites occupied for each species (i.e. basic site occupancy) was calculated for the eDNA and kick samples separately, to provide semi-quantitative estimates of abundance in the community samples. We used 'vegan' v2.5 R-package (Oksanen *et al.*, 2018) to analyse the influence from four environmental factors on gut contents (presence/absence) and community data (site occupancy). Specifically, we analysed the datasets with PERMANOVA (N permutations = 9,999) using Euclidean (gut contents) and Jaccard (community) distances with lakes, predator id (gut contents only), sample type (for community only), and season as factors. This was to determine which, if any, of the environmental factors were driving the observed gut contents and community composition; and how much of the observed variance in the data could be explained by these factors. Following, we used non-metric multidimensional scaling (NMDS) to observe potential differences and similarities in how the factors group the data based on species composition. To better describe the community

composition and their differences from the NMDS, we also calculated the species richness from the eDNA and kick samples.

Finally, we used the envfit function from the 'vegan' R-package to investigate whether the community composition (eDNA plus kick samples) from each lake were directly or indirectly related to the environmental variables (pH, conductivity (µS) and Temperature) or abundance of *D. villosus*. To do this we combined data from environmental variables and numbers of *D. villosus* collected from the kick-samples plus the number of *D. villosus* used for gut contents analysis collected in October 2017 at all three locations (Environmental variables measured are reported in Appendix 3.3). To ensure reproducibility all scripts have been deposited (Github: https://github.com/mbenucci/Dv_Gz_MATI).

3.3 Results

Raw sequencing outputs of the two libraries generated respectively 14,008,944 reads passing filter, 92.1% bases above Q30, and an error rate of 0.33% (±0.27) for the first library; and 13,443,878 reads passing filter, 92.2% bases above Q30, and an error rate of 1.31% (±0.31) for the second. These translated into an average raw read depth of 19,356.79 (±1493.18), and 24,176.63 (±1796.22) for the two libraries respectively. By splitting the read depths by sample types (eDNA and gut contents), the first run eDNA samples had a mean (±se) read depth of 9591.5 (±4154.7), and gut contents 22124.5 (±1665.8). The second run eDNA samples had mean read depth of 32317.9 (±5625.5), while gut contents 26271.7 (±2038.9).

Following read depth filtration and contamination QC, 96 (30%) of the remaining amphipods showed presence of DNA associated with potential prey DNA in their guts (N=65 from May, N=31 from October). For the 36 eDNA samples collected (N=18 in May, N=18 in October), 34/36 (94%) were successfully sequenced and passed all filters. The two dropped eDNA samples were both from Rockland Broad (eRB3, May and October, Fig.1). Three of the 36 kick-samples collected had insufficient material in the net (GW5 and GW6 from May, and GW5 from October) and were excluded from downstream analyses.

Molecular analysis of trophic interactions in D. villosus and G. zaddachi

From the 218 *D. villosus* collected across both seasons in Grafham Water and Wroxham Broad (N=104 for Grafham Water, N=114 for Wroxham Broad), the total number of interactions was 31 (14%) and involved 16 different prey taxa (Table 1). We detected 9 unique taxa across 17 individuals (~16% of N=104) from the guts of *D. villosus* collected in Grafham Water, and 10 unique taxa across 14 individuals (~12% of N=114) from *D. villosus* collected in Wroxham Broad (Table 1). The proportion of prey reads in the gut contents of *D. villosus* was very low (mean proportion of prey reads = 1.03% ±0.45). In contrast, a much higher rate of positive detections was found in native *G. zaddachi*, with 18 different prey taxa detected across 65 individuals (~61% of N=107). *G. zaddachi* also had a ten-fold higher mean proportion of gut content prey reads (mean proportion of prey reads = 10.54% ± 1.67).

Table 1. Summary of the detections of prey taxa in the gut contents of *Dikerogammarus villosus* (Dv) and *Gammarus zaddachi* (Gz).

		Gut Contents					
Sample type		*D. villosus*				*G. zaddachi*	
Predator ID		GW		WB		RB	
Site		May	Oct	May	Oct	May	Oct
Time		52	52	67	47	58	49
N shrimps		9	8	12	2	44	21
N shrimps with interactions		17.3%	15.4%	17.9%	4.3%	75.9%	42.9%
% shrimps with interactions		9	8	15	3	86	23
N total interactions							
Cladocera	*Chydorus brevilabris*	0	0	1	0	0	0
	Daphnia sp.	0	0	1	0	0	0
	Polyphemus pediculus	0	0	2	0	0	0
	Sida crystallina	0	0	0	0	0	1
Amphipoda	*Corophium multisetosum*	0	0	0	0	4	0
	Crangonyx floridanus	0	1	0	0	18	10
	Crangonyx pseudogracilis	0	0	0	0	27	7
	Gammarus tigrinus	4	0	6	0	0	0
	Gammarus zaddachi	0	0	1	0	n/a	n/a
Diptera	Chironomidae	0	0	0	0	0	2
	Chironomus luridus	1	0	0	0	0	0
	Chironomus sp.	0	0	0	0	1	0
	Cricotopus sp.	0	0	1	0	0	0
	Cricotopus laricomalis	3	0	0	0	0	0
	Tanytarsus medius	0	0	0	0	0	1
Hydrozoa	*Hydra sp.*	0	0	0	0	7	0
	Hydra oligactis	0	0	0	0	20	0
Mollusca	*Dreissena polymorpha*	1	0	2	0	0	0
	Physella acuta	0	1	0	0	0	0
Oligochaeta	*Bothrioneurum vejdovskyanum*	0	0	1	1	0	1
	Allolobophora chlorotica	0	1	0	0	0	0
	Chaetogaster diastrophus	0	0	0	0	1	0
	Limnodrilus hoffmeisteri	0	1	0	1	0	1
	Stylaria lacustris	0	0	0	0	1	0
	Tubifex tubifex	0	4	0	0	0	0
	Potamothrix moldaviensis	0	0	0	1	0	0

Platyhelminthes	*Stenostomum sthenum*	0	0	0	0	1	0
Rotifera	*Asplanchna sieboldi*	0	0	0	0	2	0
	Euchlanis dilatata	0	0	0	0	3	0
Trichoptera	*Lype phaeopa*	0	0	0	0	1	0

We compared the interactions detected in *D. villosus* and *G. zaddachi* using presence/absence data and detected a wide range of interactions across our study sites, including both extra-guild and intra-guild predation (Table 1 and Fig. 2). For *D. villosus,* most interactions were detected only once, except for *Cricotopus laricomalis* (detected 3 times in Grafham Water), *Tubifex tubifex* (detected 4 times in Grafham Water), and *Polyphemus pediculus* (detected twice in Wroxham Broad). In contrast, eight interactions were found more than once in *G. zaddachi* with some interactions detected in over 20 individuals (e.g. Hydrozoa detected 27 times, *Crangonyx pseudogracilis* 34 times, and *Crangonyx floridanus* 28 times).

IGP interactions (i.e. of other amphipods) were detected in both species, but the total number and proportion of IGP interactions was much higher in *G. zaddachi* (65/109 interactions) than in *D. villosus* (12/35 interactions) despite the fact that half as many *G. zaddachi* individuals were analysed compared to *D. villosus* (N=107 and 218 respectively, Table 1). Specifically, *Gammarus tigrinus, G. zaddachi* and *Crangonyx floridanus* were detected in *D. villosus,* with only *G. tigrinus* detected on more than one occasion, and no IGP interactions were found in both seasons (Table 1, Fig. 2). By contrast, in *G. zaddachi* 28 interactions were detected with *Crangonyx floridanus* (18 in May, 10 in October), 34 with *Crangonyx pseudogracilis* (27 in May, 7 in October) and 4 with *Corophium multisetosum* (May only, Table 1, Fig. 2).

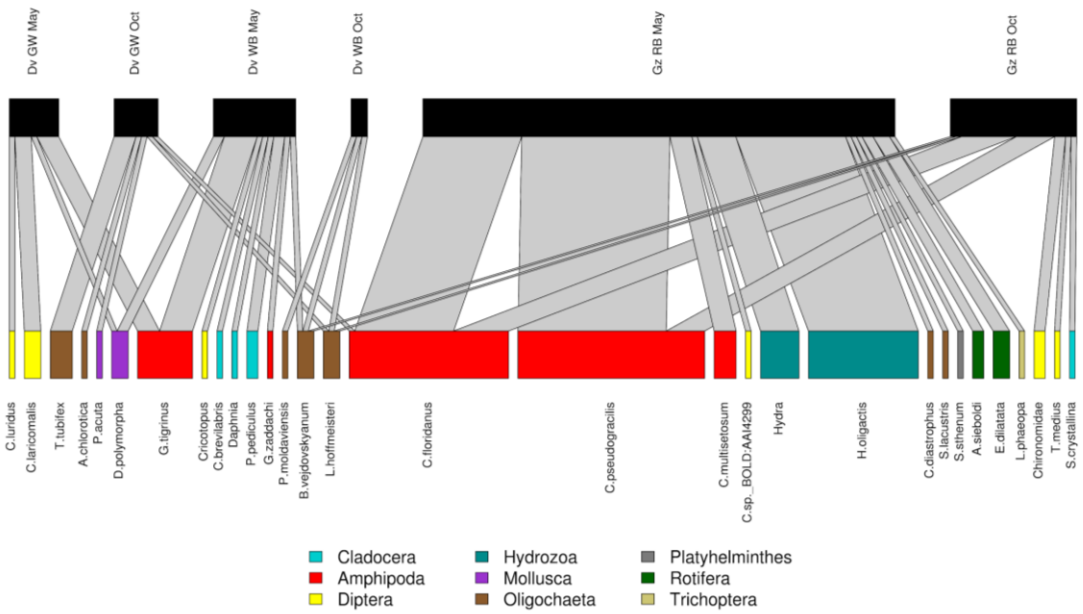


Figure 2. Bipartite network of *D. villosus* (Dv) from Grafham Water (GW), and Wroxham Broad (WB), and from *G. zaddachi (*Gz) from Rockland Broad (RB). IGP interactions are highlighted in red.

The bipartite network (Fig. 2) and NMDS of the gut contents (Fig. 3) from the two focal amphipods illustrate a small overlap in interactions, with both species predating on *C. floridanus*, *C. pseudogracilis*, *B. vejdovskyanum*, and *L. hoffmeisteri*. PERMANOVA analyses indicate that interactions were strongly influenced by both season (ADONIS: Pr(>F) = 0.0002, R^2 = 0.03998), and predator ID (ADONIS: Pr(>F) = 0.0001, R^2 = 0.12631); but not by the location (ADONIS: Pr(>F) = 0.1080, R^2 = 0.01389). Levin's and Shannon indices of niche breadth for both species are shown in Table 2. The values of the Shannon index are very similar for both *D. villosus* and *G. zaddachi* across all three lakes, while the standardised Levin's index appears marginally higher for *D. villosus* than for *G. zaddachi.* Calculating the niche overlap using Pianka index of similarity, we obtained a 53.6% overlap in the resources consumed by *D. villosus* in Grafham Water and Wroxham Broad. The overlap was in comparison much lower for the resources consumed between *D. villosus* in either Grafham Water or Wroxham Broad, and *G. zaddachi* (0.83 and 8.6% respectively).

Table 2. Values of diet breadth from the observed interactions observed in the gut contents of *Dikerogammarus villosus* and *Gammarus zaddachi* across all lakes from both seasons. Values were calculated from presence/absence data across the gut contents from both species.

Predator	Site	Shannon-Wiener	Standardised Levins	Shannon-Wiener	Standardised Levins
D. villosus	Grafham Water	1.986967	0.1514393	2.431874	0.2030361
	Wroxham Broad	2.06207	0.1470588		
G. zaddachi	Rockland Broad	1.988229	0.1169214	1.988229	0.1169214

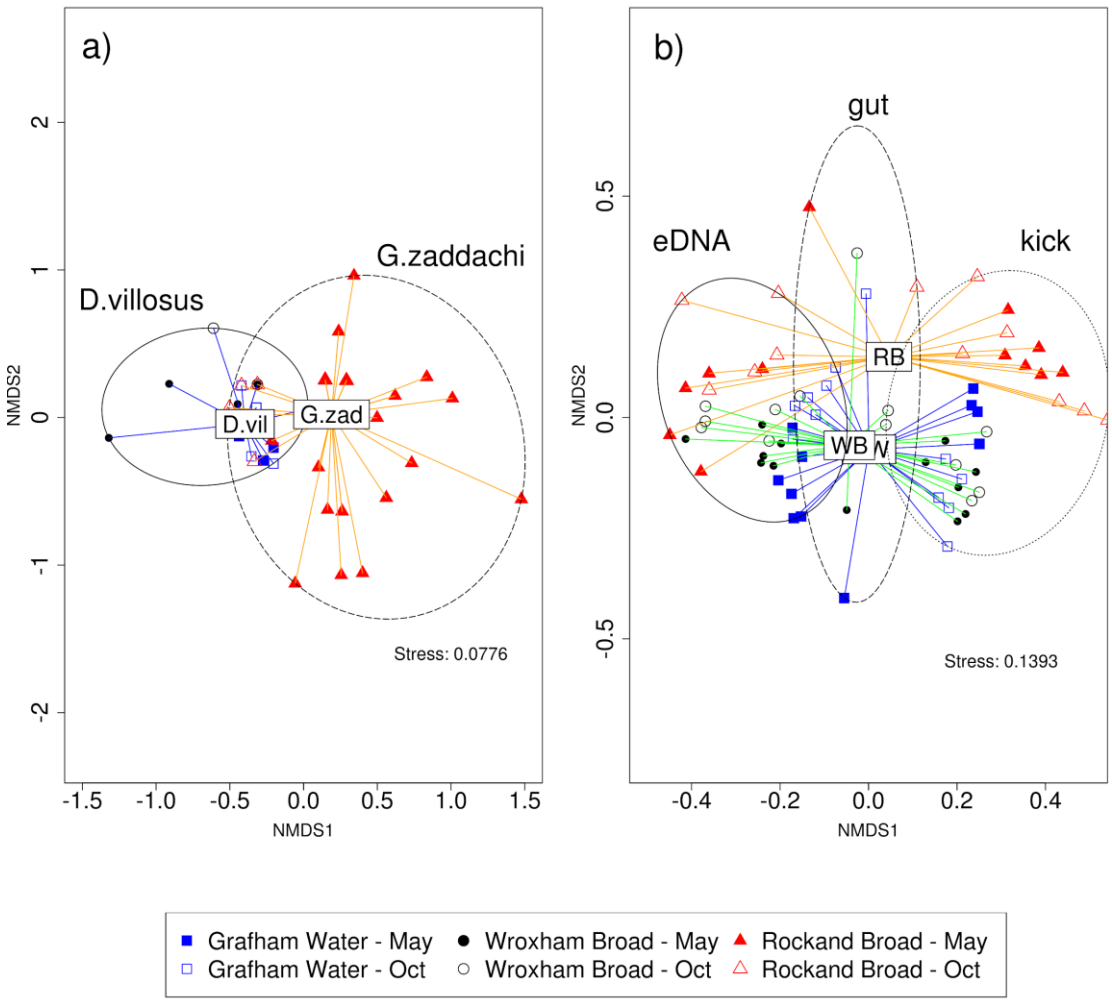


Figure 3. a) NMDS using Euclidean distance of gut contents of *D.villosus*, against native *G. zaddachi*. b) NMDS using Jaccard distance of gut contents of both amphipods against community from eDNA and kick samples.

We collated the community information (eDNA plus kick sample) and the target amphipods gut contents data to understand how much of the available resources the target amphipods were using. The PERMANOVA results using the Jaccard distance show a strong influence from all three factors: sample type ($Pr(>F)=0.0001$, $R^2=0.21179$), site ($Pr(>F)=0.0001$, $R^2=0.09698$), and time ($Pr(>F)=0.0001$, $R^2=0.03814$).

The NMDS shows a strong overlap between the three different sample types with the gut contents central and equidistant to the communities (eDNA and kick samples) (Fig. 3b). All three lakes, described by both communities and gut contents, show a pattern similar to that identified from the community analysis

alone; with Grafham Water and Wroxham Broad (the two invaded lakes) showing a higher similarity, while Rockland Broad (control lake) well separated along the second axis (Fig. 3b).

Community composition in invaded and non-invaded sites

Using both eDNA and kick sampling we detected respectively 88 and 84 unique taxa in the communities. Specifically, with eDNA we detected 55 unique taxa in Grafham Water, 41 in Wroxham Broad and 33 in Rockland Broad; while in the kick samples 37, 43 and 55 respectively (See Appendix 3.5 for the complete table). Across the two seasons, the species richness values we quite similar across all 3 lakes (Table 3).

Table 3. Summary of the Species Richness values in the communities from eDNA and kick-samples.

	Species Richness	
Site	eDNA	kick-samples
GW May	12.667 (±4.393)	9.5 (±2.179)
GW Oct	10.833 (±0.792)	9.8 (±1.463)
WB May	8.333 (±2.028)	7.0 (±1.125)
WB Oct	9.167 (±2.151)	8.333 (±1.801)
RB May	7.8 (±2.035)	12.333 (±0.919)
RB Oct	8.0 (±1.304)	9.0 (±0.775)

We identified important differences in the community composition between sample types and between lakes (Fig. 3 and 4). Our eDNA assay captures mostly micro- and meso-invertebrates taxa such as Cladocera, Cyclopoida, Hydrozoa, Oligochaeta and Rotifera, and only to a minor extent macro-invertebrates like Diptera and Amphipoda. The kick samples instead appear to have captured more of the macro-invertebrates in the community including Zygoptera, Hemiptera, Coleoptera, Diptera and Amphipoda. (Fig. 4a).

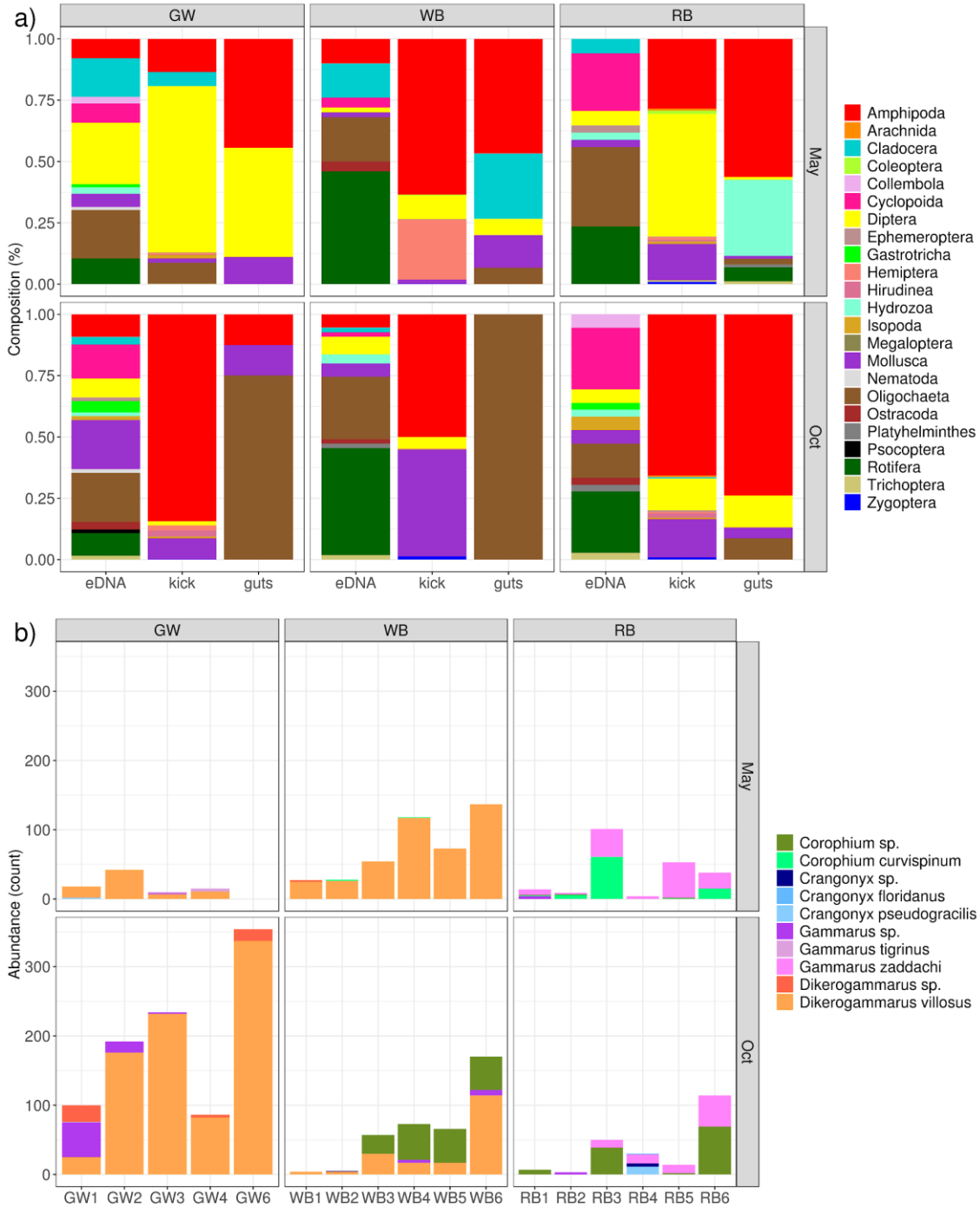


Figure 4. a) Community composition and gut contents composition across the three sampling sites and both seasons. Taxa composition was calculated using species site occupancy. The only Amphipoda detected with eDNA was *D. villosus* in GW and WB. b) Amphipoda abundance collected from the kick-samples across the sites and the two seasons. GW5 (May and Oct), and GW6 (May) missing from dataset. Taxa identified to genus level included damaged specimens.

Focussing on the community composition of intraguild predators (i.e. amphipods), we found that in the invaded sites (GW and WB), *D. villosus* was by far the dominant species particularly in Grafham Water in October and Wroxham Broad in May (Figure 4b). *Gammarus sp.* (including *G. tigrinus)* were detected at generally low frequency with the exception of site GW1 in October; and *Corophium sp.* which were detected in high abundance in WB in October (Fig 4b). In Rockland Broad (RB), *Gammarus zaddachi* and *Corophium sp.* (including *C. curvispinum*) were present in similar numbers, with *Crangonyx sp.* (including *C. floridanus* and *C. pseudogracilis*) also detected in small numbers (Fig 4b). The eDNA samples provided little information regarding the amphipods composition, with the only Amphipoda detected being *D. villosus* in GW and WB, while no Amphipoda at all were detected in RB in either sampling time (Fig. 4a).

The results of PERMANOVA on the eDNA showed the communities across the 3 lakes were significantly influenced by both the location and the season (season: Pr(>F) = 0.0001, R^2 = 0.09878; site: Pr(>F) = 0.0001, R^2 = 0.27794); with the NMDS showing slightly more overlap between the two geographically closer lakes (WB and RB)(Fig.5a). A similar influence of location and season was found on the kick sample community compositions (PERMANOVA by season: Pr(>F) = 0.0001, R^2 = 0.07245; by location: Pr(>F) = 0.0001, R^2 = 0.28602). However, in this case the NMDS shows a greater overlap between the two invaded lakes (WB and GW) than between WB and RB (Fig.5b). We also detected a significant influence of the sampling methods on the community composition (PERMANOVA sample type: Pr(>F) = 0.0001, R^2 = 0.12604), with a significant pattern also across seasons and locations (season: Pr(>F) = 0.0001, R^2 = 0.03638; site: Pr(>F) = 0.0001, R^2 = 0.08547, Fig 5c). The corresponding NMDS demonstrates greater overlap between the two invaded lakes (WB and GW) compared to RB (Fig.5c).

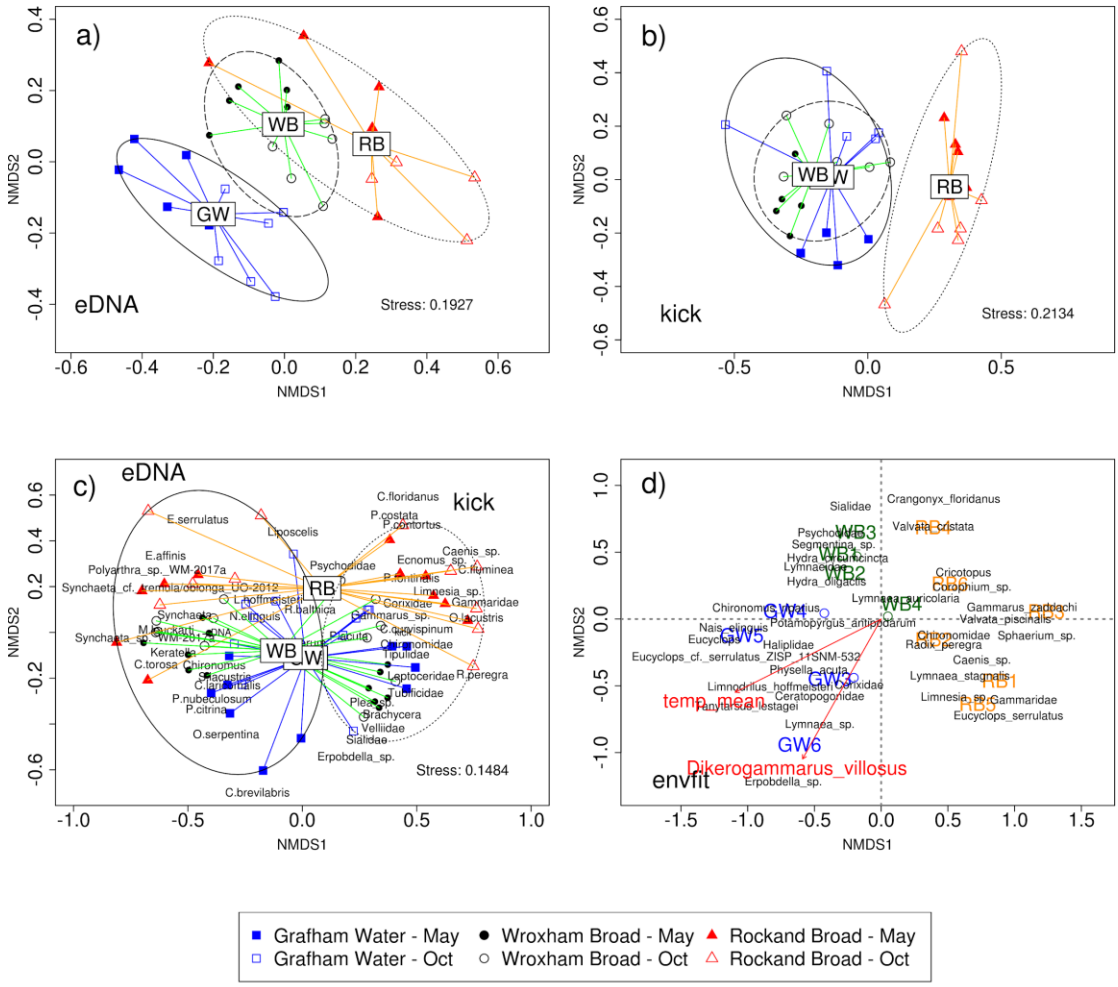


Figure 5. a) NMDS of eDNA samples using Jaccard distance. b) NMDS of kick-samples using Jaccard distance. c) NMDS using Jaccard distance comparing kick-samples and eDNA communities composition. d) ordination plot fit with environmental variables (envfit) using October communities (eDNA and kick-samples combined), only significant variables are shown (P-value < 0.01). GW corresponds to Grafham Water, WB to Wroxham Broad, and RB corresponds to Rockland Broad (control site). Solid symbols correspond to May, hollow to October.

The ordination analysis (envfit) performed to investigate the influence of environmental variables and abundance of *D. villosus* on community composition, demonstrates that communities cluster by lake (Fig. 5d). We detected a significant influence of temperature (Pr(>r) = 0.0009, R^2=0.6174), and

abundance of *D. villosus* (Pr(>r)=0.0016, R^2=0.5950); while pH and Conductivity were not significant (pH: Pr(>r)=0.14 R^2=0.2506; Conductivity: Pr(>r)=0.7874, R^2=0.0377). Only the community composition from Grafham Water sites (GW1-6) appear to follow the gradient of the two significant variables (Temperature and *D. villosus*), this is in contrast with the two Norfolk Broads (Wroxham Broad and Rockland Broad); especially for Wroxham Broad (invaded site) which was expected to follow the gradient of *D. villosus*.

3.4 Discussion

We investigated the molecular trophic interactions of invasive *Dikerogammarus villosus* in two UK lakes across two seasons and compared interactions to those of the native amphipod *Gammarus zaddachi* at a non-invaded site. In contrast to our hypothesis, we found that the number of interactions detected for *D. villosus* was low compared to *G. zaddachi*, and the level of intra guild predation was also considerably lower.

Trophic interactions and IGP in D. villosus and G. zaddachi

We detected trophic interactions in less than a fifth of *D. villosus* individuals out of the total collected from Wroxham and Grafham respectively. The main prey taxa we detected for *D. villosus* were Amphipods (including *Gammarus* and *Crangonyx)*, Diptera (Chironomids), Cladocerans, Oligochaetes, and Molluscs (including invasive *Dreissena polymorpha*); and only a third of these interactions detected were with with intraguild prey (i.e. 34.3% of total interactions). By contrast we detected interactions in more than half of the *G. zaddachi* individuals collected from Rockland Broad; with intraguild interactions with other Amphipoda that were by far the largest fraction of prey items in *G. zaddachi* with more than half of the interactions detected in total (i.e. 61% of all interactions). Of these, *Crangonyx pseudogracilis* and *C. floridanus* were the most dominant prey items. We also detected Hydra in a quarter of *G. zaddachi* individuals, while Rotifera, Diptera (Chironomidae), Molluscs, Oligochaetes, Platyhelminthes and Trichoptera were detected in a small number of individuals (3-5%).

This comparison in IGP levels, and overall interactions is striking, given that we hypothesized higher levels of IGP in invasive *D. villosus* compared to native *G.*

zaddachi. We can rule out methodological issues such as low prey detection rates introduced by our metabarcoding assay, since this type of issue would have generated similar low detections in *G. zaddachi* as well. The results for *D. villosus* are therefore more likely to accurately reflect the species ecology rather than any failures in our experimental pipeline. Interestingly, low rates of prey detection in *D. villosus* are in agreement with previous studies which used species-specific DNA assays (Koester, Bayer and Gergs, 2016). For example, Koester *et al.* (2016) found prey DNA in only 33 of 206 individuals (16%) with frequently detected prey that included Chironomidae, Gastropoda, and Mysidae, while only 8 (3.9%) of those interactions were IG interactions with other Amphipods; also the stable isotopes signal detected from *D. villosus* was lower than what was expected from a top predator which further supported those low detection rates (Koester, Bayer and Gergs, 2016). Taken together, these results suggest that *D. villosus* may not be as voracious an intra guild predator as is often assumed from mesocosm experiments and from indirect field based studies (Dick, Platvoet and Kelly, 2002).

Our low levels of IGP may reflect the fact that *D. villosus* is a particularly effective intraguild predator and competitor, to the extent that potential intraguild prey have been effectively extirpated at our field sites. *D. villosus* have been present in the sites included in this study for 7-9 years and are well established (MacNeil *et al.*, 2010) so it is plausible that interactions of *D. villosus* have changed during the invasion stages, from predation (including intraguild) in the early stages of the invasion, to detritivory and cannibalism (this latter that we cannot currently be detected with DNA metabarcoding) in well-established populations which would explain the low abundance of other amphipods and other species in the eDNA and kick samples in invaded sites (Bacela-Spychalska and Van Der Velde, 2013; Hellmann *et al.*, 2016; Koester *et al.*, 2018).

An investigation of IGP by *D. villosus* along an invasion transect, from the invasion core to the invasion front, and along a temporal transect is warranted to investigate this further. Also in consideration of the known flexible omnivory of this invasive amphipod, the dietary changes in *D. villosus*, different from predation and that might be at play as for example detritivory (MacNeil *et al.*, 2011), will require further investigation to understand their source. Regarding the high IGP interactions found in the native amphipod, Wang *et al*. (2019) reported how IGP interactions can promote biodiversity by releasing pressure on resources from

consumers and thus potentially opening up new niches (Wang, Brose and Gravel, 2019). This might explain the reason of the differences detected in our target species, and how this process could play a more important role for the functioning and stability of ecological communities than we originally thought.

Improving our understanding of the proportion of omnivory, cannibalism, predation and phytoplanktivory that constitute the diet of amphipods might help in grasping a better picture of the interactions of these species with their communities, whether in relation to biological invasions or not. This might require using a larger temporal resolution, accounting also for potential dietary changes during the different life-stages. Similarly, IGP interactions should also be further investigated to verify their role in community composition and in community networks, both in invaded and non-invaded sites (Aebi *et al.*, 2011; MacNeil *et al.*, 2011).

Dietary overlap between D. villosus and G. zaddachi

We detected significant differences in the niche overlap index (Pianka's index) between the two target amphipods across our sites, which is in agreement with the potential omnivory of both species which should indicate that they broadly use the resources available to them in the individual sites (Macneil, Dick and Elwood, 1999; Platvoet *et al.*, 2009). We detected a low similarity (<9%) in the niche overlap index between *D. villosus* from either invaded lake with *G. zaddachi*, while we detected instead a high overlap among *D. villosus* (~56%). These results appear reflected also in the distance-based ordination analysis (NMDS) comparing the gut contents, and in the distance ordination of gut contents, eDNA and kick samples together. The similarities detected in the *D. villosus* gut contents analysis, appears to follow the same distance obtained from the community composition detected across our study sites during both seasons; although these results need to be put in the perspective of being representative of a small percentage of the individuals collected. In consideration with the lack of agreement linking *D. villosus* invasion and the modifications it has on invaded benthic communities (Hellmann *et al.*, 2016; Koester *et al.*, 2018), we cannot fully explore the actual direction of influence; whether the community is influencing *D. villosus* diet, whether the opposite, or whether other processes are happening.

Community differences in invaded and non-invaded sites

Regarding the communities associated with both amphipods, we detected few differences related to whether the sampling was carried out using molecular or standard sampling methods. The results we obtained indicate the communities across the two invaded lakes appear to be more similar than the control site. We detected two main environmental parameters, and only the benthic communities collected in Grafham Water were following the increasing gradient of those two environmental parameters. The integration of both sampling methods increases the overall picture we obtained about the available resources for the amphipods to predate upon. In our study, this provided us with a more comprehensive understanding of the communities available across the three lakes by describing micro- and meso-community using eDNA, and a more macro-community using the kick sampling.

In addition, the ordination analysis shows that only the benthic communities from Grafham Water (GW) were found along the environmental gradients of Temperature and *D. villosus* abundances; the other two sites, Wroxham Broad (WB) and Rockland Broad (RB), don't seems to be arranged along the gradients from either environmental variables. We acknowledge that, in agreement with Hellmann *et al.* (2016), we have demonstrated a partial view in this since one season is not a suitable time frame for accurately assessing the influence of these parameters on the communities, and that a much longer temporal transect would be necessary (Hellmann *et al.*, 2016).

Strengths and limitations of the methods

We recognise that previous studies demonstrated that there could be a correlation between bulk-samples and eDNA (Elbrecht *et al.*, 2017; Elbrecht, Peinert and Leese, 2017). However, we used morphology and abundance data from kick samples directly, without processing for DNA metabarcoding due to limitations in our consumables, so we cannot fully compare our results with those studies. In our case eDNA collected with a Phil-sampler appeared to detect DNA from a wider range of taxa including Oligochaeta, Hydrozoa and more mobile taxa like Cladocera and Cyclopoida that tend to be associated with the water column; while the kick sampling appeared to provide a picture more involving macro-invertebrates like Zygoptera, and Hemiptera. Regarding the gut contents, the

molecular method allowed us to describe trophic interactions without restriction towards only known preys (Koester, Claßen and Gergs, 2013).

Overall, DNA metabarcoding allows for a more comprehensive analysis of trophic interactions and communities than conventional kick sampling. However, the prey to predator DNA ratio when using metazoan universal primers is greatly biased towards the predator DNA, which dominates each sample. In the cases where prey and predator can be amplified by the same primer assay, the use of blocking primers (Vestheim and Jarman, 2008), might be considered to increase the prey signal from gut contents. Blocking primers might not be a “silver bullet” in solving the issue, and may introduce further issues at the development stage, such as trying to find a species-specific region overlapping with a universal region (Vestheim and Jarman, 2008), but they could help improve the DNA ratio in each sample by reducing the predator DNA. Ultimately however, despite the low rate of prey DNA we detected, we still managed to generate enough information from the HTS to detect trophic interactions across both species.

3.5 Conclusions

Here we demonstrated the use of broad-spectrum molecular methods, such as DNA metabarcoding, in conjunction with thorough community information, to detect direct trophic interactions in two species of amphipods, an invasive and a native species in the UK across two seasons.

We detected different levels of trophic interactions created by these two species, which appears in agreement with the ecology and omnivory expected from these amphipods, that would be expected to follow the resources found in each site. However, our data show that, despite the similarities in the diet breadth of the target amphipods, their way of using the communities available to them had important differences. As mentioned, we suspect multiple processes are shaping the diet of the invasive *D. villosus*, therefore field studies aiming at describing the contribution of predation, detritivory and phytoplanktivory in the same populations are needed. We showed on a spatial and temporal scale the overlap in niche of two populations of *D. villosus*, while keeping into consideration the communities from which either population was collected from. We attempted to highlight the importance of IGP in the diet of omnivorous high priority INNS. Our results contradicted our initial hypothesis that IGP was higher in the invasive amphipod

rather than the native amphipod, and instead we detected the opposite trend. The importance of IGP in both invaded and non-invaded communities is still not fully explored, and we might suspect, in agreement with current literature, that this interaction plays an important role in shaping species communities.

In conclusion we have brought direct evidence of prey-predator interactions that *Dikerogammarus villosus* creates in field conditions in two long-term invaded sites using DNA metabarcoding. Equally, we confirmed that predator-prey interactions in field conditions, particularly IGP, can also more frequently form a relatively large proportions of the interactions in the UK native *Gammarus zaddachi*.

Chapter 4 - Seasonal and spatial patterns in molecular trophic interactions of *Harmonia axyridis* (Pallas, 1773), in the UK.

Abstract

Detecting species interactions has interested ecologists since the early 1900s; however, it has often proved challenging, and a number of methods have been developed in an attempt to describe interactions in the wild. Currently the development of High-Throughput Sequencing is enabling ecologists in using DNA metabarcoding to describe species interactions. Although the current opportunities offered by DNA metabarcoding, one of the technical issues arises from the use of “universal” PCR primers which amplify the same DNA region across a wide range of taxa. This property offers a great advantage in molecular trophic interactions, but it can also represent a limiting factor by often having the predator DNA to be completely overrepresented in the amplicon pool. Reducing the overrepresentation of consumer DNA in invertebrate-invertebrate trophic interactions is not easily solved, and a few studies have addressed the problem with the development of consumer-specific blocking primers, which rely on the principle of binding target DNA and inhibiting amplification. Preliminary description of the diet of *H. axyridis* has been recently done using targeted molecular approach, with an emphasis on detection of IGP; however little is known about the more general diet of *H. axyridis* in field conditions and the relative amount of IGP versus non IG diet over seasons and across the invasive range. In this study, we firstly investigated the use of blocking primers for recovering a greater proportion of prey to predator reads in *H. axyridis,* and then used DNA metabarcoding to investigate the diet of *H. axyridis* across two seasons in the UK, focussing our sampling on the invasion core (Oxford) and closer to the invasion front (Yorkshire). Here we demonstrated that blocking primers improved the rate of detection and proportion of prey reads, but also increased the variance in predator reads in our DNA metabarcoding experiment. In field trials, we found no evidence of IGP by *H. axyridis,* but we detected aphids and barklice at high proportions in the ladybirds gut contents. The results mirrored the seasonal availability of these prey in the respective communities. With minor improvements to the method and a finer scale temporal and spatial resolution, DNA metabarcoding could provide additional, important insights into the trophic ecology of *H. axyridis* and other invasive invertebrates.

4.1 Introduction

Molecular trophic interactions

Understanding how species interact has interested ecologists from different perspectives since the early 1900s (e.g. predator-prey relationships, energy flow, network stability, network modelling) (Layman *et al.*, 2015). Particularly relevant is the focus on how, and to what extent, species interactions can influence the structure and composition of the wider community, which in turn defines the stability of the wider ecosystem, its functioning and health (Post *et al.*, 2008; Zou *et al.*, 2016). Detecting species interactions has however often proved challenging, and a number of methods have been developed in an attempt to describe interactions in the wild (Sheppard and Harwood, 2005). These include direct observations of the interactions, both in the form of eye-witness accounts, e.g. bees visiting flowers on fruit trees (Free, 1960), or through inferring the interaction *a posteriori*, which entails detecting the interaction after it has happened. *A posteriori* approaches include analysis of fragments and materials in gut contents, stomach regurgitates, or faeces (McIntosh, Page and Goldsworthy, 2006; Klimaszewski *et al.*, 2013). The methods used for the *a posteriori* approach included monoclonal antibodies, which allows for mass-screening of predators, as exemplified by the study on Linyphiidae spiders diet (Harwood *et al.*, 2007); and since the 1990s the use of stable isotopes, which captures the trophic role of species based on the carbon and nitrogen isotope ratios of the diet from the last few months (Rothhaupt, Hanselmann and Yohannes, 2014; Koester, Bayer and Gergs, 2016).

More recently, the development of High-Throughput Sequencing (hereafter HTS), which allows the sequencing of millions of DNA sequences (Deiner *et al.*, 2017), has dramatically modified the way ecologists are monitoring and investigating ecosystems and their processes (Baird and Hajibabaei, 2012; Lawson Handley, 2015; Deiner *et al.*, 2017; Ruppert, Kline and Rahman, 2019). In particular, DNA metabarcoding, a technique that allows for DNA barcoding of whole communities by simultaneously sequencing a targeted region of DNA, is increasing our ability to investigate whole communities to a greater depth and with a reduced cost per sample (Creer *et al.*, 2016). Most work in this emerging field has focused on

aquatic ecosystems (Hänfling *et al.*, 2016; Gorički *et al.*, 2017; Lobo *et al.*, 2017; Coble *et al.*, 2019), although DNA metabarcoding has been applied in terrestrial systems, and also aerial monitoring (Creer *et al.*, 2016; Deiner *et al.*, 2017). An advantage of DNA metabarcoding is its use to describe communities from the bulk tissue samples (Elbrecht and Leese, 2017), and to describe communities from their eDNA released in the environment (Deiner *et al.*, 2017). Examples of the applications of this technology span across a wide range of ecological questions; from species detection as in the case of threatened or invasive species (Biggs *et al.*, 2015/3; Dejean *et al.*, 2012), species distribution and quality assessment (Hänfling *et al.*, 2016; Coble *et al.*, 2019; Li *et al.*, 2019), population genetics (Tringe and Rubin, 2005; Hooper *et al.*, 2019), and trophic interactions (Kitson *et al.*, 2018; Littlefair *et al.*, 2018; Sint *et al.*, 2019). This latter field, in particular, is witnessing a major benefit from DNA metabarcoding which is enabling to go beyond the description of the composition of a community, and into the investigation on how the species within a community are interacting (Clare, 2014).

As the study of trophic interactions moves from stable isotopes analysis to DNA-based methods, which include species-specific assays and DNA metabarcoding, the investigation of diet breadth, niche overlap and use of resources took a stronger network perspective (Pompanon *et al.*, 2012; Roy and Lawson Handley, 2012; Evans *et al.*, 2016; Zarzoso-Lacoste *et al.*, 2016). The basis of this increased perspective is that although DNA-based methods cannot provide the same temporal resolution obtained from stable isotopes (Carreon-Martinez and Heath, 2010; Alberdi *et al.*, 2018), they can allow mass-screening of predator species, thus reducing the costs per sample. Species-specific assays and DNA metabarcoding also differs in the information that can be obtained and in their applications. Both these methods are applicable to detect prey species in gut contents analysis, however while species-specific assays provide a presence/absence result and require knowing *a priori* the species to target (Rondoni *et al.*, 2015), DNA metabarcoding allows the analysis of species diet and niche breadth with an information on species composition, but also without knowing the potential prey species *a priori* (Clare, 2014; Evans *et al.*, 2016; Porter and Hajibabaei, 2018). Furthermore, DNA metabarcoding is highly sensitive and can provide a high taxonomic resolution of the prey identity to species level (Wirta

et al., 2015). Applications of DNA metabarcoding to trophic interactions can already be found across a broad range of systems: e.g., herbivory in both marine and terrestrial species (Leal *et al.*, 2014; Pansu *et al.*, 2018), parasite/parasitoid-host interactions (Kitson *et al.*, 2018), predator-prey interactions in vertebrates like bats, carnivorous plants, and invertebrates alike (Clare *et al.*, 2014; Wirta *et al.*, 2015; Littlefair *et al.*, 2018).

Molecular trophic interactions applied to Invasive non-native species

Applying DNA metabarcoding to investigate trophic interactions in biological invasions has the potential to be a game changing method to assess the impact of Invasive Non-Native Species (INNS) (Schönrogge and Crawley, 2000; Blanchet, 2012; Furlong, 2015; Lawson Handley, 2015). Because INNS are a driver of biodiversity loss (Hulme, 2009), understanding how they interact with the hosting communities and their impacts on resident and native species is a priority in ecology (Booy *et al.*, 2017). Several studies have successfully used DNA-based methods to investigate molecular trophic interactions of INNS, but mostly with single-target PCR assays (Staudacher *et al.*, 2011; Koester, Claßen and Gergs, 2013; Thomas *et al.*, 2013). The use of DNA metabarcoding instead appears to potentially be able to improve our understanding of the trophic interactions of INNS on a large scale. For example Harms-Tuohy *et al.* (2016) demonstrated the direct impacts from the invasive lionfish (*Pterois volitans*) on the native marine communities in Puerto Rico using DNA metabarcoding (Harms-Tuohy, Schizas and Appeldoorn, 2016). Therefore the use of DNA metabarcoding to study these trophic interactions by INNS, in particular those with generalist or unknown diet, has great potential (De Barba *et al.*, 2014).

DNA metabarcoding is offering many advantages and new opportunities for discovering species interactions, although it also introduces its own set of challenges, both technically in the amplification of the diet targets (Clare, 2014), and analytically in the interpretation of DNA results (Deagle *et al.*, 2018). One of the technical issues arises from the use of so-called “universal” PCR primers in metabarcoding which amplify the same DNA region across a wide range of taxa. This property offers a great advantage in molecular trophic interactions for accurately describing the niche breadth of a species; but it can also represent a

limiting factor if the amplification range of the primers includes the predator/consumer, in which case the predator DNA can become completely overrepresented in the amplicon pool (Vestheim and Jarman, 2008; Piñol *et al.*, 2015). Such an overrepresentation is common when predator and prey are in the same taxonomic group, as in the case of invertebrate-invertebrate predation (Pinol *et al.*, 2014; Piñol *et al.*, 2015). Reducing the overrepresentation of consumer DNA in invertebrate-invertebrate trophic interactions is not easily solved and can represent a major challenge in molecular trophic interactions, and a few studies have addressed the problem with the development of consumer-specific blocking primers (Vestheim and Jarman, 2008; Leray, Agudelo, *et al.*, 2013; Su *et al.*, 2018). Blocking primers, which rely on the principle of binding target DNA and inhibiting amplification, have been successfully applied by Vestheim and Jarman (2008) who were able to completely remove the consumer DNA (Australian Krill) from their amplicon pools (Vestheim and Jarman, 2008). The benefits of reducing predator DNA lies in the ability to better describe the diet and the identity of consumed species, in particular by detecting rare interactions that might otherwise remain hidden (Leray, Agudelo, *et al.*, 2013). Although agreeing with the usefulness of blocking primers, Piñol *et al.* (2014) manage to describe Linyphiid spiders diet using the 3% of prey DNA sequences they obtained from High-Throughput Sequencing without using blocking primers (Piñol *et al.*, 2014). The methodological drawbacks of blocking primers are not limited to only the accidental co-blocking, but also in the number of mismatches that can be present between the blocking primers and the universal primers (Piñol *et al.*, 2015).

Despite these challenges, DNA metabarcoding can potentially provide important insights into the impact of invasive species, particularly when combined with an ecological network approach (Roy and Lawson Handley, 2012). The use of the ecological network perspective with DNA metabarcoding targeting INNS can improve our understanding of the processes underlying the success or failure of invasion, and what changes are induced by the introduction of a novel species across the whole network (e.g. parasites, parasitoids, herbivory, etc...) (Roy and Lawson Handley, 2012; Hohenadler *et al.*, 2019).

Ecology of Harmonia axyridis and its impacts

Predatory ladybirds (Coleoptera: Coccinellidae) are an important group of predators in the aphidophagous community (Sloggett and Majerus, 2000). Coccinellidae are known to have developed a varied diet breadth, feeding on plant material, insects and in some instances on mould and mildew (Sloggett and Majerus, 2000). The Coccinellini tribe mainly include predatory species, with a diet composed mostly by aphids and scale insects, while few species are more generalist whose diet include a wider range of preys (Sloggett and Majerus, 2000; Brown and Roy, 2018). For example, in the UK, the majority of generalist Coccinellini feed predominantly on aphids and scale insects, although mites, adelgids, barkflies and other groups are also preyed upon (Sloggett, 2008). These ladybirds are also important intraguild predators, and readily consume eggs, larvae and pupae of other coccinellids, syrphids and chrysopids (Gagnon *et al.*, 2011; Katsanis *et al.*, 2013; Ingels *et al.*, 2015). Intraguild predation (IGP) is described as the predatory interaction between two competing species that share similar resources (Polis, Myers and Holt, 1989). IGP occurs naturally across communities (Hall, 2011), and is relevant in shaping communities and trophic networks (Wang, Brose and Gravel, 2019). It is also thought to be important in invasion success, providing opportunity for IG predators to remove their competitors, and a key factor in the success of the invasive harlequin ladybird, *Harmonia axyridis* (Ware, Yguel and Majerus, 2009; Brown, Frost, *et al.*, 2011). However the role of IGP in the impact of *H. axyridis* has not been universally supported, with some studies finding little evidence for IGP in the field (Smith and Gardiner, 2013).

Harmonia axyridis (Pallas, 1773) is a globally distributed invasive ladybird (Brown and Roy, 2018) originally from Eastern Asia and Japan, with a diet consisting mainly of aphids and scale insects, which are known global agricultural pests (Morales-Hojas, 2017), although it feeds also on honeydew, adelgids and larvae of other insects including IG prey such as other coccinellids (Koch *et al.*, 2003; Pell *et al.*, 2008; Brown, Frost, *et al.*, 2011; Brown, Thomas, *et al.*, 2011; Roy *et al.*, 2016). Because of its diet preference for aphids and other insect pests, *H. axyridis* has been widely used as a biocontrol agent in agriculture across the globe (Koch, 2003; Brown, Thomas, *et al.*, 2011; Camacho-Cervantes, Ortega-Iturriaga and Del-Val, 2017), with first recorded introductions in North America in 1916 where it took approximately half a century and suspected multiple releases

before viable populations were recorded in the wild in late 1980s (Koch, 2003; Brown, Thomas, *et al.*, 2011; Roy *et al.*, 2016). From the late 1960s introductions also took place in Eastern Europe, starting from the 1980s and throughout all of the 1990s the same took place in Western Europe, North and South Africa (Brown *et al.*, 2007), and from the 2000s *H. axyridis* was detected in South America (Grez *et al.*, 2016, 2017). The UK witnessed it first record of *H. axyridis* in 2004 (Majerus, Strawson and Roy, 2006), and thanks to a long term monitoring programme (Brown, Frost, *et al.*, 2011; Brown and Roy, 2018) there are good records of its dispersal both westwards, occupying most of Wales, and northwards reaching as far north as Yorkshire and Cumbria (Roy and Brown, 2015; Brown *et al.*, 2018). UK populations of *H. axyridis* are generally bivoltine (Koch, 2003; Pell *et al.*, 2008; Honek *et al.*, 2014), while native UK ladybirds species and main competitors (such as *Adalia bipunctata* and *Coccinella septempunctata*) are univoltine (Brown, Frost, *et al.*, 2011). This difference in reproductive strategies is considered as one of the elements contributing to the success of *H. axyridis* invasion in the UK (Brown, Frost, *et al.*, 2011). Another important element contributing to *H. axyridis* success in the UK is the lack of its natural enemies (Ceryngier *et al.*, 2018), and although there are records of parasites and parasitoids now targeting the invader, their contribution in limiting *H. axyridis* populations is still not fully explored.

A number of recent studies have investigated the diet of *H. axyridis* using a targeted molecular approach, with an emphasis on detection of IGP. For example, using prey-specific PCR assays, evidence of IGP towards *Adalia decempunctata, A. bipunctata,* and *Episyrphus balteatus* has been detected in *H. axyridis* collected from several European countries at rates of 2.8-9.6% (Thomas *et al.*, 2013; Brown *et al.*, 2015; Rondoni *et al.*, 2015). However little is known about the more general diet of *H. axyridis* in field conditions and the relative amount of IGP versus non IG diet over seasons and across the invasive range. In this study, we firstly investigated the use of blocking primers for recovering a greater proportion of prey to predator reads in *H. axyridis,* and then used DNA metabarcoding to investigate the diet of *H. axyridis* across two seasons in the UK, focussing our sampling on the invasion core (Oxfordshire) and closer to the invasion front (Yorkshire). Since IGP is thought to be important in facilitating establishment, we hypothesized that IGP would be greater at the invasion front.

Associated arboreal communities were also sampled to investigate the opportunities for IGP and other forms of predation.

4.2 Methods

a. Study sites

Samples of *H. axyridis* were collected from 12 sites across two locations in May and October 2017. Specifically, six sites were selected in Oxfordshire which is considered part of the invasion core area (Abingdon-on-Thames Fields, Raleigh Park, Sutton Courtenay Common, Streatley Commons, The Kidneys, Wallingford Riverside Park), and six sites in Humberside and the East Riding of Yorkshire, which is at the northern front of the range expansion (Beverley Westwood, Oakfield Park, Pearson Park, The Lawns, Thwaite Gardens, University of Hull campus) (Fig. 1).

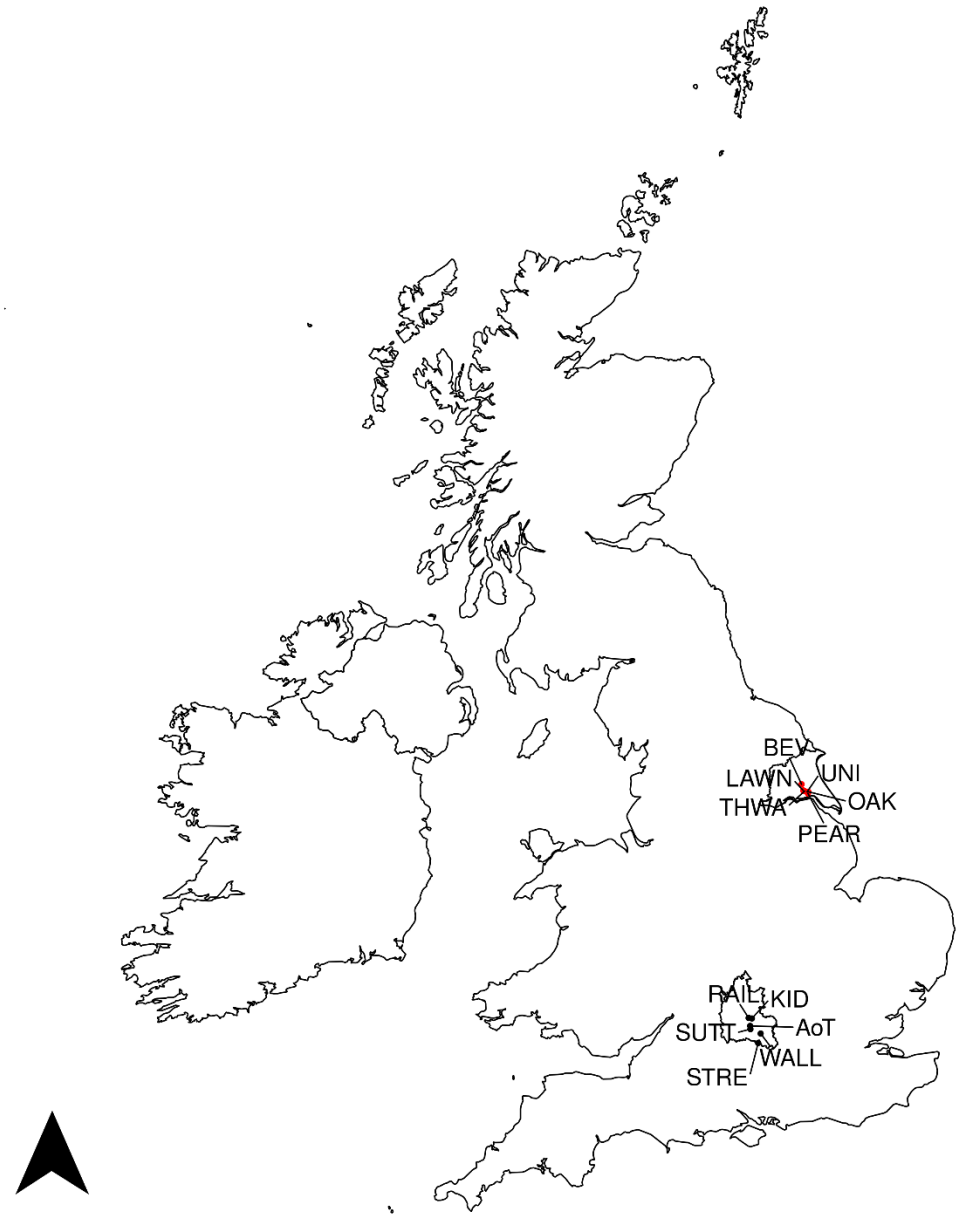


Figure 1. Location of study sites for *H. axyridis*. Oxfordshire sites: AoT (Abingdon-on-Thames Fields), RAIL (Raleigh Park), SUTT (Sutton Courtenay Common), STRE (Streatley Commons), KID (The Kidneys, Oxford), WALL (Wallingford Riverside Park). Yorkshire sites: BEV (Beverley Westwoods), OAK (Oakfield Park), PEAR (Pearson Park), LAWN (The Lawns, Cottingham), THWA (Thwaite Gardens), UNI (University of Hull campus).

In each site three trees were sampled from a mix of *Betula pendula* (Silver Birch) and *Tilia platyphyllos* (Large-leaved Lime). *Harmonia axyridis* specimens (N total = 352; of which 11 were larvae and 341 adults) were collected together with their

associated arthropod communities (N=72). Collection was carried out using a standard beating tray (110cm x 86cm) and two separate suction pooters in order to avoid DNA cross-contamination: one pooter for *H. axyridis* specimens only, and one for the arboreal community only. Both pooters were fitted directly with sterile 50 ml Falcon tubes (Fisher Scientific Ltd, UK) that were changed for each sampling tree, to minimise the handling of the specimens. Upon collection, the communities were stored in 100% ethanol, while the *H. axyridis* specimens were first placed on ice in an insulated container to reduce their metabolism, until they could be frozen back in the University of Hull facilities and transferred to individual tubes containing RNAlater. All samples were ultimately stored at -20°C.

b. Blocking primers design

Following Vestheim and Jarman (2008), we designed blocking primers that were 26bp long each, that were partially overlapping with the Leray (COI) binding site for the metabarcoding primers, and to which was added the C3 (3 hydrocarbon) spacer at the 3' end to prevent elongation (Vestheim and Jarman, 2008; Leray, Yang, *et al.*, 2013). The primers were developed *in silico* by using COI sequences belonging to UK coccinellidae species mined from GenBank, aligned with MAFFT v7 (Katoh *et al.*, 2002) and manually inspected with AliView v (FIG. alignment). The candidate blocking primers were selected if a *Harmonia*-specific region could be found overlapping with the Leray binding region, both in the forward and reverse directions (Vestheim and Jarman, 2008; Leray, Yang, *et al.*, 2013). Five candidate sets of blocking primers met this criteria and were tested using Primer-BLAST against the whole BLAST nt/nr database to verify that no Coccinellidae, and no potentially prey species or other guild member species were going to be coblocked by the primers (Ye *et al.*, 2012). One combination of primers (Ha-blk-F1 5-CCCTCCTCTTTCTTCTAATTTAACAC-3-C3; Ha-blk-R4 5-AATCCTGGGAGAATTAAAATATAAAC-3-C3) met all these criteria and was tested *in vitro*. This primer combination was able to potentially block DNA from a list of non-target taxa; however, most of the taxa were terrestrial species from the Americas, or marine species. Since none of the potentially blocked taxa are found associated with *H. axyridis* in the UK, we continued with *in vitro* testing. The *in vitro* testing involved single-taxa tissue DNA templates of *Harmonia axyridis* (N=3), *Adalia decimpunctata* (N=3), *Halyzia sedecimguttata* (N=2), *Propylea*

quattordecimpunctata (N=1) that were amplified in parallel with and without blocking primers in triplicate samples. PCRs were performed in 25 µl reaction volumes, using 1x MyFi™ Mix *Taq* polymerase (Bioline, UK) and 0.4 µM (final conc.) of each Leray-Geller primer (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013). The blocking primers were added to the reaction in 0.4 µM final concentration for each primer. Both sets of samples were run with the same conditions: an initial step at 98°C for 3 mins, followed by 40 cycles of 98°C for 1 min, 53°C for 30 sec, and 72°C for 90 sec, plus a final step at 72°C for 10 mins. Amplifications were visualised on an Agilent TapeStation using High Sensitivity D1000 Screen Tape kit (Agilent Technologies Inc., US) (Tapestation results have been added in the Appendix 4.1). Following the *in silico* and *in vitro* tests, the blocking primers were applied on gut contents of wild caught *H. axyridis* and sequenced via metabarcoding to check for their efficacy. The samples used in this test comprised 50 *H. axyridis*, equally split between Thwaite Gardens (N = 25) and The Lawns (N = 25) (both Cottingham, East Riding of Yorkshire, UK) collected in May 2017. These samples were sequenced without (N = 50) and with blocking primers (N = 50).

c. DNA metabarcoding

H. axyridis guts were dissected from the rest of the body in order to minimize the ratio of predator to prey reads (Total N = 352). Dissection of *H. axyridis* guts were performed under a stereomicroscope using single-use scalpel blades and petri dishes, and the whole digestive tract was removed from each individual. Scalpel blades, blades and forceps were sterilised by immersion in 10% v/v commercial bleach for few minutes and rinsed with milliQ water before each sample. Scalpel blades were discarded after each specimen. All materials and surfaces were wiped with 10% v/v commercial bleach before and after each dissection. Each specimen was also rinsed with milliQ water and briefly dried with blue roll prior to dissection. All dissected materials were transferred immediately into individual sterile 1.5 mL Eppendorf tubes stored on ice and processed for DNA extraction within 2 h after dissection.

DNA extractions followed the Mu-DNA tissue extraction protocol described by Sellers *et al.* (2018), with few modifications (Sellers *et al.*, 2018). The gut samples

were ground with pestles that had been sterilised with 10% v/v commercial bleach for 5 minutes, rinsed in milliQ water and exposed to UV-light for 2 hours. The grinding was carried out after the addition of the tissue lysis solution, directly in the 1.5 mL tubes used for the dissection. All gut samples were digested on an Eppendorf ThermoMixer (Fisher Scientific Ltd) at 56 °C and 700 rpm for 4 to 6 h. Tissue extraction blanks (N=7) were included using 500µl of milliQ water. The extraction blanks were treated in the same way as the tissue samples.
The community samples from the three tree species were pooled together by site and sampling time (i.e. N = 72/3 = 24) and dried under fume hood for 2 hours under fume hood to remove any traces of ethanol. The grinding step was performed with UV-sterilised 15 ml falcon tubes using 5 g of UV-sterilised 1 - 1.4 mm garnet beads. The volumes for the community extractions were scaled up proportional to the volume to allow the digestion of the material. Digestion took place overnight using 3 ml of Lysis solution mix on a rocking incubator at 56 °C. From each of the community samples we recovered between 2 and 2.5 ml of Lysate, which was transferred into new sterile 15 ml Falcon tubes. We then extracted 600 µl for the downstream steps. The remaining steps for both gut content and community samples followed the Mu-DNA protocol (Sellers *et al.*, 2018).

Three separate sequencing libraries were generated: the first library included the 50 samples from the blocking primer experiment (together with 280 samples from a separate experiment). The second library included 83 wild *H. axyridis* samples (together with 256 samples from a separate study); while the third library included the remaining 351 samples from the present study. To minimise contamination, samples from different studies were processed in separate plates. PCRs were prepared in 96 well plates, each of which contained a minimum of two *Osmia bicornis* (Linnaeus, 1758) and *Lumbricus rubellus* (Hoffmeister, 1843) genomic DNA as positive controls (N = 8 first library, N = 10 second library, N = 11 third library), and a minimum of two PCR negative controls (2 µl of molecular grade water) per plate (N = 11 first library, N = 20 second library, N = 23 third library). PCRs targeted a 313 bp region of COI using metazoan universal primers mtCOIintF - jgHCO2198 (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013). All three libraries followed the same protocols, with the only exception that the gut contents samples in the second and third library had the blocking primers added to the

reactions. The community samples in the third library were run without blocking primers. The protocol included an initial PCR using 0.4 µM of each COI primer, 0.4 µM of each blocking primer (for the gut contents samples in the second and third library only), 1x MyFi™ Mix *Taq* polymerase (Bioline, UK), and 2 µl of DNA template. PCR conditions were 95 °C for 3 mins, followed by 40 cycles of 95 °C for 15 sec, 53 °C for 30 sec, 72 °C for 30 sec, and a final extension step of 72 °C for 10 mins. The library preparation followed a two-step protocol with nested barcodes, so the primers used in the first PCR were modified by adding 20 different barcodes (12 forwards and 8 reverse), and 8 different heterogeneity spacers for a total of 96 barcodes combinations (Kitson *et al.*, 2018) (Appendix 4.2). The PCR products were individually cleaned using magnetic beads as described by Quail, *et al.* (2009), with minor modifications as described below (Quail, Swerdlow and Turner, 2009). The initial ratio of beads (Mag-Bind RxnPure Plus, Omega Biotek, US) was changed to 0.5x per 20 µl PCR product and allowed to stand for 15 min before transferring the supernatants to new wells. The second ratio was changed to 0.12x of magnetic beads per initial volume of PCR products and allowed to stand for 5 mins to allow target fragments to bind to the beads. The supernatants were then discarded, and the beads bound with DNA were washed twice with 200 µl of 80% ethanol. The DNA was ultimately eluted in 15 µl of 10 mM Tris-HCl (pH 8). The individual plates were then uniquely tagged with the Illumina MiSeq adapter primers in a second PCR, which followed the protocol and reaction volumes as described in the “Amplicon, Clean-Up and Index, 2013” (Illumina technologies, online) using MyFi™ Mix *Taq* polymerase (Bioline, UK). Following the second PCR and gel visualisation, the PCR products were cleaned again with magnetic beads as described above. All PCR and clean-up products were visualised on 2% agarose gels. PCR products from the individual plates were quantified with a Qubit fluorometer (ThermoFisher Scientific Inc., UK) and pooled together in equimolar amounts. The pooled libraries were then quantified with qPCR using the NEBNext Library Quant Kit (New England BioLabs Ltd., UK) and the fragment size was further checked on a TapeStation using a High Sensitivity D1000 Screen Tape kit (Agilent Technologies Inc., US). The final libraries were denatured and diluted following Illumina “MiSeq System Denature and Dilute Libraries Guide” (Illumina). All 3 libraries were sequenced on an Illumina MiSeq using a V2 kit at 250 cycles per reads, loading 15 pM libraries with 10% PhiX.

d. Bioinformatics

Illumina raw sequences (NCBI SRA: PRJNA578363) were processed using the custom pipeline metaBEAT v0.97.10 (https://github.com/HullUni-bioinformatics/metaBEAT). This includes trimming raw Illumina sequences with Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014) using a Phred score of 30 or higher and a 5-base sliding window. The sequences were then merged with FLASH v1.2.11 (Magoč and Salzberg, 2011), checked for chimeras and clustered at 97% similarity with VSEARCH v1.1.0 (Rognes *et al.*, 2016). The centroids of the clusters that contained more than 2 sequences were taxonomically assigned using BLASTn v2.2.28+ (Altschul *et al.*, 1990) and the Lowest Common Ancestor (LCA) approach using Taxtastic v0.8.5 (https://github.com/fhcrc/taxtastic). Sequences were assigned with 97% identity, and a minimum of 80% alignment length. The taxonomic assignment was first run against curated databases of macroinvertebrate COI sequences mined from GenBank and EMBL, and curated using SATIVA v0.9 (Kozlov *et al.*, 2016) (more details about database creation and taxa coverage is reported in Appendix 4.3). Sequences that remained unassigned were queried against the NCBI nt database (updated at the end of August 2018), with the same BLASTn and LCA approach described above. The final output was a collapsed table of DNA reads by taxonomy and the samples.

e. Statistical analysis

Statistical analysis was done in R v3.5.1 (R Core Team, 2018). The bioinformatic output containing read counts with the OTU summed by taxonomic ID, was initially quality controlled (hereafter referred to as QC) to remove sequencing fails and low coverage samples. Each library was treated separately at this step, as we calculated the mean number of reads for each library, and then samples that had read counts smaller than the mean-SD were removed. Following this QC, the retained samples were then further cleaned from contamination detected in the positive controls. We detected low levels of contamination in the positive controls belonging to each sequencing libraries, so we applied fixed thresholds, specific for each dataset, of 0.1%, 0.3% and 0.1% respectively. In other words, for the first data set, sequences were discarded from downstream analyses if they were found below a threshold of 0.1% across the entire sample set. Sequence reads were normalised to avoid bias from different coverage between libraries. DNA

reads belonging to samples from the blocking primer experiment (N=50 with blocking primers, N=50 without) were normalised by calculating:

$$Taxon\ normalised\ reads\ =\ (n\ reads\ sample/N\ total\ reads) * 1x10^{6}\ .$$

We then tested whether there was a difference between predator and prey (normalised) read count with and without blocking primers using a paired Wilcoxon rank sign test (R Core Team, 2018), and we tested whether there was an association between the two variables (prey and predator normalised DNA reads) and the two categories (blocking and no blocking primers) using a Chi-Square (χ^2) test.

Read count data from the field samples was converted into presence/absence. A tripartite network was constructed from gut contents data to quantify the interaction strengths between predator and prey using the 'bipartite' v2.11 R-package (Dormann, Gruber and Fründ, 2008). The overlap between *H. axyridis* gut contents and the wider community was analysed with PERMANOVA (N permutations = 9,999) using the gut contents or communities as dependent variables, and seasons and locations as factors. Euclidean distances was used for the gut contents to measure the absolute distance between *H. axyridis* diet from the two counties and the two seasons (Sint *et al.*, 2019); while the Jaccard distances was used for community samples as they converted into presence/absence data. Differences between sample types were visualised using non-metric multidimensional scaling ordination plots (NMDS). Both PERMANOVA and NMDS were from the 'vegan' v2.5 package (Oksanen *et al.*, 2018). To ensure reproducibility all scripts have been deposited (Github: https://github.com/mbenucci/MATI_Harmonia-axyridis).

4.3 Results

The three MiSeq libraries, the first containing the 50 gut samples without blocking primers, the second and third with the gut samples with blocking primers and the community samples, produced respectively: 14,008,944 reads passing filter with 92.12% bases >Q30 and 1.70% error rate, 13,443,878 reads passing filter with 92.18% bases >Q30 and 1.29% error rate, 15,812,350 reads passing filter with 86.29% bases >Q30 and 1.84% error rate.

Blocking primers

In vitro tests of the blocking primers were estimated using a TapeStation (Agilent Technologies Inc., US) which demonstrated a reduction in the mean concentration of amplified *H. axyridis* DNA of approximately 50% (mean concentration without blocking primers = 4876.67 ±316.67; mean concentration with blocking primers = 2170 ±261.59); while the mean concentration of the amplified DNA from the other Coccinellid species tested had no reduction (mean concentration without blocking primers = 6845 ±939.54, mean concentration with blocking primers = 7038.33 ±704.24) (See Appendix 4.2 for further details).

In the field test instead all of samples sequenced without blocking primers had read depth greater than the QC threshold and were included in the analysis, whereas 5/50 samples sequenced with blocking primers failed to pass the read depth QC threshold and were excluded. The normalised read counts were: 99.815% (±0.0691) predator to 0.185% (±0.0691) prey without blocking primers, and 96.005% (±1.549) predator to 3.996% (±1.549) prey with blocking primers. Hence, blocking primers slightly improve the proportion of prey to predator reads, but also introduce greater variance in the normalised DNA reads for both predator and prey (Fig.2). There was a significant difference between the ratio of predator to prey reads (Chi-squared = 13925, df = 1, p-value < 2.2e-16, Fig.2). We also found a significant difference in normalised read count between treatments for prey (Wilcoxon Test U = 1, p-value < 0.001) but not for the predators (U = 495, p-value = 0.804).

Using blocking primers increased the number of samples that were positive for prey from 13/50 (without) to 20/50 (with blocking primers). Four prey taxa were detected in the experiment (*Euceraphis betulae*, Chrysomelidae, *Pyrralta vilburni*, and *Drepanosiphum platanoidis). E. betulae* was detected in 12 *H. axyridis* individuals without blocking primers, and an additional six individuals with blocking primers (Fig. 3b). Chrysomelidae were detected in different single individuals in the two treatments (Fig. 3b). *D. platanoidis* was detected in one *H. axyridis* individual only when blocking primers were used, and then at very low read count (<20 reads, Fig. 3a). Conversely, *P. vilburni* was detected in one individual without blocking primers, but not with (<70 reads, Fig 3a). The range of

predator read count is greater with blocking primers than without, as demonstrated in Fig. 3c.

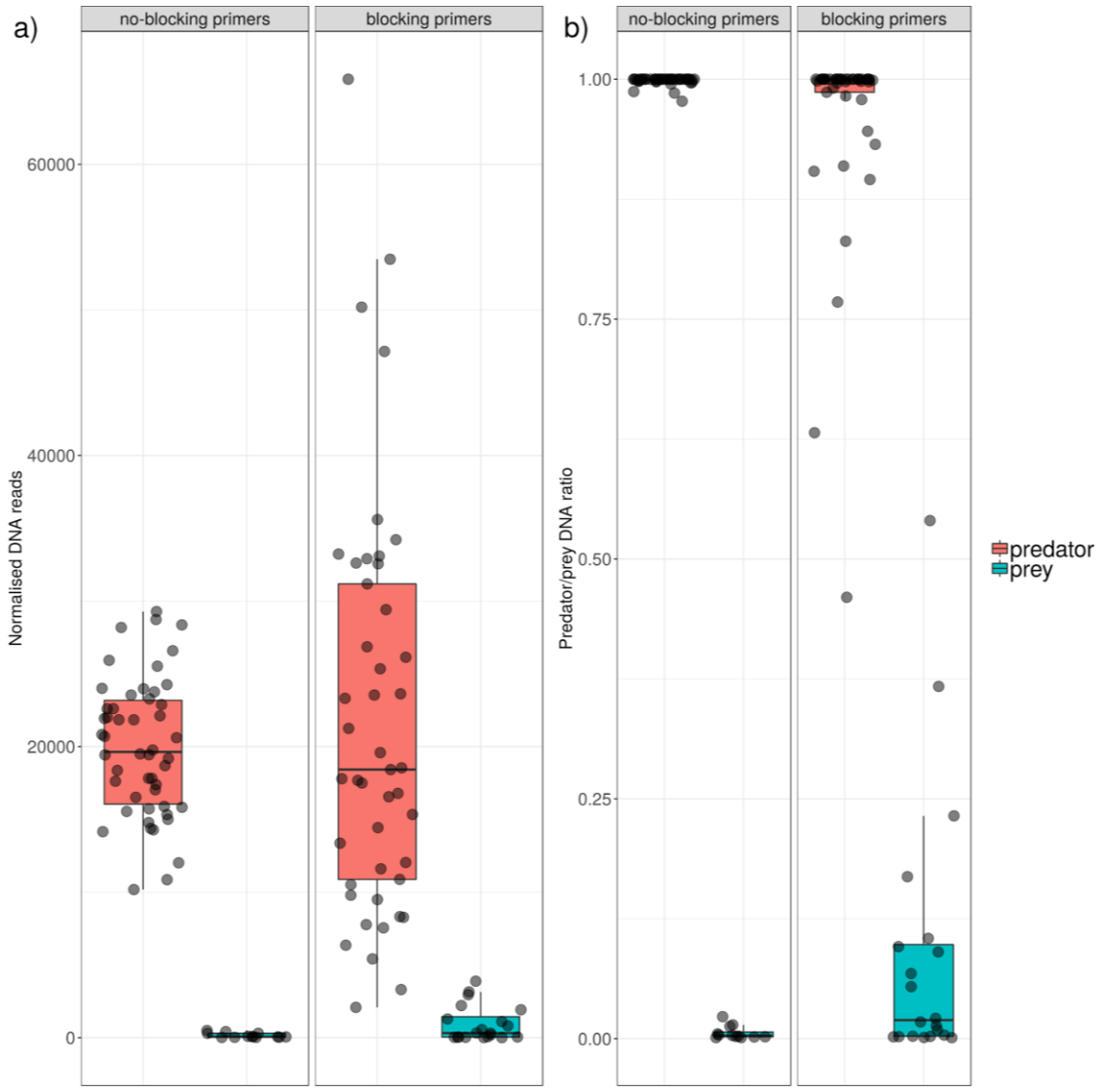


Figure 2. Boxplot of a) normalised DNA reads sequenced without (N=50) and with (N=45) blocking primers; and b) the predator-prey ratio of the normalised DNA reads. For preys, only values greater than 0 are shown.

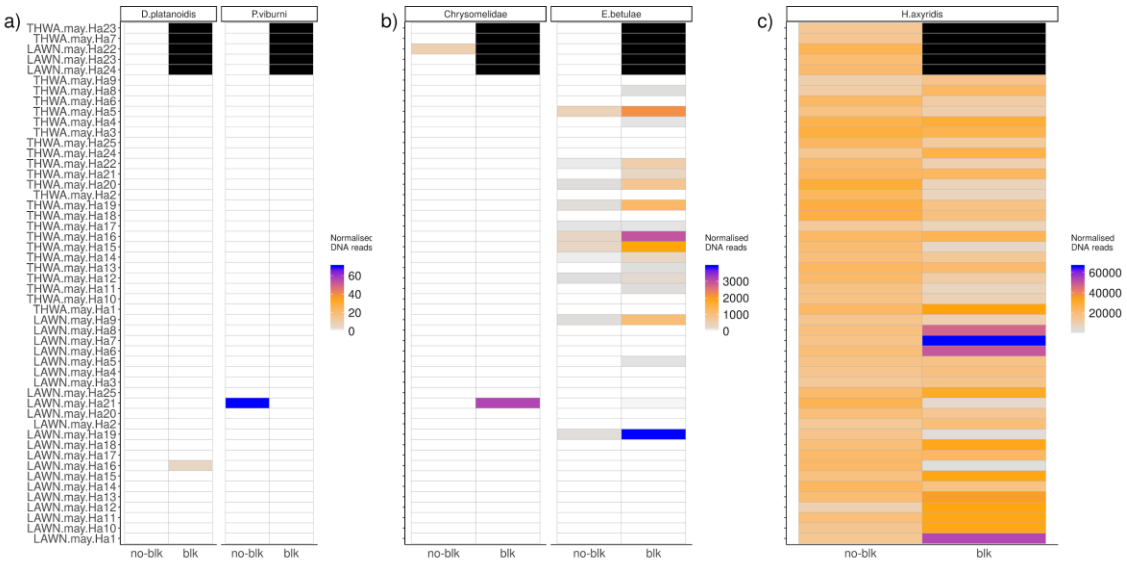


Figure 3. Normalised DNA reads across all 4 prey taxa detected and across paired samples (N=50) without and with blocking primers. The taxa were divided based on their scale of normalised DNA reads: a) Scale range 0-70 reads, b) scale range 0-4000 reads, c) scale range 0-70000 reads. "no-blk" corresponds to samples sequenced without blocking primers, "blk" corresponds to samples sequenced with blocking primers. In black are samples that failed to sequence, or failed to pass QC. On the Y axis, “THWA” corresponds to samples from Thwaite Gardens, while “LAWN” corresponds to The Lawns.

Gut contents analysis from field samples

A total of 352 samples were successfully sequenced, of which 325 passed QC (i.e. 92.9%, Table 1). Of the Oxford samples passing QC, 16 in May (25.4%) and 10 samples in October (16.95%) were positive for interactions. For the Yorkshire samples, 23 (22.12%) from May and 17 (17.17%) from October were positive for interactions (Table 1). The detection rates of interactions were therefore similar across counties, and slightly higher for May than October.

Of these interactions, the majority were potential prey taxa including Aphididae (*Callipterinella spp.*, *Euceraphis spp.*, *Drepanosiphum platanoidis*, *Eucallipterus tiliae*), Tachinidae (*Ocytata pallipes*), Chrysomelidae, Chironomidae (*Chironomus nuditarsis*), and one taxon of mites (Trombidiformes). *Euceraphis spp.* was by far the most common prey species in both regions. In May 84% and 91% of the interactions detected in both Oxfordshire and Yorkshire respectively

were *Euceraphis*; while in October it was detected in Yorkshire only (45% of the interactions Table 1), Psocoptera (Psocoptera and *Ectopsocus californicus*) were detected in Yorkshire only, and comprised 8/19 (45%) of the interactions. Additional detections were of other Aphid species (*Eucallipterus tiliae*), Diptera (*Chironomus nuditarsis*) and leaf beetles (Chrysomelidae) in single individuals only (Table 1, Fig. 4). Intraguild predation was not detected. However, the common parasitoid wasp, *Dinocampus coccinellae*, a common parasitoid of Coccinellidae, was detected in 12 *H. axyridis* from Oxfordshire (3 from May, 9 from October) and one May individual from Yorkshire (Table 1, Fig. 4). *Ocytata pallipes,* a parasitoid fly (Diptera: Tachinidae) was detected in one individual from Oxfordshire in October. This species is a parasitoid of the earwig, *Forficula auricularia,* and it is therefore unlikely to be parasitizing a coccinellid. With this possible exception, no other prey interactions were detected in Oxfordshire in October. These results appear also consistent with our observation during dissection that the gut contents seemed to be empty compared to other samples.

Table 1. Summary of the N detections of potential prey and parasitoid taxa in the gut contents samples. N total of specimens sampled~~s~~ equal 352.

	county	Oxfordshire		Yorkshire	
	time	May	October	May	October
	N specimens sequenced	72	63	105	112
	N specimens passing QC	63	59	104	99
	N specimens with detected interactions	16	10	23	17
	Detection rate	25.4%	16.9%	22.1%	17.2%
	Total N interactions	16	10	24	19
	(of which prey + parasitoid)	(13 + 3)	(1 + 9)	(23 + 1)	(19 + 0)
Prey	*Callipterinella sp.*	1	0	0	0
	Euceraphis sp.	11	0	21	8
	Trombidiformes	1	0	0	0
	Ocytata pallipes	0	1	0	0
	Chironomus nuditarsis	0	0	0	1
	Chrysomelidae	0	0	1	1
	Drepanosiphum platanoidis	0	0	1	0
	Ectopsocus californicus	0	0	0	3
	Eucallipterus tiliae	0	0	0	1
	Psocoptera	0	0	0	5
Parasitoid	*Dinocampus coccinellae*	3	9	1	0

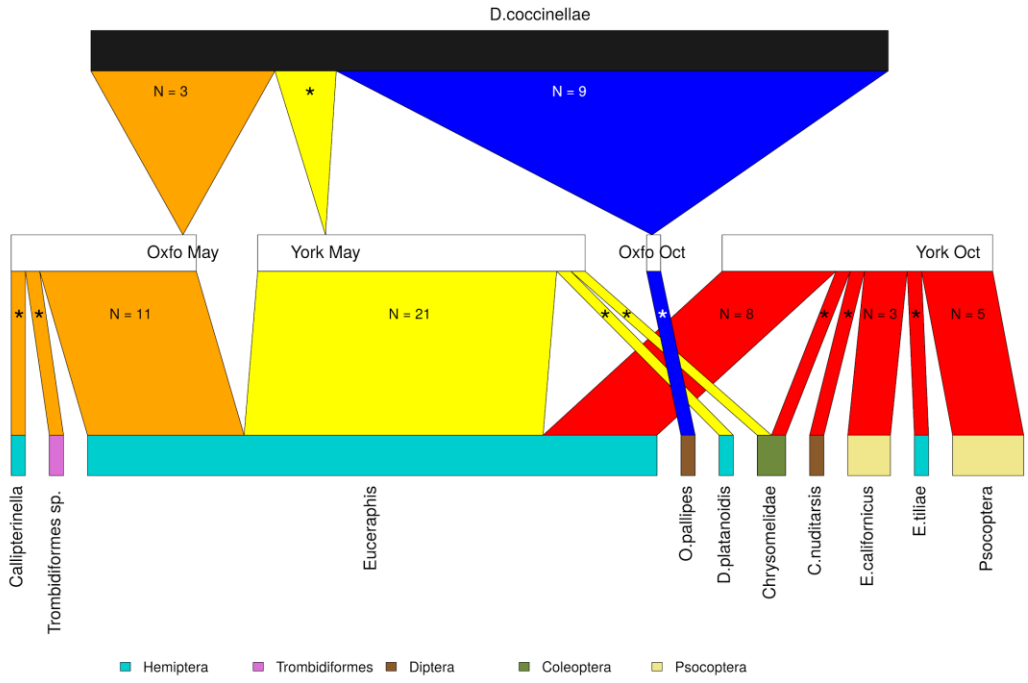


Figure 4. Tripartite networks from gut contents detections. Values show the strength of the interactions detected; '*' corresponds to 1 interaction.

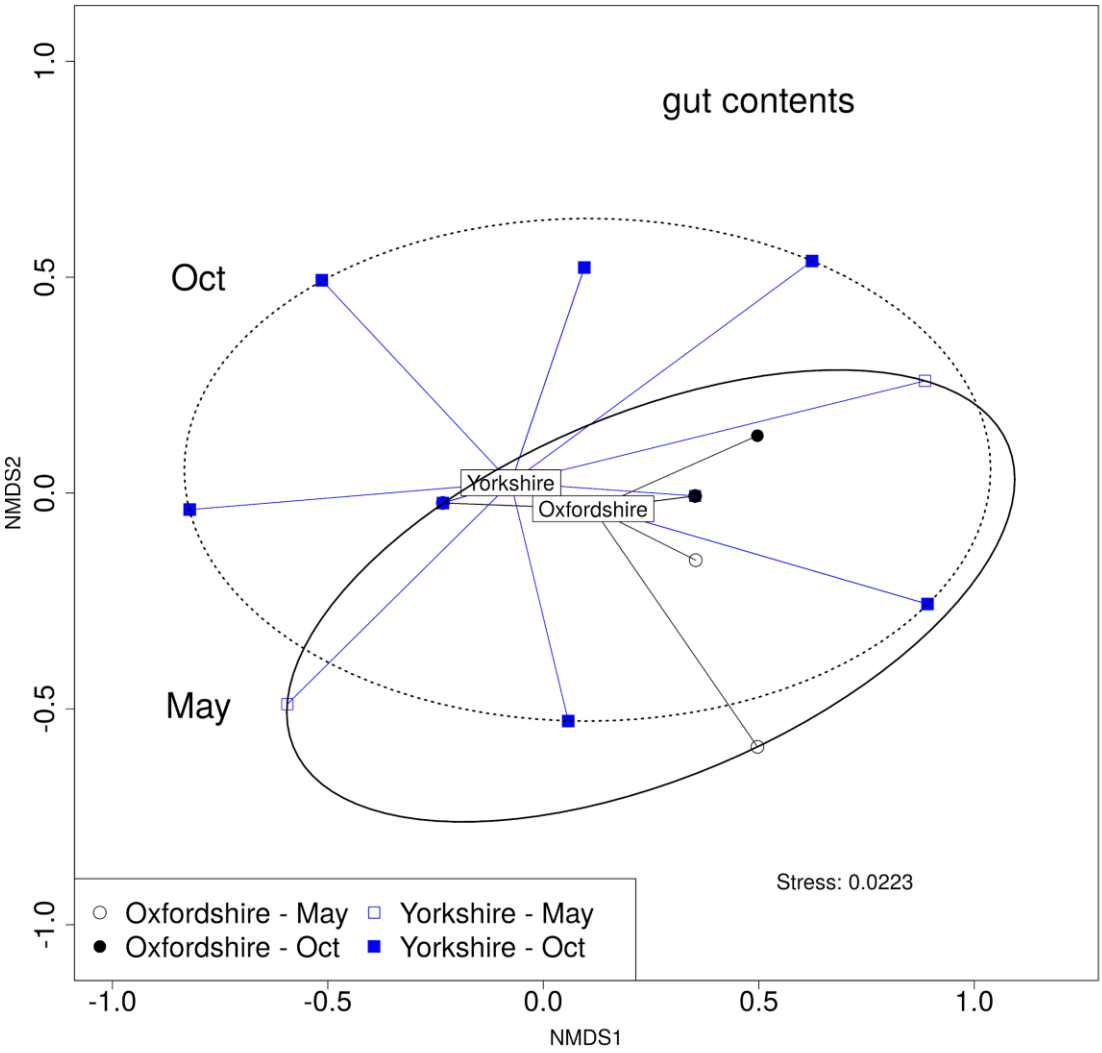


Figure 5. NDMS of the gut contents samples. Black corresponds to Oxfordshire, blue to Yorkshire. Solid line corresponds to May, dashed line to October.

There was a high overlap, between gut contents between location and season (NMDS Fig. 5), and the corresponding PERMANOVA was not significant (PERMANOVA county*time: Pr(>F) = 0.0876, R^2 = 0.02485). However when seasons and locations were considered separately, there were differences in prey species composition, with prey differing significantly between seasons (PERMANOVA time: Pr(>F) = 0.0001, R^2 = 0.17361), and between locations (PERMANOVA county: Pr(>F) = 0.0037, R^2 = 0.06144). In total we could explain 25.99% of the observed variance by comparing season and location.

Relationship between H. axyridis and the wider arboreal community

In total we detected 60 unique species in the communities from both counties and both seasons (N Oxfordshire = 40, N Yorkshire = 33). Oxfordshire communities showed similar number of species in both seasons (N May = 23, N Oct =21), while the number of species in Yorkshire communities was greater in October than May (N May = 11, N Oct = 24). In total we detected 3 species shared between gut contents and the communities, which were detected only in Yorkshire (*Drepanosiphum platanoidis* (Aphididae), *Eucallipterus tiliae* (Aphididae), and *Ectopsocus californicus* (Psocoptera)), while in Oxfordshire we detected no shared species between gut contents and community.

The community metabarcoding demonstrated that aphids were the dominant component of the arboreal communities associated with *H. axyridis* at both sampling sites and seasons (Fig. 6). Psocoptera were also highly abundant in both counties in October. The *H. axyridis* gut contents closely mirror the arboreal communities for these dominant taxa, with the exception of Oxfordshire in October (Fig. 6).

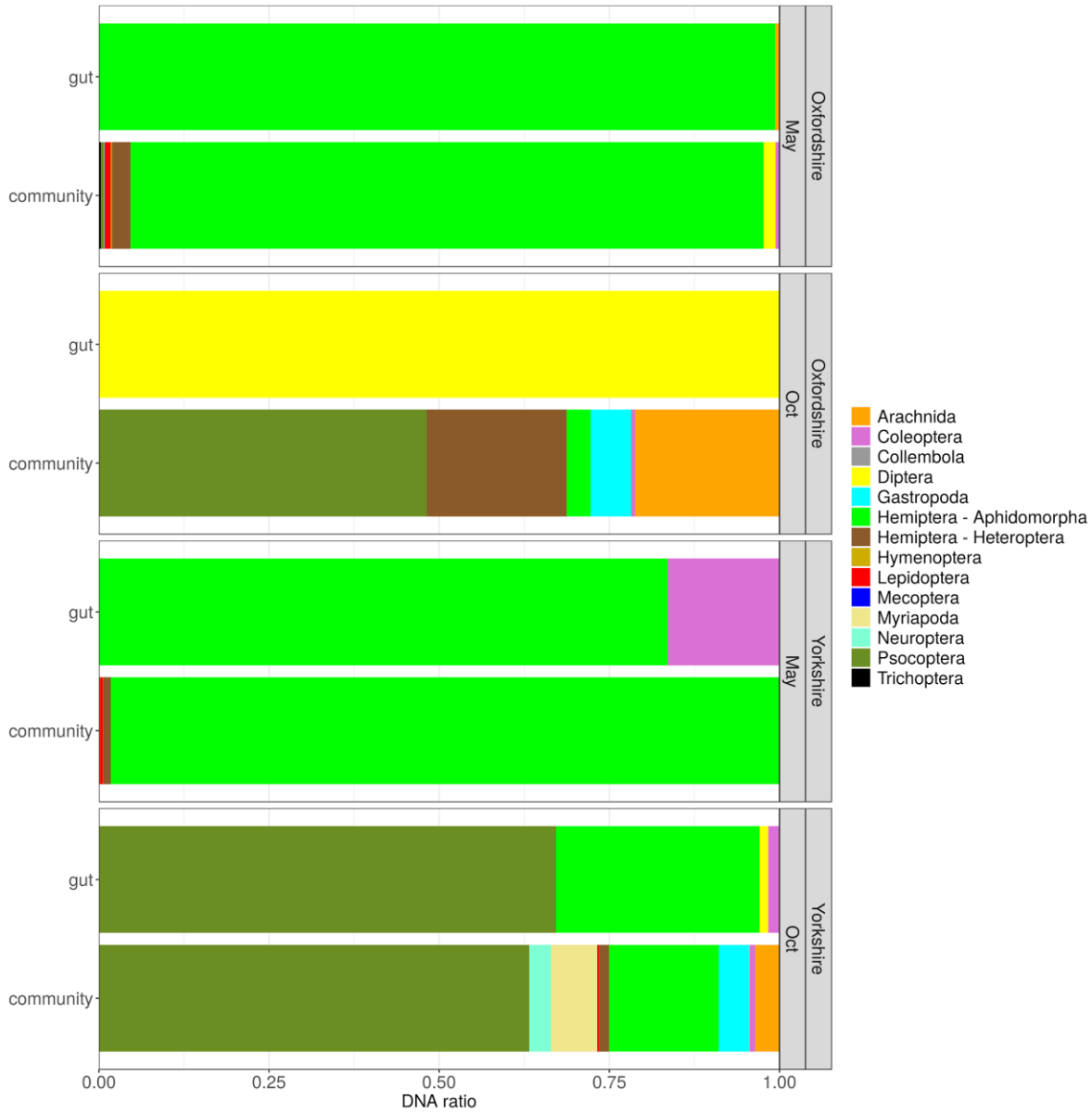


Figure 6. Barplot of gut contents and communities compositions based on DNA reads counts. *Dinocampus coccinellae* was not been included in the composition of the gut contents samples.

There was a significant difference between seasons in terms of the wider community (PERMANOVA time: Pr(>F) = 0.0001, R^2 = 0.32097, which explains 32.1% of the observed variance), but no difference between sampling locations (PERMANOVA county: Pr(>F) = 0.1899, R^2 = 0.05003, Fig 7a). We also found that there was no significant difference when location and county were combined (PERMANOVA county*time: Pr(>F) = 0.1652, R^2 = 0.06001). The NMDS plot shows that May communities from both counties appear also more similar than the October samples (Fig. 7a).

There were significant differences between gut contents and community sample types (type: Pr(>F) = 0.0001, R^2 = 0.1847 with a total of 24.32% of the variance explained), and a significant influence on the taxa compositions from the seasons (time: Pr(>F) = 0.0001, R^2 = 0.05848) and from the sample type (type: Pr(>F) = 0.0001, R^2 = 0.1847). A significant influence of the location (county: Pr(>F) = 0.0014, R^2 = 0.02422) was also detected (Fig. 7b). The combination of sampling time and sample type (whether gut contents or communities) had significant influence (time*type: Pr(>F) = 0.0001, R^2 = 0.09469) on communities composition, and this explains a further 9.47% of the observed variance. In total the combination of time, sample types and sampling location with their interactions explains 44.18% of the total observed variance. The gut contents fully overlap with the communities (NMDS, Fig 7b) as expected from the barplot (Fig 6).

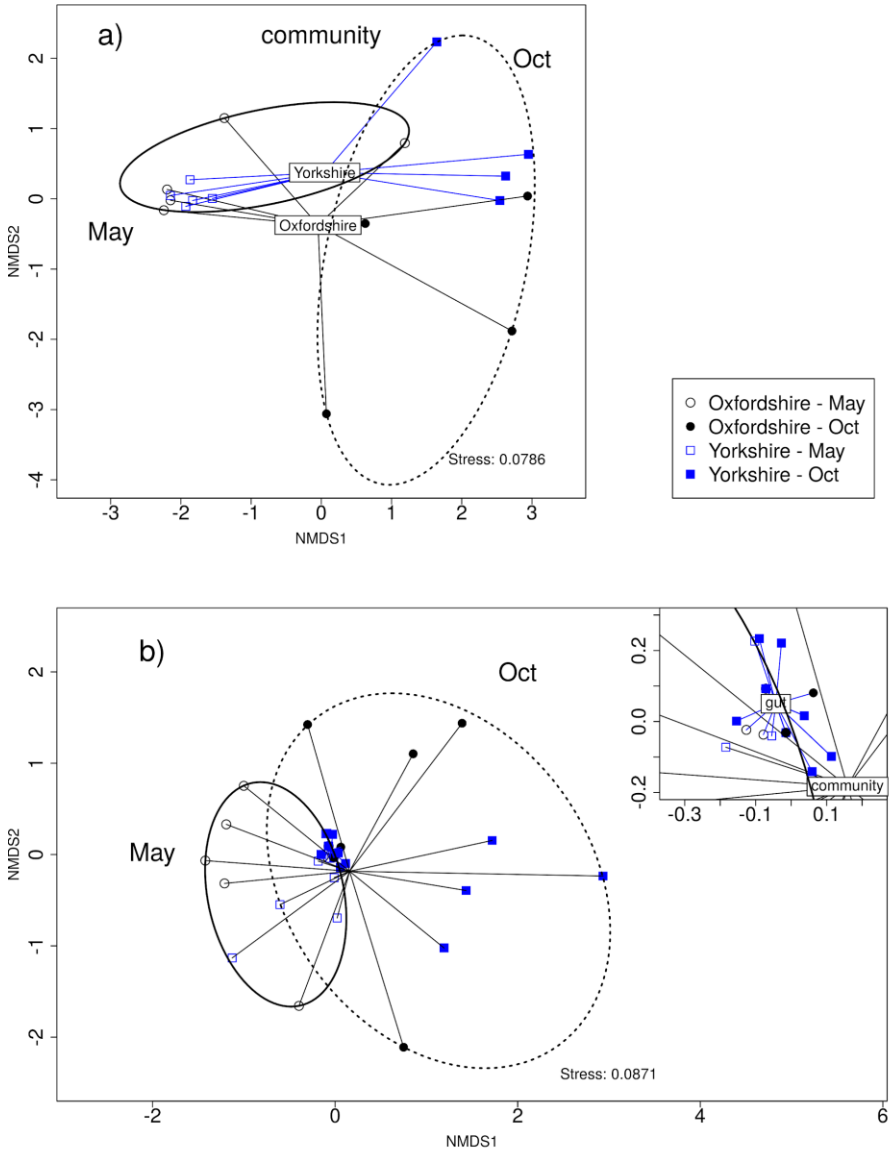


Figure 7. NDMS plot of a) invertebrate communities obtained from bulk tissue DNA sequencing, and b) the comparison between communities and gut contents samples. Inset shows plot b) magnified gut contents samples.

4.4 Discussion

We firstly developed blocking primers with the aim of reducing the ratio of *H. axyridis* to prey DNA and tested their efficacy in a paired experiment with 50 wild-caught *H. axyridis* samples. Blocking primers increased the number of interactions detected, and the proportion of prey reads, but they also increased the variance of predator reads in the dataset. Secondly, we investigated the

trophic interactions of *H. axyridis* in the field, at the core (Oxfordshire) and front (Yorkshire) of the UK invasion in two seasons, by metabarcoding of gut contents and associated communities. The gut contents data mirror the available communities, with aphids and Psocoptera dominating both sample types. Contrary to our expectations, we did not detect IGP interactions against other UK ladybirds or other aphidophagous species. As discussed below, this is likely explained by the absence or rarity of intraguild competitors within the immediate communities at the time and place of sampling.

Blocking primers

Blocking primers successfully reduced the yield of PCR product for *H. axyridis* whilst not impacting the yield for all three non-target coccinellid species tested. However, in the metabarcoding experiment, the results were more variable. In our paired experiment on 50 wild-caught *H. axyridis,* using blocking primers increased the overall number of interactions detected from 13 to 20 and increased the ratio of prey to predator reads. Detection rate improved with blocking primers for *Euceraphis betulae,* but we did not see a notable improvement for the three other taxa detected (*Pyrralta vilburni, Drepanosiphum platanoidis* and Chrysomelidae). Addition of blocking primers increased the variance in the predator reads, with some samples showing reduced predator read counts, but others having up to three-fold higher reads than without.

Despite the success of the blocking primers in reducing PCR product yield for predator but not potential prey DNA, the prey read counts from the metabarcoding experiment showed only minor improvement: from ~0.2% to ~4% when blocking primers were used. The increase in prey relative to predator read counts we obtained is on a similar scale to, and in some cases better than, that found in other studies. For example, a recent study that developed Aranae-specific blocking primers improved the proportion of Hexapoda reads in spider gut contents from 0.9 to just 1.46% (Toju and Baba, 2018).

Other studies have reported considerable challenges to the design of effective blocking primers for DNA metabarcoding studies especially when amplifying with universal primers. In some cases the use of blocking primers can also result in

the introduction of amplification biases, as Piñol *et al.* (2014) report, with unwanted co-blocking of potential prey species that can be closely related to the consumer species (Vestheim and Jarman, 2008; O'Rorke *et al.*, 2012; Piñol *et al.*, 2014, 2015). Despite these challenges, we managed to account for and limit the risk of co-blocking of potential prey species and of other members of Coccinellidae family both *in silico* and *in vitro*, before applying the blocking primers on wild field samples. Possible next steps for improving the prey:predator read count proportions include 1) increasing the ratio of blocking primers to the target primers (e.g. from 1:1 used here to potentially 10:1 as used by Vestheim and Jarman 2008) or 2) enriching the prey DNA by selectively removing high molecular weight DNA (which has been shown to improve prey detection rates in spiders, (Krehenwinkel *et al.*, 2017)), 3) extracting from the mid and hindgut only, and only including samples in which stomach contents seem full, or 4) digestion of predator-specific DNA and subsequent size selection for example using Crispr/Cas9 (Jiang and Doudna, 2017).

Harmonia trophic interactions

Harmonia axyridis is well known for its generalist feeding behaviour and as a strong intraguild predator (Michaud and Grant, 2003; Snyder, Clevenger and Eigenbrode, 2004; Pell *et al.*, 2008; Katsanis *et al.*, 2013; Roy *et al.*, 2016; Brown and Roy, 2018). A number of studies have demonstrated IGP of native competitors by *H. axyridis* in field conditions using prey specific PCR assays in both the invasive (Ingels *et al.*, 2013; Thomas *et al.*, 2013; Brown *et al.*, 2015; Rondoni *et al.*, 2015; Howe *et al.*, 2016) and native ranges (Yang *et al.*, 2016), and IGP by *H. axyridis* has been correlated with declines in native ladybird populations (Brown, Frost, *et al.*, 2011; Roy *et al.*, 2012). Other studies have challenged this widely accepted hypothesis, finding no evidence for IGP in the field (Smith and Gardiner, 2013). Collectively, studies that have documented IGP by *H. axyridis* in the field have found relatively low detection rates, with the highest levels of IGP <10% (Ingels *et al.*, 2013; Thomas *et al.*, 2013; Brown *et al.*, 2015; Rondoni *et al.*, 2015; Howe *et al.*, 2016). Despite this relatively low detection rate, we were surprised to not find evidence of IGP in our 352 samples of *H. axyridis.* There are a few possible reasons for this finding. Firstly, most of our samples were adults, whereas IGP is likely to be most common at the fourth larval instar

stage (Ware and Majerus, 2008; Ware, Yguel and Majerus, 2009; Thomas *et al.*, 2013), although IGP of the predatory flowerbug, *Anthocoris nemoralis* by adult *H. axyridis* has been documented in the field (Howe *et al.*, 2016). Very few *H. axyridis* larvae were found during our sampling campaign, so it was not possible to restrict our experiment to larvae, or to perform a fair comparison between adults and larvae. Secondly, phenology is important for coccinellid population dynamics and predator-prey interactions (Adriaens, Gilles San Martin and Maes, no date). Our sampling was carried out in early summer (May) and autumn (October) whereas previous studies that have detected IGP in UK *H. axyridis* populations sampled in late June and early July (Thomas *et al.*, 2013; Brown *et al.*, 2015). Our sampling did not overlap with opportunity for IGP since native ladybirds are univoltine and their eggs and immature stages are present in the peak of summer. Thirdly, it is evident from our community samples that non-IG prey - i.e. aphids and barkflies - were the dominant components of the community at time of sampling, therefore the opportunity and requirement for IG predation was low. More temporal replicates, during the summer months would be necessary to fully investigate the dynamics of IGP.

Non-IG interactions were detected in 17-25% of individuals that were successfully sequenced. The majority (78%) of these interactions involved aphids, and these were almost entirely *Euceraphis spp.,* with one detection each of *Callipterinella spp., Drepanosiphum platanoidis* and *Eucallipterus tiliae.* Barklice (Psocoptera, including *Ectopsocus californicus*) were detected in 14.5% of individuals but only in October in Yorkshire. The dominance of aphids and barklice in the gut contents can be explained by their dominance in the arboreal communities at the time of sampling. Indeed, with the exception of Oxfordshire in October, the gut content samples mirror the availability of these two dominant taxa in the community. *Chrysomelidae* were detected in two individuals, while Trombidiformes, *Ocytata pallipes* and *Chironomus nuditarsus,* were detected in single individuals. *O. pallipes* is a parasitoid of the European earwig *Forficula auricularia* (Linnaeus, 1758) (Kuhlmann, 1993, 1994; Maczey *et al.*, 2016)~~)~~. Considering the host-specificity of *O. pallipes*, and its strong association with *F. auricularia*, we consider the interaction detected of *H. axyridis* with *O. pallipes* as a predator-prey interaction, which raises the question of whether *H. axyridis* is potentially impacting a parasitoid-host network. The coccinellid parasitoid, *Dinocampus*

coccinellae, was detected in 13 individuals and also visually confirmed in one during dissection (Supplementary materials - S1). Interactions between *H. axyridis* and *D. coccinellae* have been investigated in lab experiments and in field surveys throughout the overlapping range of the two species. Generally it is thought that *H. axyridis* is a poor host for *D. coccinellae* (de Castro Guedes, Dios and de Almeida, 2018) and rates of parasitism are generally low, but may be increasing as the parasitoid adapts to a novel host (Berkvens *et al.*, 2010; Comont *et al.*, 2014; den Berg *et al.*, 2014). Our study illustrates that metabarcoding can be used to obtain estimates of the rates of both predation and parasitism simultaneously in the field.

Prey composition was strongly influenced by season, and again mirrored the prey availability, with aphids dominating the community and gut contents in May, and barklice dominating communities and (Yorkshire only) gut contents in October. Spatially, the communities and gut contents overlapped in May, but there was an important difference between sites in October, when virtually no positive prey interactions were detected in Oxfordshire, but Yorkshire *H. axyridis* were consuming aphids and barklice. This is in spite of the observation that barklice and aphids were still present in the Oxfordshire community at the time of the October sampling. One possible explanation is that Oxfordshire *H. axyridis* were ahead of their northern counterparts, and had stopped feeding in preparation for overwintering (Labrie, Coderre and Lucas, 2008). Despite the lack of IGP interactions, the molecular results seem to further support the potential presence of competitive exclusion that *H. axyridis* can play against native Coccinellidae, which Kenis *et al.* (2016) included in their risk assessment of Coccinellidae species across Europe. Aphidophagous Coccinellidae are known to lay eggs near a food source; this trait together with *H. axyrydis* mutlivoltinism and bigger body size of native and invasive ladybirds larvae at similar life stage, could indicate that competitive exclusion might play a bigger role in the success of this invader.

Overall, a finer scale for temporal and spatial replicates would be needed to test both these hypotheses. In the specific situation of *H. axyridis*, IGP and competitive exclusion seem to be influenced by the timing of emergence of the different ladybird species. In the native range, smaller ladybirds appear to have anticipated the emergence time, which allows them to match *H. axyridis* larvae

body size. On the contrary in the invaded range, smaller ladybirds seems to have similar emergence time than the invasive Coccinellid, leaving them vulnerable to predation by the invasive larvae which have a bigger body size (Pell *et al.*, 2008; Ware and Majerus, 2008). The multivoltinism ultimately could also reinforce the exclusion of native ladybirds from food sources in the invasive range, but without a comparative study between native and invasive species we cannot fully confirm whether competitive exclusion is happening and what is its influence.

4.5 Conclusions

Here we demonstrated that blocking primers improved the rate of detection and proportion of prey reads, but also increased the variance in predator reads in our DNA metabarcoding experiment. In field trials, we found no evidence of IGP by *H. axyridis,* but we detected aphids and barklice at high proportions in the ladybirds' gut contents. The results mirrored the seasonal availability of these prey in the respective communities. The parasitoid wasp, *D. coccinellae,* was also detected, both using DNA metabarcoding and through microscopy. With minor improvements to the method and a finer scale temporal and spatial resolution, DNA metabarcoding could provide additional, important insights into the trophic ecology of *H. axyridis* and other invasive invertebrates.

Chapter 5 - Reciprocal intra-guild predation between newly detected *Crangonyx floridanus* and established *Crangonyx pseudogracilis* in a UK lake.

Abstract

The early detection of invasive non-native species (INNS) before they become established is challenging, and traditional approaches for detection (e.g. kick sampling) can sometimes miss non-native species that are cryptic or at very low densities. An even greater challenge is to infer the interactions between INNS and resident species, which is important for assessing and quantifying the ecological impact of INNS. Molecular methods, and particularly environmental DNA (eDNA) metabarcoding, have enormous potential for the detection of INNS and understanding of their trophic interactions. In this study we used a combination of DNA barcoding, morphology and eDNA metabarcoding to confirm the cohabitation of two closely related INNS: established *Crangonyx pseudogracilis* and newly detected *C. floridanus* in the UK. We then used DNA metabarcoding of gut contents (ingested DNA or "iDNA") to investigate the trophic interactions of the two *Crangonyx* species. We detected high levels of reciprocal intraguild predation (IGP) between *Crangonyx* species, but with more IGP towards the established *C. pseudogracilis* than vice versa. The results open further questions about the dynamics of the interaction between the two species, their relationship with the wider community, and their role in ecological networks. Our study also illustrates the combined power of kick sampling, eDNA and iDNA for providing a more holistic description of the community than any method in isolation.

5.1 Introduction

Invasive non-native species (INNS) are one of the main threats to global biodiversity (Hulme, 2009), so early detection and understanding their impacts on invaded communities is key to their management, and an important field of research (Sheppard *et al.*, 2018). The early detection of non-native species (NNS) before they become established is particularly challenging, and traditional approaches for detection (e.g. kick sampling in freshwater systems) can sometimes miss species that are cryptic or at very low densities (Blackman, 2018). Molecular methods, and particularly environmental DNA (eDNA), have enormous potential for detection of INNS during the pre-establishment stages of the invasion process (Lawson Handley, 2015; Blackman, 2018). The first published application of contemporary eDNA to detect INNS was carried out on American bullfrogs, *Lithobates catesbeiana* (Ficetola *et al.*, 2008). Since then, a rapidly growing number of case studies have used eDNA to monitor pathways (e.g. ballast water (Shaw *et al.*, 2019)), detect new species, and monitor established species (Lawson Handley, 2015; Blackman, 2018). DNA based methods are particularly promising for detection of cryptic invasive species (Hebert *et al.*, 2004; Bastos *et al.*, 2011; Simmons *et al.*, 2016; Blackman *et al.*, 2017; Mauvisseau *et al.*, 2018), which often remain undetected and establish in new systems (Geller, 1999; Bickford *et al.*, 2007; Teske *et al.*, 2011). An even greater challenge than species detection is to infer the interactions between INNS and resident species, which has important implications for quantifying their ecological impact (Ricciardi and Atkinson, 2004; Van der Putten, Macel and Visser, 2010).

The application of DNA-based species identification to gut content analysis can improve our understanding of the trophic interactions created by INNS and the impacts they cause on resident communities which would be too difficult to detect (Symondson, 2002). This rapidly growing field, called Molecular Analysis of Trophic Interactions or “MATI”, has been applied across different trophic systems, such as carnivorous plants (Littlefair *et al.*, 2018), omnivorous vertebrates (De Barba *et al.*, 2014), and scavenging invertebrates (Siegenthaler, Wangensteen, Benvenuto, *et al.*, 2018). The description of interactions among a limited number of species can be carried out using single-target assays, like for example Koester

et al. (2013) who developed group specific assays to target the expected major contributors to *Dikerogammarus villosus*' diet (Koester, Claßen and Gergs, 2013). However with the introduction of DNA metabarcoding, MATI improved the description of niche and diet to high resolution without knowing the identity of putative prey species *a priori* (De Barba *et al.*, 2014; Wirta *et al.*, 2014, 2015). This approach opens up the analysis not only to the detection and description of the main components of species diet but also to the detection of rarer interactions. Although it can be argued that rare interactions are less influential on the nutrition of the predator (Piñol *et al.*, 2014), they can still be indicative of a wider resource usage from the INNS with potential biodiversity loss and destabilisation of the trophic network (Borrvall, Ebenman and Tomas Jonsson, 2000).

The niche similarities between closely related species and the resulting interactions in field conditions have often interested ecologists across wide range of ecosystems, also with a focus on whether niche similarity can linked to phylogeny (Valiente-Banuet and Verdú, 2008; Rafferty and Ives, 2013). Exploring the niche breadth and trophic interactions of INNS have important implications on the impact and risk assessments during invasion, which can include understanding how the introduced species can change the receiving ecosystem and community (Thiele *et al.*, 2010), and evaluating the invasion potential of INNS (Penk *et al.*, 2017). The potential overlap in the niche breadth among closely related species and the potential competition that can result from the shared resources (Burns and Strauss, 2011), can also provide important information on the INNS success and their potential impact, based on how the invasive and closely related species interact within an invaded system (Dick, Ian Montgomery and Elwood, 1999; Raso *et al.*, 2014). In the context of biological invasions, understanding competition and interactions in closely related species can have important implications during impact assessment because it can the presence of closely related native can indicate the availability of suitable niches for the invasive species (Li *et al.*, 2015), and sequently it can highlight the degree a site can be susceptible to invasion or not (Strauss, Webb and Salamin, 2006). Arguably, one of the most important trophic interactions between competing species is intraguild predation (IGP) (Arim and Marquet, 2004; MacNeil and Dick, 2014), which happens when predation is directed towards a competitor from the same trophic guild (Polis, Myers and Holt, 1989). IGP can influence the

composition and functioning of invertebrate communities (Arim and Marquet, 2004), for example, by releasing predation pressure on shared prey species (Grason and Buhle, 2016/6; MacNeil, Elwood and Dick, 1999) which causes a positive influence on the overall community biodiversity (Wang, Brose and Gravel, 2019). While IGP happens under normal conditions across communities, during biological invasions IGP can influence the success or failure of INNS establishment (Alexander and Edwards, 2010).

There are examples in which IGP facilitates INNS establishment by removing resident competitors (MacNeil and Platvoet, 2005), or conversely, has limited INNS populations (MacNeil, Elwood and Dick, 1999). Despite the influence towards INNS, whether facilitating or limiting, observing IGP in field conditions is challenging; and most of the information currently available has been obtained from lab conditions where direction of interaction and mortality can be quantified. For example Grason and Buhle (2016) described the case of the invasive Oyster Drill (*Ocenebra inornata*), which has major impacts on populations of Oysters (*Ostrea lurida*); however it gets strongly reduced by native species of Crabs (*Cancer spp.*), which are able to reduce the INNS abundance, hence releasing Oysters from predation pressure (Grason and Buhle, 2016/6). Aquatic ecosystems are characterised by strong trophic links within their communities, and for this reason even the introduction of a single INNS can cause deep changes in the trophic networks of the invaded aquatic ecosystems (Gallardo *et al.*, 2016).

Crangonyctidae (Amphipoda) is an exclusively freshwater family of which 80% of species are hypogean (Väinölä *et al.*, 2008). There are 47 known species belonging to the genus *Crangonyx*, of which 42 occur in North America (Zhang and Holsinger, 2003; Svavarsson and Kristjansson, 2006). In comparison, Eurasia only has five known native species, four of which occur in subterranean waters of Europe (Zhang and Holsinger, 2003; Svavarsson and Kristjansson, 2006). *Crangonyx pseudogracilis* (Bousfield, 1958), is a North American amphipod which has a wide non-native distribution across Europe. It was first recorded in Europe, in England in 1936 (Crawford, 1937), then later in Ireland in 1975 (Holmes, 1975), and subsequently across Western Europe. Early reports pointed towards a low survival of *C. pseudogracilis* following introduction, mostly because of being predated by other bigger amphipod species, like *G. pulex*

(MacNeil, Elwood and Dick, 1999; Van der Velde *et al.*, 2000). However, *C. pseudogracilis* can now be widely found throughout the UK and European freshwater systems. *C. pseudogracilis* has no known detrimental effects on native species, and some authors have suggested it may have positive ecological impacts including fulfilling the important trophic role of detritivore (MacNeil and Dick, 2014). The closely related *Crangonyx floridanus* (Bousfield, 1963) is also indigenous to the North America but from the eastern and east-central United States, including Florida and Louisiana, with non-native populations being found in western USA (Colorado, Oregon and California) (Toft, Cordell and Fields, 2002; Zhang and Holsinger, 2003), as well as Japan (Kanada *et al.*, 2007), and recently the UK (Mauvisseau *et al.*, 2018). Little is known about the population in the native range, and most information on *C. floridanus* comes from Japan, where it was first recorded in 1989 (Nagakubo *et al.*, 2011). *C. floridanus* was only recently discovered in the UK using a combination of microscopy and DNA barcoding (Mauvisseau *et al.*, 2018). The timing of introduction into the UK, its colonisation pathway and current distribution are currently unclear, but it was identified in two locations (Windermere in Cumbria and Smestow Brook in the West Midlands) separated by 200 km; suggesting that it is a widespread, and established population (Mauvisseau *et al.*, 2018). *C. floridanus* and *C. pseudogracilis* are morphologically highly similar, and although recent analysis has not fully resolved their phylogenetic relationship, they are still classified as separate species (Slothouber Galbreath *et al.*, 2010; Nagakubo *et al.*, 2011).
Due to the highly similar morphology between *C. floridanus* and *C. pseudogracilis,* we cannot exclude that *C. floridanus* remained undetected in the UK for a long time. Current knowledge regarding the impacts of *C. floridanus* are restricted to Japan, where this species has rapidly dispersed across the river network in the past decades, facilitated by its ability to utilise a wide variety of microhabitats, its high fertility rates and ability to withstand conditions of low oxygen (Nagakubo *et al.*, 2011). However, direct evidence of the impacts caused by the invasion by *Crangonyx floridanus* are still sparse (Tojo *et al.*, 2010).

In this study we firstly used DNA barcoding, microscopy and phylogenetic analysis, including Maximum Likelihood (ML) tree construction and haplotypes network, to confirm the identification and cohabitation of *Crangonyx floridanus* and *Crangonyx pseudogracilis* in the UK. Secondly, we selected one single lake

in the UK and carried out DNA metabarcoding of *C. floridanus* and *C. pseudogracilis* gut contents to investigate the trophic interactions that these species have with the benthic community of the site, and to investigate potential intra-guild predation involving the focal species and other guild member species. Finally, we collected eDNA and kick samples to describe the benthic invertebrates associated with the two *Crangonyx* species across two seasons. Our objectives were 1) to confirm the presence of both *Crangonyx floridanus* and *Crangonyx pseudogracilis* in our target site, 2) to carry out molecular analysis of trophic interactions on the gut contents of these two cohabiting species to investigate the breadth of their trophic interactions with the benthic community and with each other, and 3) to describe the community that is available to these amphipods using a combination of eDNA and kick sampling. Given the lack of previous reports on direct interactions of these two species, and considering the similarities in their ecology; we hypothesised *C. pseudogracilis* and *C. floridanus* to possess similar diet breadth, which would lead to a high niche overlap and a possibilities of competition over shared resources; however we predicted greater IGP interactions from the newly introduced *C. floridanus* towards *C. pseudogracilis*, than the opposite.

5.2 Methods

a. Study sites

Sampling took place in 6 locations across the UK (Fig. 1). Community and gut content analysis were carried out at Chasewater Reservoir (1. Staffordshire, UK; Coord: Lat 52.661732, Long -1.9426501), while the remaining 5 sites (2. Bourne Stream Lat 50.728599, Long -1.9039807; 3. Kings Dyke Lat 52.560337, Long -0.21853879; 4. River Nar Lat 52.698221, Long 0.68570137; 5. Thwaite Garden Pond Lat 53.780629, Long -0.4003787; 6. Upton Broad Lat 52.665578, Long 1.5313521) were selected based on recent samples collected by the Environment Agency that included specimens of *Crangonyx spp.*; so their inclusion was to further expand our understanding of the current distribution of *C. floridanus* (Fig. 1).

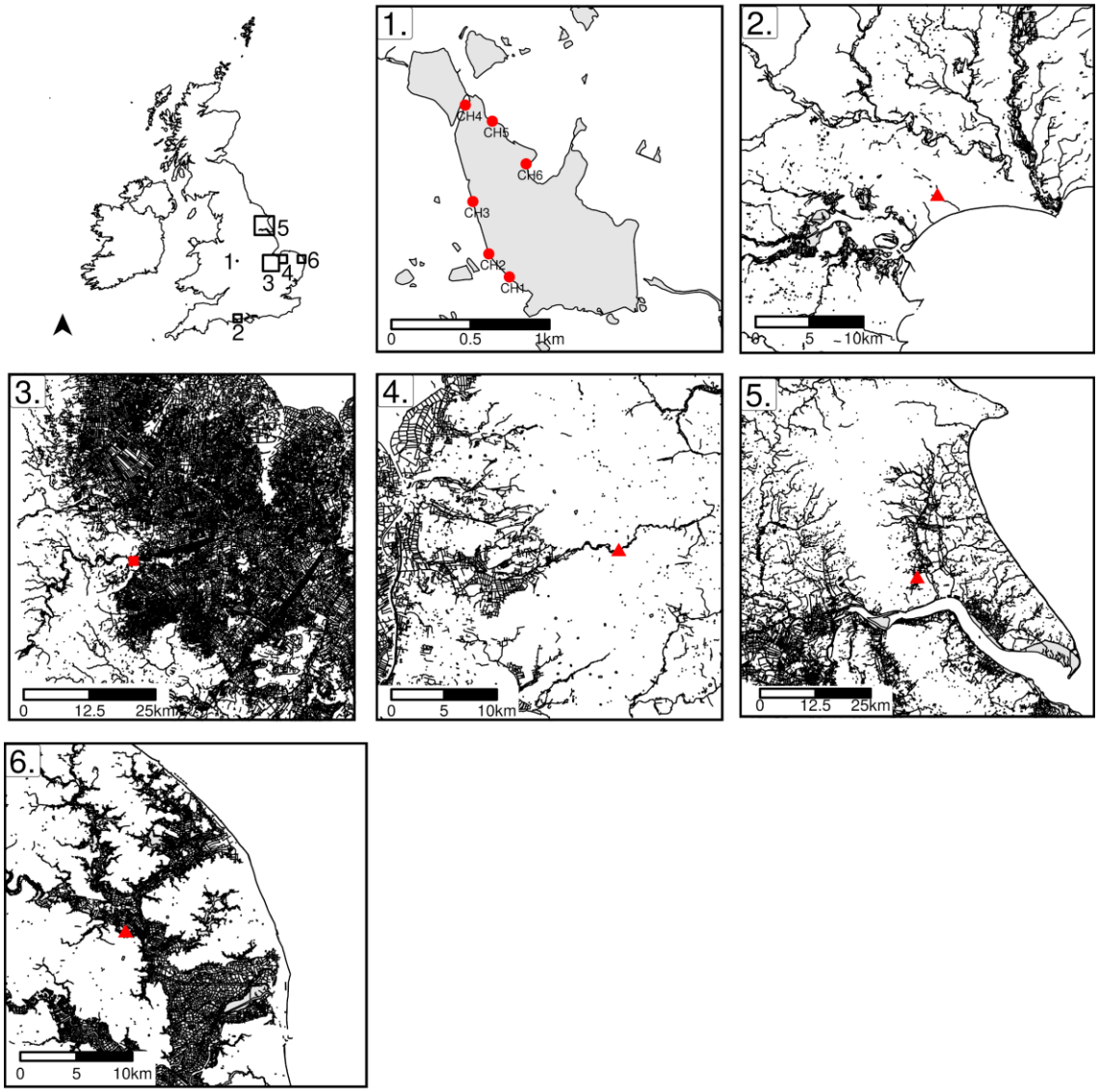


Figure 1. Sampling locations for Crangonyx specimens (site 1 for gut contents, community and DNA barcoding; sites 2-6 only for DNA barcoding). 1. Chasewater Reservoir (Staffordshire, UK); 2. Thwaite Garden Pond (East Riding of Yorkshire, UK); 3. River Nar (Cambridgeshire, UK); 4. Kings Dyke (Cambridgeshire, UK); 5. Bourne Stream (Dorset, UK); 6. Upton Broad (Norfolk, UK). Squares indicate locations with detected *Crangonyx floridanus*, triangles indicate locations with detected *Crangonyx pseudogracilis*, circles indicate detection of both species.

b. Sample collection and preparation

To investigate the distribution of *C. pseudogracilis* and *C. floridanus,* during Summer 2017 *Crangonyx* individuals were collected by kick-sampling from Chasewater Reservoir (N = 50), Thwaite Gardens (N = 10), River Nar (N = 10) , Kyngs Dyke (N = 5), Bourne Stream (N = 5), and Upton Broad (N = 5) (Fig. 1)

and stored in 80-100% Ethanol. Out of these a total of 15 *Crangonyx* specimens were sexed (Constable, 2014), then morphologically identified using Chapman (2007) identification key (Chapman, 2007), and then DNA barcoded using standard protocols (See Appendix 5.1 for the details on the DNA barcoding protocol).

For the community and trophic interactions analyses, sampling took place in 6 locations at Chasewater Reservoir only, in May and October 2017 (Fig. 1). Three types of samples were collected,in the same order, at each location: i) 2 L water samples for eDNA analysis, ii) kick-net samples for morphological identification of freshwater macroinvertebrate communities, and iii) collection of *Crangonyx* specimens for gut content analysis.

The water samples (N = 6 in May, N=6 in October) were collected from each location using a Phil Sampler (Hydro Technologies Inc.) and 6 Pyrex-media bottles (500 mL), which were sterilised in the lab using 10% v/v commercial bleach solution, 5% v/v lipsol solution and rinsed with purified water. Before sampling, each Pyrex bottle was rinsed twice using lake water at the sampling location to remove any possible remaining bleach or detergent. Field blank samples (2 L of milliQ water) were taken into the field on each sampling visit at each lake (N=1 in May and N=1 in October). These blank samples were treated in parallel with other samples. Samples were stored on ice and filtered within 24 h of collection. Filtration took place in a dedicated eDNA laboratory at the University of Hull, UK. The samples were filtered through 0.45 µm cellulose nitrate membrane open filters (Whatman, GE Healthcare, UK) and sterilised Nalgene units (Thermo Fisher Scientific) attached to a vacuum pump (15~20 in. Hg, Pall Corporation). Filters were placed dry in petri dishes, sealed with parafilm and stored at -20 ^{o}C until DNA extraction.

Following the eDNA sampling, standardised 3-minute kick-samples were collected from each sampling location (N = 6 in May, N=6 in October). The kick-samples with the communities were placed in Whirl Pak sealed plastic bags (Nasco, US) and preserved in 100% Ethanol. Invertebrates from the kick-samples were identified to the lowest taxonomic level possible. If species identification was not possible, due for example to damage, then genus or family level were used. From these kick-samples, 50 *Crangonyx* specimens per sampling event (N tot=101; N=51 in May, N=50 in October) were manually picked out for gut

contents analysis and immediately stored in sterile 1.5 mL screw-cap tubes with 1 mL of RNAlater and then frozen at -20 ºC upon return to the laboratory. Guts were dissected from *Crangonyx* individuals to maximize the ratio of prey to predator DNA, using single-use scalpel blades and petri dishes to minimize cross-contamination. Scalpel blades and forceps were sterilised by immersion in 10% v/v commercial bleach for few minutes and rinsed with milliQ water before being used and discarded after use. All other materials (e.g. petri dishes) and working surfaces were wiped with 10% v/v commercial bleach before and after each dissection to further prevent cross-contamination. Each specimen was also rinsed with milliQ water and patted dry with blue roll before being dissected. The dissected material was transferred immediately into sterile 1.5 mL Eppendorf tubes stored on ice and processed for DNA extraction within 2 h after dissection.

c. DNA extraction, DNA barcoding and DNA metabarcoding

DNA extractions followed separate protocols based on the type of sample material. For the DNA barcoding we removed a pereopod (P5, 6 or 7) for DNA extraction from each specimen (N tot = 15) used in species confirmation. Pereopods were placed in individual sterile 1.5 mL Eppendorf tubes and air dried to remove any traces of ethanol. DNA extraction was modified from the Mu-DNA tissue extraction protocol as described by Sellers *et al.* (2018), with the additions described in Supplementary materials (Supplementary materials - S1) (Sellers *et al.*, 2018). The membrane filters for the eDNA samples were processed using MoBio Power Water kit (Qiagen) following manufacturer's protocol. The DNA from the gut contents was extracted following the Mu-DNA tissue extraction protocol as described by Sellers *et al.* (2018), with the addition of a grinding step with individual sterilised pestels to allow a better digestion by the Proteinase-K (Sellers *et al.*, 2018). The grinding was done after the addition of the tissue lysis solution, and directly into the 1.5 mL tubes used for the dissection. The individual plastic pestles used to grind the material were sterilised with 10% v/v commercial bleach, stored in 100% ethanol and exposed to UV light for 3 h. All tissue samples were digested on a Thermo mixer (Fisher) at 56 ºC and 700 rpm for 4 h, until all material was digested.

Two separate DNA metabarcoding sequencing libraries were generated: the first library contained gut contents and eDNA samples collected in May, and the second library contained the samples collected in October. Each 96 well plate contained *Osmia bicornis* (Linnaeus, 1758) genomic DNA positive controls (Ntot = 4 first library, and Ntot = 7 second library), and PCR negative controls containing 2 µl of molecular grade water instead of DNA template (Ntot = 6 first library , and Ntot = 10 second library). PCRs targeted a 313 bp region of COI using metazoan universal primers mtCOIintF - jgHCO2198 (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013). The primers were designed to enable a nested-barcode approach using a 2 step protocol for library preparation, in which the first PCR tags each individual within a 96 well plate using 96 unique primer combinations (N = 12 forward indexes, and N = 8 reverse indexes), and the second PCR indexes the individual plate (Kitson *et al.*, 2018) (See Appendix 5.2 for details on the sequencing primers). The first PCR used 0.4 µM of each Primer, 1x MyFi™ Mix *Taq* polymerase (Bioline, UK), and 2 µl of DNA template in a 25 µl reaction volume. PCR conditions were 95℃ for 3 mins, followed by 40 cycles of 95 ^{o}C for 15 sec, 53 ^{o}C for 30 sec, 72 ^{o}C for 30 sec, and a final extension step of 72 ^{o}C for 10 mins.

Products from the first PCR were individually purified with Mag-Bind RxnPure Plus (Omega Biotek, US) on a 96 well magnetic stand to remove primer dimers and to isolate the target fragments. The clean-up protocol was optimised from the size-selection protocol described by Quail, *et al.* (2009) with a few modifications, as described below (Quail, Swerdlow and Turner, 2009). An initial ratio of 0.5x magnetic bead per 20 µl PCR product was used and allowed to stand for 15 min before transferring the supernatants to new wells. The second ratio of 0.12x magnetic beads per initial 20 µl of PCR products, was then added to the supernatant and allowed to stand for 5 mins to allow target fragments to bind to the beads.

The supernatants were then discarded, and the beads were washed twice with 200 µl of 80% ethanol. The DNA bound to the beads was ultimately eluted in 15 µl of 10mM Tris-HCl (pH 8). After visualisation on 2% agarose gels, PCR products from the same 96 well plate were pooled. The individual plates were then further tagged with the Illumina MiSeq adapter primers in the second PCR, which followed the protocol and reaction volumes as described in the “Amplicon, Clean-

Up and Index, 2013" (Illumina technologies, online) using MyFi™ Mix *Taq* polymerase (Bioline, UK). Following the second PCR and gel visualisation, the PCR products were cleaned again with magnetic beads as described above. Gels were performed after each step, using 2 µl of PCR or clean-up products on 2% Agarose gel in 1x Sodium Boric Acid buffer (Brody and Kern, 2004) at 200 V for 20 min.

After the clean-up of the second round PCR products, the individual plates were quantified for dsDNA with a Qubit fluorometer (ThermoFisher Scientific Inc., UK) and pooled together in equimolar amounts, taking into account the number of samples in each plate. The pooled libraries were then quantified with qPCR using the NEBNext Library Quant Kit (New England BioLabs Ltd., UK) and the fragment size was further checked on a TapeStation Automated Electrophoresis (Agilent Technologies Inc., US) using a High Sensitivity D1000 Screen Tape kit. The final libraries were denatured and diluted following Illumina "MiSeq System Denature and Dilute Libraries Guide" manufacturer's instructions (Illumina Inc., United Kingdom). Both sequencing libraries were sequenced on an Illumina MiSeq using a V2 kit at 250 cycles per reads, loading 600 µl of 15 pM template libraries with 10% PhiX.

d. Bioinformatics for *Crangonyx* species confirmation

To confirm the identity of the two *Crangonyx* species we constructed a Maximum Likelihood (ML) tree and a haplotype network using DNA sequences from barcoded specimens, DNA metabarcoding experiments, and published sequences available on NCBI. The DNA sequences obtained from the barcoded specimens were manually inspected and quality controlled using CodonCode Aligner v5.1.3 (CodonCode Corp., Dedham, MA, USA) to trim the primer sequences and the low quality bases, and to merge the forward and reverse sequences. The barcoding sequences were also dereplicated at 100% identity within Reprophylo before being used for the ML tree and haplotype network.

For the tree identity confirmation experiment the Illumina raw sequences (NCBI SRA: PRJNA575704) were processed using the custom pipeline metaBEAT v0.97.10 (https://github.com/HullUni-bioinformatics/metaBEAT), with the

workflow that was adapted from Blackman *et al.* (2017) (Blackman *et al.*, 2017). metaBEAT v0.97.10 includes Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014), which was used to identify the read pairs from the barcodes from the first PCR, and to retain the portions of reads with a Phred score of 30 or higher using a 5-bases sliding window. Following that, VSEARCH v1.1.0 (Rognes *et al.*, 2016) was used to check for chimeras among the paired sequences, and then to cluster the sequences at 97% similarity. The sequences were clustered using VSEARCH v1.1.0, and the minimum number of sequences for a cluster to be retained was set equal to 5. Because in this part of the experiment we were only interested in confirming the identity of only the 2 *Crangonyx* species, we performed the taxonomic assignment against published sequences of *Crangonyx floridanus*, *C. pseudogracilis*, and *C. islandicus* downloaded from NCBI. The assignment was performed at 97% identity with 85% minimum alignment against the reference sequences using BLASTn v2.2.28+ (Altschul *et al.*, 1990) with a Lowest Common Ancestor (LCA) approach using Taxtastic v0.8.5 (https://github.com/fhcrc/taxtastic). The published sequences from NCBI included 50 records for *Crangonyx floridanus* (Nagakubo *et al.*, 2011; Mauvisseau *et al.*, 2018), 12 for *Crangonyx pseudogracilis* (Slothouber Galbreath *et al.*, 2010), and 4 records for *Crangonyx islandicus* as an outgroup taxa (Kornobis *et al.*, 2011). From the NCBI records, 8 published sequences (AJ968905, AJ968906, AJ968907, AJ968908, AJ968909, AJ968910, AJ968911, EF570296) were identified as potentially misassigned due to the number of differences in the alignment compared to other records in GenBank and our own data. Including or excluding them did not influence the topology of the main branches in the ML tree.

Following taxonomic assignment we extracted only the clusters belonging to either *C. floridanus* or *C. pseudogracilis* from the whole metabarcoding dataset, clusters that needed to be supported by at least 2% of the total reads per sample to remove low coverage sequencing and PCR errors. The reads associated with these centroids were then aligned and dereplicated with VSEARCH v1.1.0 to check for presence of haplotypes.

Crangonyx floridanus, *C. pseudogracilis* sequences obtained from metabarcoding, barcoding and published literature were then all used for the ML tree construction. The tree construction was performed using Reprophylo

(https://github.com/HullUni-bioinformatics/ReproPhylo) (Szitenberg *et al.*, 2015). The ML tree building included the 313 bp metabarcoding dataset (N = 121), the 658 bp barcoded sequences (N = 8), and the 658 bp COI sequences from NCBI (N = 42). Prior to tree construction, all the sequences were aligned with MAFFT v7 (Katoh *et al.*, 2002) and then trimmed using TrimAl v1 (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009) the sequences to remove bases that were not supported in at least 90% of the 171 sequences (metabarcoding, barcoding and literature sequences together). This resulted in a minibarcode ML tree based on 299 bp sequences. The tree was then constructed using RAxML v8 (Stamatakis, 2014). The same alignment that was used to construct the tree was also used to construct the haplotype network using 'adegenet' (Jombart, 2008), 'ape' (Paradis and Schliep, 2018), and 'pegas' (Paradis, 2010). The haplotypes that were represented by only one sequence (singletons) from our metabarcoding data, were filtered out as they were suspected to be sequencing errors.

e. Bioinformatics for community and gut contents analysis

The metabarcoding raw sequences were then processed again for the gut contents and eDNA experiment. This used metaBEAT v0.97.10, as above, however the centroids of the clusters were retained if they contained more than 2 sequences. Following the taxonomic assignment was performed again with BLASTn v2.2.28+ (Altschul *et al.*, 1990) and the Lowest Common Ancestor (LCA) with the same parameters as above. However, the first the taxonomic assignment was run against a set of curated databases of macroinvertebrates COI sequences mined from NCBI and EMBL (See Appendix 5.3 for details on the database creation). The sequences unassigned against this set of curated databases were ran again on BLASTn and LCA approach against the NCBI nt database (updated at the end of August 2018). The outputs of both assignment steps were merged into a single table of DNA reads for each sample collapsed by taxonomy, so that different OTUs assigned to a single specie were merged together. Downstream analysis was performed in R v.3.5.1 (R Core Team, 2018). The bioinformatic output containing read counts collapsed by taxonomic ID, were initially quality controlled and filtered to remove low coverage samples that contained less than 1000 reads. The retained samples were then further cleaned from contamination that was detected in the positive samples which should have contained only

Osmia bicornis DNA. Low levels of contamination were detected in both May and October sequencing data. In the first run with the sample from May we detected a total of 22 reads not belonging to *O. bicornis* from all positive samples (7 reads from *D. villosus*, 8 reads from *G. zaddachi*, 7 from human DNA). In the second run with October samples instead we detected from all positive samples a total of 73 contaminating reads (31 from Cyclopidae, 18 from *D. villosus*, 2 from Diptera and 22 from human DNA). Based on the ratio of these contaminations in the positive samples we applied 0.03% and 0.39% thresholds respectively for first and second runs.

f. Ecological data analysis

Following the QC steps, the read counts files from the eDNA and gut contents datasets, and the kick samples dataset, were transformed into presence/absence data, which for eDNA and kick samples this represented the detection of species at each location within the lake; while for the gut contents it represented the species detected in the gut of the individual specimens. Site occupancy across the six sites within Chasewater and the two sampling seasons was calculated for the eDNA and kick-samples datasets.

To analyse trophic interactions, we first assigned the predator ID using the relative proportion of DNA. In agreement with Piñol *et al.* (2014), predator DNA constitute the majority of the relative proportion of DNA in each sample (Piñol *et al.*, 2014). In our case we used a relative proportion of DNA reads in each sample that was greater than 90% to define the predator identity. Only 6 samples from the second run needed to be assigned using a relative proportion of DNA greater than 80% individual samples. Another important aspect to be taken in consideration is the potential bias introduced from tag-jumping (Schnell *et al.*, 2015). We acknowledge the potential presence of 2.1% and 2.6% of the sequences that might have potential error in the assignment due to tag-jumping; however, we minimised this bias using a chimera detection on all DNA reads data through a using VSEARCH v1.1.0 (Rognes *et al.*, 2016) before the taxonomic assignment.

To investigate trophic interactions in *C. pseudogracilis* and *C. floridanus* we used the interaction strength, defined as the number of links that we could detect

between each *Crangonyx* species, and their prey from DNA (Berlow *et al.*, 2004). To visualise these links we used a bipartite network that was then constructed from the gut contents data for *C. pseudogracilis* and *C. floridanus* using the bipartite package (Dormann, Gruber and Fründ, 2008). To investigate the potential competition between the two *Crangonyx* species, and the similarities in their diet we calculated niche breadth and niche overlap using the standardised Levin's and Shannon indices (Krebs and Others, 1989; Lyngdoh *et al.*, 2014; McClenaghan *et al.*, 2015), and the Pianka index for similarity from the 'spaa' package (Zhang, 2016).

To investigate the composition of the wider communities within Chasewater and to understand which environmental factors might be driving the community compositions, the presence/absence data (dependent variables) from gut contents, eDNA and kick samples were analysed with PERMANOVA (N permutations = 9,999), and non-metric multidimensional scaling (NMDS) from the 'vegan' package (Oksanen *et al.*, 2018). Regarding the driving environmental factors we ran both analysis against predator ID (for gut contents only), sample type (for community only), and season (for both gut contents and community) as factors (Shiels *et al.*, 2013; Sint *et al.*, 2019). For the gut contents PERMANOVA and NMDS we used the Euclidean distance, while for the community PERMANOVA and NMDS we used Jaccard distance. Species richness within lake was calculated for the kick-samples and eDNA samples. To ensure reproducibility, all scripts regarding DNA barcoding, haplotypes and ecological analysis are deposited at (Github: https://github.com/mbenucci/Cohabiting_C.floridanus_and_C.pseudogracilis).

5.3 Results

Data summary

We obtained DNA barcode sequences from 13 of the 15 specimens (2 sequences were excluded from downstream analysis due to poor quality in both forward and reverse).

The first metabarcoding library (May) generated 14,008,944 reads passing filter, 92.1% bases above Q30, and an error rate of 0.33% (±0.27). This translated into

an average read depth per sample of 31742.43 (±7102.63) reads (eDNA: 18017.14 ±5519.37; gut contents 38603.73 ±9216.21). The second library (October), generated 13,443,878 reads passing filter, 92.2% bases above Q30, and an error rate of 1.31% (±0.31). This translated into an average read depth per sample of 13361.64 (±1233.33) reads (eDNA: 20136.14 ±7191.82; gut contents: 16163.04 ±1088.02). Out of the 101 Crangonyx specimens collected for gut contents analysis, 40 were identified based on relative ratio of DNA reads as *Crangonyx pseudogracilis* (N May = 33, N Oct = 7), and 61 as *Crangonyx floridanus* (N May = 18, N Oct = 43). After QC based on the read depth, we retained 87 (~86%) of the gut contents samples (N May = 37, N Oct = 50), of which 30 (54.5% of 87 specimens) were *C. pseudogracilis*, and 57 (65.5% of 87 specimens) were *C. floridanus* (Tab. 1). From the 12 eDNA samples collected (N May = 6, N Oct = 6), 9 (75%) remained following QC; with CH6 in May, and CH1 and CH6 in Oct that didn't pass the QC thresholds.

Detection and confirmation of C. floridanus and C. pseudogracilis

The 13 *Crangonyx* specimens collected and barcoded were morphologically identified as either *C. floridanus* or *C. pseudogracilis*, based on the presence (*C. pseudogracilis*) or absence (*C. floridanus*) of spines on the Uropod 2 (Fig. 2).

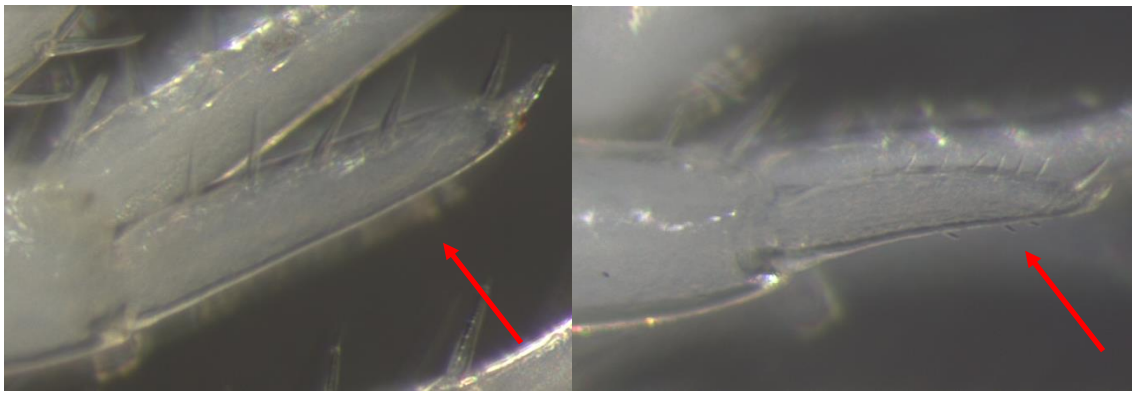


Fig. 2. Microscopy images of Uropod 2 of male specimens of *Crangonyx floridanus* (left) and *C. pseudogracilis* (right). Red arrows point at Uropod spines, absent in male *C. floridanus* and present in male *C. pseudogracilis*. (Photos by M. Benucci).

Following the morphology, we provisionally identified 5 specimens to *C. pseudogracilis* and 8 specimens to *C. floridanus*. Our morphological identification was confirmed by the Sanger sequences for all 13 specimens. The sequences

obtained from the DNA barcoding were then aligned, and we found 18% divergence (123bp) between the two Crangonyx species across the COI barcoding region (Folmer *et al.*, 1994) (See Appendix 5.4 for barcode sequence alignment). Furthermore, we observed a low sequence divergence within each species, with no difference in the Folmer region within the 5 *C. pseudogracilis* sequences, and within the 8 *C. floridanus* sequences (Appendix 5.4). In the metabarcoding data we identified only one haplotype for each *Crangonyx* species because the sequences were clustered at 97% identity.

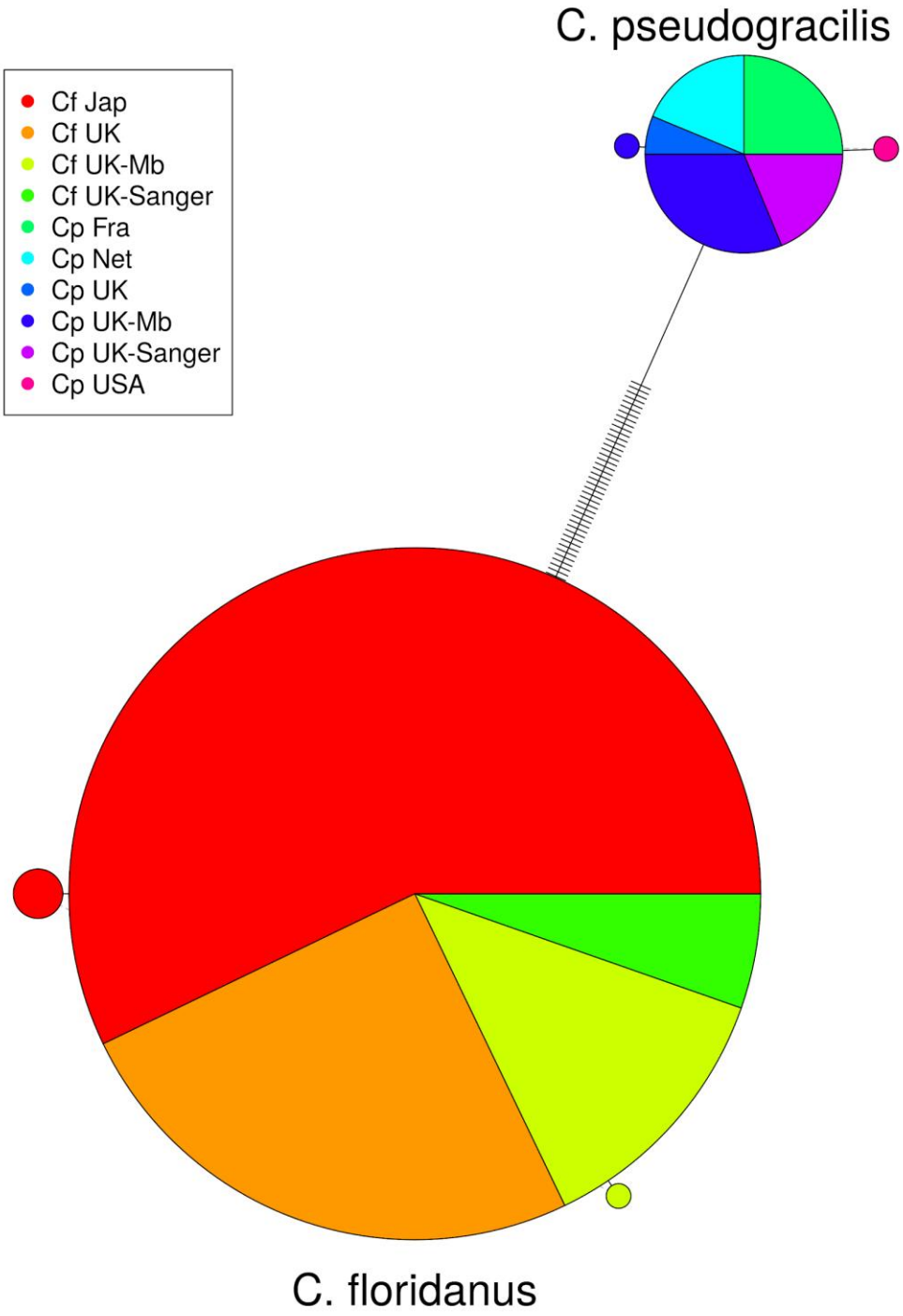


Figure 2. Haplotype network of *C. floridanus* and *C. pseudogracilis* COI sequences. Cf-Mb/Cp-Mb and Cf-/Cp-Sanger corresponds to the metabarcoding (Mb) and Sanger sequences generated in this study respectively. The remaining sequences (Cf UK, Cf Jap, Cp UK, Cp Net, Cp Fra, Cp USA) correspond to the sequences produced by previous literature.

The ML tree generated from the COI sequences includes records from the literature (Slothouber Galbreath *et al.*, 2010; Nagakubo *et al.*, 2011; Mauvisseau

et al., 2018), aligned with the barcoding sequences (658bp) and the metabarcoding sequences (313bp) generated in this study (See Appendix 5.5 for complete RAxML tree). The tree shows a strong support (>99%) for the node splitting into the two branches of the tree which contains published *C. pseudogracilis* and *C. floridanus* sequences. The COI barcoding and metabarcoding sequences produced in this study, clustered following the species ID assignment as *C. floridanus* or *C. pseudogracilis*. *C. floridanus* sequences we generated cluster with the Japanese (Nagakubo *et al.*, 2011) and other UK sequences (Mauvisseau *et al.*, 2018) with the same species ID. Newly generated *C. pseudogracilis* sequences cluster with *C. pseudogracilis* from Europe and USA (Slothouber Galbreath *et al.*, 2010). Of particular interest are the DNA barcoding sequences from the specimens collected in Chasewater Reservoir (See Appendix 5.5). The assignment of these sequences and their position in the tree are found to agree with the morphological identification that we carried out.

Table 1. Detection of prey DNA in the gut contents of the target *Crangonyx* species from both seasons.

Sample type		Gut contents	
Predator id		*C. floridanus*	*C. pseudogracilis*
N total Crangonyx		61	40
N Crangonyx with known interactions		57	30
N total interactions detected		58	37
N taxa in gut contents		8	11
Amphipoda	*Corophium multisetosum*	1	2
Amphipoda	*Crangonyx floridanus*	NA	22
Amphipoda	*Crangonyx pseudogracilis*	49	NA
Cladocera	*Diaphanosoma brachyurum*	1	0
Cladocera	*Sida crystallina*	2	2
Cladocera	*Chydorus brevilabris*	1	0
Copepoda	Cyclops	1	0
Copepoda	*Cyclops abyssorum*	0	1
Diptera	*Endochironomus albipennis*	0	1
Gastropoda	*Potamopyrgus antipodarum*	2	2
Oligochaeta	*Limnodrilus hoffmeisteri*	0	1
Oligochaeta	*Lumbriculus variegatus*	1	3
Oligochaeta	*Tubifex tubifex*	0	2
Tricladida	*Schmidtea polychroa*	0	1

Crangonyx trophic interactions, niche breadth and overlap

In both *Crangonyx* species the relative proportion of DNA from the gut contents samples contained mostly predator DNA, with *C. floridanus* gut contents from May and October that were made respectively for 98.49% (±0.64) and 91.37% (±1.15) by predator DNA, while 1.04% (±0.53) and 8.27% (±1.17) was prey DNA. Similarly, *C. pseudogracilis* gut contents from May and October contained respectively 99.09% (±0.40) and 80.18% (±1.70) predator DNA, with 0.66% (±0.40) and 19.65% (±1.71) of prey DNA.

We detected 95 predator-prey interactions in the 87 *Crangonyx* specimens. Thirty-seven interactions were detected in the 30 *C. pseudogracilis*, and 58 interactions were detected in the 57 *C. floridanus*. Eleven different taxa were

detected in *C. pseudogracilis*, and 8 different taxa in *C. floridanus* (Table 1). In both species the majority of interactions were reciprocal IGP between the two *Crangonyx* species, with 53% of *C. pseudogracilis* positive for *C. floridanus* (56.8% of all interactions), and 80% of *C. floridanus* positive for *C. pseudogracilis* (84.5% of all interactions, Table 1, Fig. 3). The congeneric species constitute the bulk of the prey detected in both May and October (*C. floridanus*: 14 in May, 7 in Oct; *C. pseudogracilis*: 14 in May, and 35 in Oct, Fig. 3). A second intraguild prey, *Corophium multisetosum* was also detected in both species, but in very few (2-5% of) individuals and only in May (*C. floridanus*: 2 individuals; *C. pseudogracilis*: 1 individual, Table 1, Fig. 3).

In terms of the extra-guild prey community, three prey taxa were shared between *Crangonyx* species (*Sida crystallina*, *Potamopyrgus antipodarum, Lumbriculus variegatus*). Three species were only detected in *C. floridanus (Diaphanosoma brachyurum, Chydorus brevilabris,* and *Cyclops),* while five taxa were detected in *C. pseudogracilis* (*Cyclops abyssorum, Endochironomus albipennis, Limnodrilus hoffmeisteri, Tubifex tubifex* and *Schmidtea polychroa,* Table 1, Fig. 3).

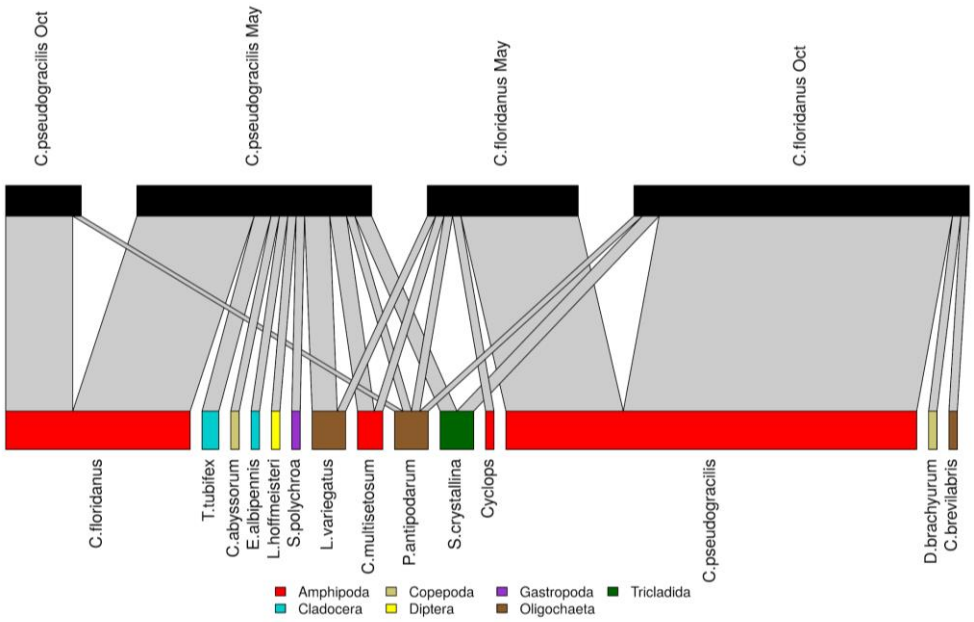


Fig. 3. Bipartite network showing DNA metabarcoding results from the gut contents of *C. floridanus* and *C. pseudogracilis* collected in Chasewater Reservoir.

The niche breadth for the 2 *Crangonyx* species (excluding cases of reciprocal IGP) was very similar, but slightly higher for *C. pseudogracilis* than *C. floridanus* (Standardised Levins Index: 0.483, and 0.374 respectively). The Pianka index of niche overlap was 66.95% between the two *Crangonyx* across both seasons. When instead we included reciprocal IGP into the Pianka index calculation, the niche overlap was 1.17%. The identity of the predator explains 39.96% of variance in gut contents (PERMANOVA Pr(>F)=0.0001, R^2=0.39959), with a small overlap between species on the NMDS (Fig. 4a); whereas we found no difference in gut communities between seasons (~1.39% of the total variance, Pr(>F)=0.0948, R^2=0.01387) (Fig. 4a).

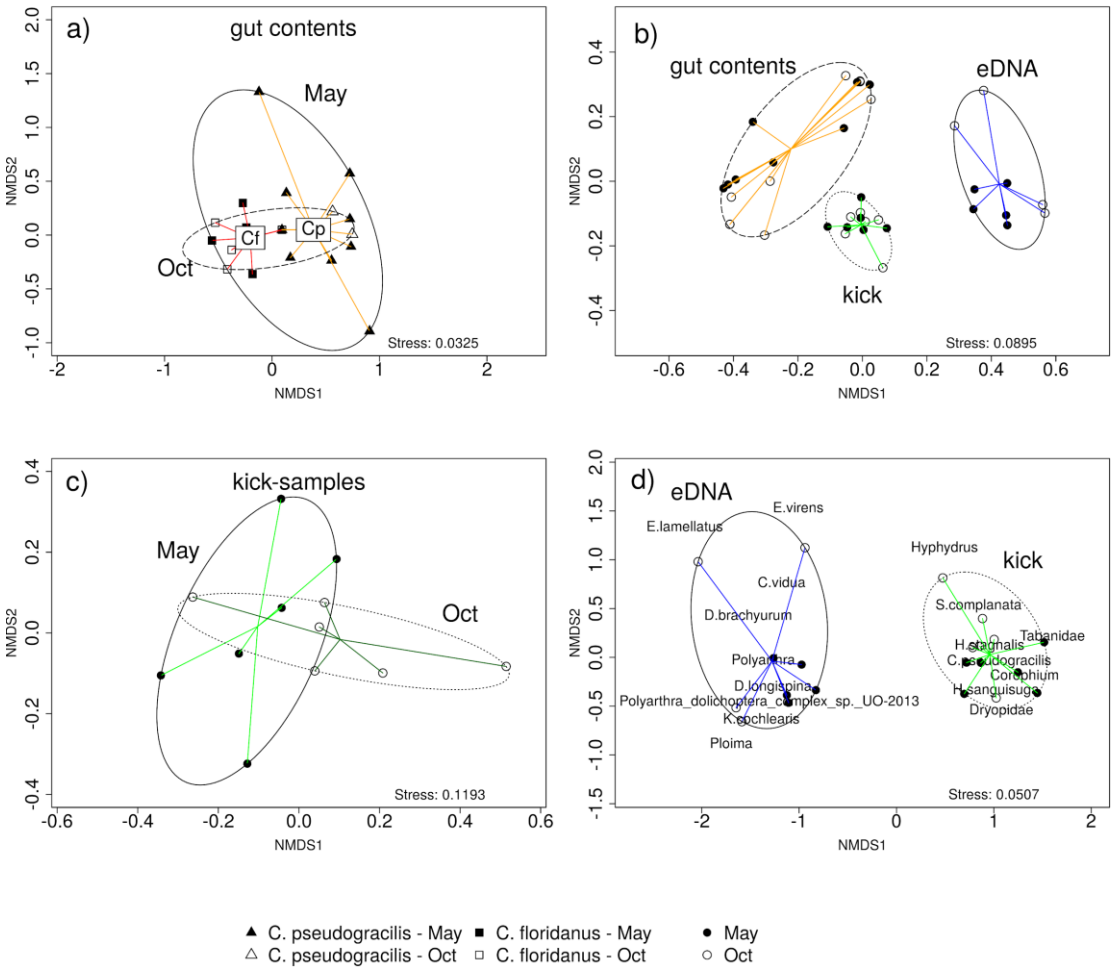


Figure 4. a) NMDS of DNA metabarcoding of gut contents of *C. floridanus* and *C. pseudogracilis*; b) NMDS comparing gut contents DNA metabarcoding, eDNA and kick-samples. Solid symbols correspond to May, hollow to Oct. c) NMDS of kick-samples by season, d) NMDS of eDNA vs kick-samples by species composition. Solid symbols correspond to May, hollow to October.

The wider benthic community available to C. floridanus *and* C. pseudogracilis

We detected 72 different taxa in the community across both seasons and eDNA and kick sampling methods, with 44 taxa uniquely detected in the kick samples and 31 taxa in the eDNA samples (Fig. 5a). Only two taxa were detected in both sample types (*Asellus aquaticus*, *Potamopyrgus antipodarum*). The 12 kick-samples (May N=6, Oct N=6) mostly included meso- and macro-invertebrates, such as Mollusca (Gastropoda), Amphipoda (Crangonyctidae and Corophidae), Coleoptera, Hirudinea, Diptera, while the eDNA samples mostly included micro- and meso-invertebrates such as Rotifers, Cladocera, Cyclopoida, and Ostracoda and macro-invertebrates like Diptera, and Gastropoda (Fig. 5a,b) (See Appendix 5.6 for the complete table). Amphipods made up approximately a quarter of the kick samples, and were almost entirely *Crangonyx,* with only 1 individual of *Corophium curvispinum* detected (Fig. 5a,d), but were not detected using eDNA (Fig. 5b). Four taxa were found in both gut contents and eDNA samples: Copepods (Cyclopoida), 2 taxa of Cladocerans (*Diaphanosoma brachyurum*, and *Sida crystallina*), and 1 Gastropod (*Potamopyrgus antipodarum*). Three taxa were detected in gut contents and kick samples: 2 Amphipods (*Crangonyx floridanus*, and *Crangonyx pseudogracilis*), and 1 Gastropod (*Potamopyrgus antipodarum*). Two taxa - Oligochaetes and Triclads - were found in the gut contents but not in the eDNA or kick samples.

The NMDS and PERMANOVA analyses demonstrate substantial differences between communities sampled by the three sample types (Fig. 4b,d). Sample type explains 41.73% of the total variance, when all three samples are analysed together (PERMANOVA Pr(>F) = 0.0001, R^2 = 0.41725, Fig. 4b), or 34.3% of the total variance when just eDNA and kick samples are included (Pr(>F)=0.0001, R^2=0.34297, Fig. 4d). By contrast, a much smaller proportion of the variance is explained by season, but this increases as the number of sample types included in the analysis decreases: i) when all three samples are included (variance explained 2.42%, Pr(>F) = 0.1396, R^2=0.02420, Fig. 4b), ii) when only eDNA and kick samples are included (6.85%, Pr(>F)=0.0345, R^2=0.06849, Fig. 3d), and iii)

when only kick samples are included (19.25%, Pr(>F)=0.0242, R^2=0.19247 Fig. 3c).

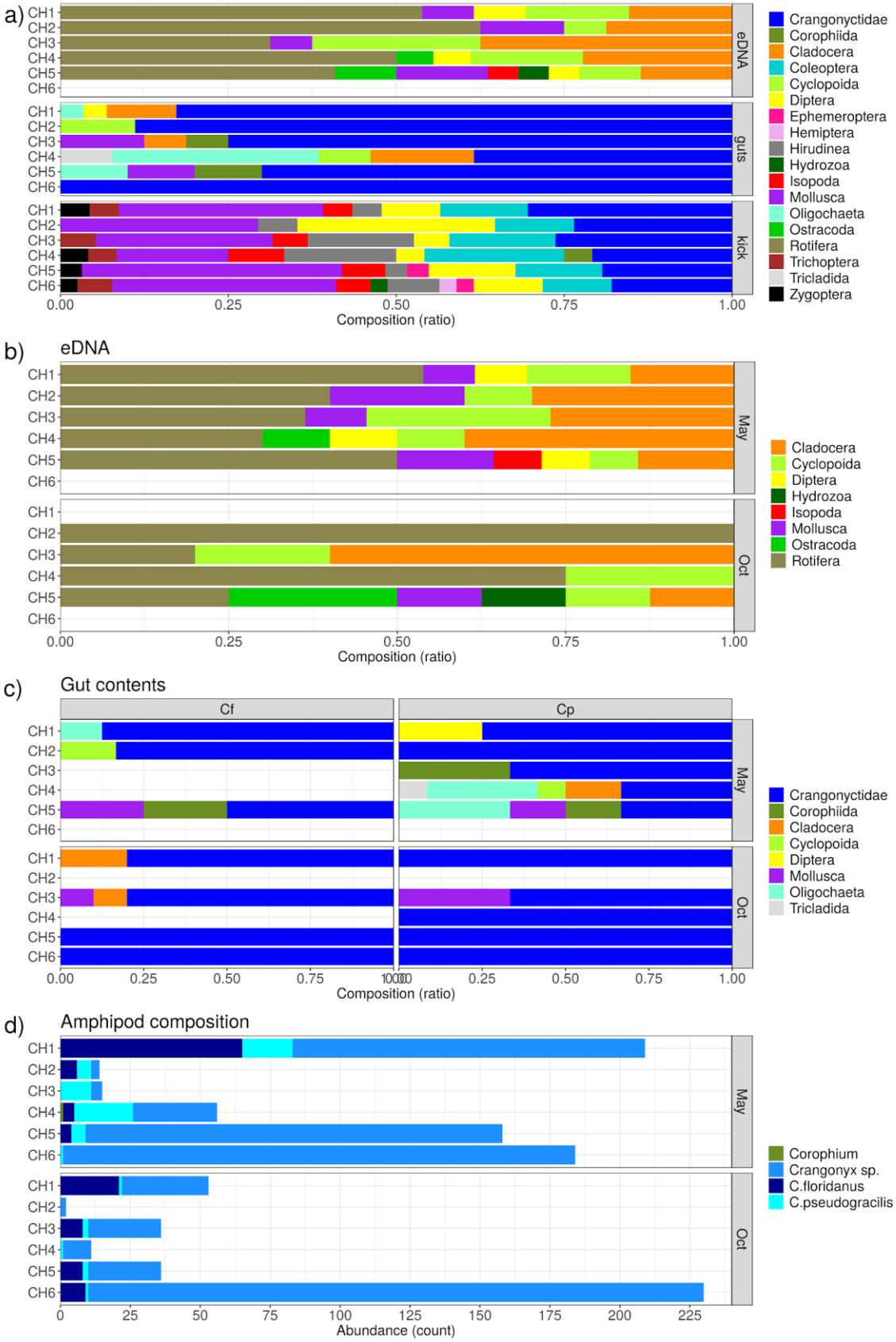


Figure 5. a) Barplot of gut contents detection (DNA read counts data), eDNA (DNA read counts data) and kick-samples community composition (count data). b) Barplot of community composition from eDNA samples (DNA read counts data). c) Barplot of DNA read counts gut contents composition excluding predator DNA (*C. floridanus* N = 15, *C. pseudogracilis* N = 23). d) Barplot of amphipoda composition from kick-samples (count data).

5.4 Discussion

In this study we confirmed that established populations of the newly recorded *Crangonyx floridanus* can be found in cohabitation with *C. pseudogracilis* in a UK lake, and that there is reciprocal IGP between the two species. The Pianka index results and the bipartite from our gut contents, excluding reciprocal IGP, show approximately 67% niche overlap between the two target species, which confirms our initial hypothesis of shared use of resources; in addition we also detected that reciprocal IGP constitute a high proportion of the species diet. The results from the IGP, supported by the bipartite and interaction strength, show also that reciprocal IGP was asymmetric with more IGP from *C. floridanus* towards *C. pseudogracilis*, than the opposite. This is in line with our initial hypothesis of greater IGP in the more recent invader. By describing the communities available to *Crangonyx* species using a combination of methods, we demonstrated that they are the dominant Amphipod in our study lake, which explains why IGP towards other species was virtually non-existent. Finally, we detected important differences between sample types in terms of community composition, and our work highlights that a combination of kick sample, eDNA and ingested DNA (iDNA) data provides a more holistic description of communities, than either method in isolation.

Cohabitation of Crangonyx floridanus and C. pseudogracilis.

We investigated the co-occurrence of *C. floridanus* and *C. pseudogracilis* using morphological identification, DNA barcoding and metabarcoding and confirmed the presence of *C. floridanus* in two locations: Chasewater Reservoir (Staffordshire) and Kings Dyke (Cambridgeshire), sites that are separated by approximately 150 km; and in only Chasewater Reservoir we could detect both species *C. floridanus* and *C. pseudogracilis* cohabiting. *C. floridanus* was recently

detected in the same county and catchment as Chasewater at Smestow Brook, and also in Windermere (Cumbria), 150 km to the north west (Mauvisseau *et al.*, 2018). These combined results suggest a widespread distribution in England, with populations distributed over at least 300 km.

The subtle morphological differences we identified between the male specimens of these two *Crangonyx* species are in agreement with previous descriptions of these species by Chapman *et al.* (2007) and confirms that both are cohabiting in Chasewater Reservoir (Chapman, 2007). Our results from the phylogenetic tree and haplotype network analysis further support the cohabitation by matching with the morphological assignment. Consistent with the results from Nagakubo *et al.* (2011) and Mauvisseau *et al.* (2018), our DNA-barcoding sequences show the presence of 18% divergence between the two species (Nagakubo *et al.*, 2011; Mauvisseau *et al.*, 2018). The topology of the tree we produced and the haplotype network analysis are also consistent with previously published COI tree from Nagakubo *et al.* (2011) about the Japanese populations of *C. floridanus* (Nagakubo *et al.*, 2011). *C. floridanus* DNA sequences produced in this study show high similarity to *C. floridanus* sequences produced from previous studies in the UK (Mauvisseau *et al.*, 2018), and Japan (Nagakubo *et al.*, 2011). We acknowledge that doubts have been raised over whether *C. floridanus* and *C. pseudogracilis* can be considered fully separate species, based on the COI and 18S trees (Nagakubo *et al.*, 2011); however, while we recognise this as an open question that needs answering, it was also beyond the scope of this study, which was aimed at confirming the identity based on the current knowledge rather than questioning the relationship of these species.

Our results from the morphology and DNA analysis confirm the identity of these two species with a high level of crypticism between them, which can explain how *C. floridanus* might have remained undetected in the UK for a long time; which differently from the detection of *Gammarus fossarum* (Blackman *et al.*, 2017), the timeframe of *C. floridanus* in the UK remains still unknown. The limited COI diversity within *C. floridanus* specimens from Japan and the UK, and the relative limited information currently available on *C. floridanus* from the native range currently makes impossible to infer the sources of the UK populations.

From a broader perspective, our results also highlight the strength, species resolution and the importance of DNA-based analysis in differentiating cryptic and closely related species (Bickford *et al.*, 2007).

C. floridanus and C. pseudogracilis trophic interactions, niche breadth and IGP

The DNA metabarcoding results we obtained from gut contents are an important first step in the understanding of dynamics between cohabiting *C. pseudogracilis* and *C. floridanus*. Niche overlap for the two species (excluding IGP interactions) was approximately 67%, however, when we included the reciprocal IGP in the analysis, the Pianka index of niche overlap resulted around 1.2% highlighting that IGP is a major component of the diet in these congeneric species. The niche breadth we calculated appeared to be higher for *C. pseudogracilis*, than *C. floridanus*.

We detected 11 and 8 prey taxa in *C. pseudogracilis* and *C. floridanus* respectively, with 4 taxa (*Corophium multisetosum, Sida crystallina, Potamopyrgus antipodarum* and *Lumbriculus variegatis*) shared between them. In addition to the shared taxa, extra guild prey included Cladocera, Diptera, Oligochaetes and Tricladida. However, the great majority of interactions were intraguild predation of the reciprocal *Crangonyx* species. Reciprocal IGP was asymmetric, with 85% of *C. floridanus* positive for *C. pseudogracilis,* compared to 57% of *C. pseudogracilis* positive for *C. floridanus*. Reciprocal IGP was documented across both seasons. The only other IGP interactions detected were towards *Corophium multisetosum* and accounted for less than 2% of *C. floridanus* and <7% of *C. pseudogracilis.* The fact that the great majority of IGP interactions involved reciprocal *Crangonyx* species can be explained by their dominance in the communities. *Crangonyx* made up approximately one quarter of the community found in our kick samples, and the only other Amphipod detected was 1 individual of *Corophium* out of 1004 of Amphipod specimens detected.

Previous studies have demonstrated both strong IGP by *Gammarus* towards *Crangonyx* and different microdistribution patterns for the two - likely resulting from predator avoidance by the latter (MacNeil, Elwood and Dick, 1999)(MacNeil, Elwood and Dick, 1999; MacNeil and Dick, 2014)(MacNeil, Elwood and Dick, 1999). It is therefore not entirely surprising that no other Amphipods (apart from small numbers of *Corophium*) were found in our kick samples. Our samples were restricted to the shoreline and it is possible that other Amphipods are present in

different microhabitats within Chasewater, and *Crangonyx* are actively avoiding them. It is also possible that establishment of *Crangonyx* was possible because of the lack of existing Amphipod intraguild predators in our study site. Indeed previous studies have suggested that *C. pseudogracilis* populations may colonize areas where native species have been lost, for example due to habitat degradation (MacNeil, Elwood and Dick, 1999; MacNeil and Dick, 2014). Further surveys of the Amphipod community and exploration of historical records are needed to distinguish between these two hypotheses.

It is well known that previous invaders may facilitate establishment of new invaders (Ricciardi, 2001) and that new invaders may displace previous ones (Dick and Platvoet, 2000). We hypothesize that the existence of *C. pseudogracilis* facilitated the establishment of *C. floridanus*, by providing an intraguild prey resource, to the extent that the newcomer is now displacing the previous invader. A next step is to investigate the dynamics of cohabiting *Crangonyx,* particularly in sites where other Amphipods (such as *Gammarus* spp.) are present as potential intraguild predators and prey.

The wider invertebrate community: comparison of sample types

Our data highlight the utility of combining kick sampling with environmental DNA and ingested DNA (iDNA) to provide a more comprehensive description of the community than can be obtained using the methods in isolation. Only one taxon was detected in all three sample types (*Potamopyrgus antipodarum*) and just two additional taxa (*Asellus aquaticus* and *Dreissena polymorpha*) were detected in both eDNA and kick samples. The low overlap in taxa detected with eDNA and kick sampling demonstrates that eDNA (at least with the assay and pipeline used here) is not a straight forward replacement for more established methods for studying macroinvertebrate communities, but instead should be considered an additional tool in the box for biodiversity assessment. However, multilocus metabarcoding (targeting groups of closely related taxa with more specific primer pairs) could soon improve comparability between eDNA and other sample types. Oligochaetes and Triclads were found in the gut contents but not in the eDNA or kick samples. This supports the use of predators (or indeed consumers and filter feeders) as natural samplers, and that iDNA can be used to detect taxa that often

go undetected using other methods (Siegenthaler et al. 2018; Schnell et al. 2018; Mariani et al. 2019).

Conclusion and next steps

We have demonstrated, using a combination of sampling methods, that non-native *C. pseudogracilis* and *C. floridanus* cohabit in the UK, and engage in high levels of reciprocal IGP. This opens further questions about the dynamics of the interaction between the two species, their relationship with the wider community, and their role in ecological networks. Presence of *C. pseudogracilis* has been suggested to benefit higher trophic levels, particularly fish such as brown trout, by providing smaller, more manageable prey items compared to *Gammarus* sp. (MacNeil, Elwood and Dick, 1999; MacNeil and Dick, 2014). Our analyses, while just a snapshot in time and place, support the idea that *Crangonyx* exert relatively little top down pressure on lower trophic levels compared to on each other. The large numbers of *Crangonyx*, and reciprocal IGP between them may therefore have positive impacts on the wider community by releasing top down pressure and providing an abundant resource for higher trophic levels. It would be interesting to investigate the temporal dynamics of this ecological network in the medium to long term to better understand the impacts of these co-invading species.

Limitations and suggestions for further research

The results we presented here are, by all means, not exhaustive because of the limitations in time replicates of the sampling, and because of the limited background information on the ecology of *Crangonyx floridanus*. The limited background information over the ecology and trophic role played by *C. floridanus* in its native range (South-Eastern U.S.) and across the invasive range needs to be further investigated to better understand the potential impacts this species can have on a longer time scale. We detected asymmetric IGP in favour of *C. floridanus*, however we could not yet infer whether *C. floridanus* is having a direct influence on the abundance of *C. pseudogracilis* and the benthic community of Chasewater Reservoir.

Based on previous studies, we expect *C. floridanus* to follow a similar invasion and establishment process as *C. pseudogracilis*; which, as reported by MacNeil

et al. (1999), was expected to be out competed by the native European Gammarid species (MacNeil, Elwood and Dick, 1999). We agree that Gammarid species might be predating on the smaller *Crangonyx*, however established populations *C. pseudogracilis* can be found widely in the UK in particular. This supports the importance of understanding the ecology and trophic interactions of species in order to evaluate how species fit into the community that surrounds them, and in case of non-native species their potential impacts on the community (Hall, 2011).

On the methodological aspect, we acknowledge the potential presence of tag-jumping in our DNA metabarcoding data, however as Schnell *et al.* (2015) report, we expect them to affect a relatively low percentage from the total number of reads, with a further mitigation from the inclusion of the chimera detection analysis. To further limit the possibility of misidentification of the two consumers, we also used a high percentage threshold (> 80%) of DNA reads within the samples to assign the consumer taxonomy. This approach is far from ideal, but the over abundance of consumer DNA in molecular data, which is often a technical challenge in molecular analysis of trophic interactions (Vestheim and Jarman, 2008; Piñol *et al.*, 2014, 2015), in this case provided extra support on the correct identification of the consumers.

The use of ingested DNA for community assessment is also a topic that can potentially expand the way we survey for biodiversity by using predators and scavengers gut contents (Schnell *et al.*, 2018; Siegenthaler, Wangensteen, Soto, *et al.*, 2018; Mariani *et al.*, 2019). The benefit of these approach is demonstrating how one sampling can appear to be applicable to different objectives, since from the gut contents we can extrapolate both the information on trophic interactions and the information on the members of the community available. The inclusion of omnivores species, which interact with multiple trophic levels at the same time, could even further expand the information we can obtain since we could use multiple molecular assays to access the multi-trophic information that omnivores possess (Stat *et al.*, 2017).

5.5 Conclusion

Here we brought direct evidence of the cohabitation of *Crangonyx floridanus* and *Crangonyx pseudogracilis* in UK lake using morphological and molecular

analysis to confirm the identity of the two species in question. We further used DNA metabarcoding on the gut contents from both *Crangonyx* species to describe their trophic interactions with the benthic community and with each other.

About the trophic interactions, our study shows an asymmetric reciprocal IGP from *C. floridanus* against *C. pseudogracilis*; however, the presence of bidirectional reciprocal IGP might potentially indicate a pressure on limiting the population growth of *C. floridanus* by *C. pseudogracilis*. The limitation of an invasive non-native species population by the presence of a closed related species appears in agreement with the expected patterns explained by part of the Darwin's Naturalization Hypothesis/Conundrum (Li *et al.*, 2015), and by studies on intraguild predation of close-related species (Ricciardi and Atkinson, 2004). IGP interactions have also the potential to influence the biodiversity of local invertebrates communities, by releasing potential shared preys (MacNeil, Elwood and Dick, 1999), and by enhancing local biodiversity (Wang, Brose and Gravel, 2019). Our results also confirm our initial hypothesis of a strong overlap in the use of resources between these two taxa, supported by the high niche overlap and the bipartite network. We ultimately provided another example of how ingested DNA could provide a broader picture in describing communities, integrating eDNA, and standardised sampling with molecular trophic interactions. We presented here an overview of trophic interactions that *Crangonyx floridanus* and *Crangonyx pseudogracilis* have with their associated community, including asymmetric reciprocal IGP interactions.

Chapter 6 - General discussion

The focus of this thesis was to investigate trophic interactions of three invasive arthropods in the UK using DNA metabarcoding of gut contents. I initially explored the possibility to detect prey DNA in predators' gut contents with DNA metabarcoding in controlled laboratory experiment using one of the three invasive non-native species (INNS): *Dikerogammarus villosus, Crangonyx floridanus* and *Harmonia axyridis* before applying the same methodologies on a larger scale in field conditions to all target INNS. The overarching idea was to describe the trophic interactions of the three target INNS, to lead to a better understanding of the impact they have on invaded invertebrate communities and, in particular, on native members of the same guild. The broad goals of this thesis were to: a) prove the concept of detecting prey DNA in the predator gut contents in controlled conditions (Chapter 2), b) investigate the predation rate of INNS towards their communities in field conditions (Chapter 3, 4), and c) investigate the predation rate of INNS towards UK native species that belong to the same guild as the INNS (defined as intra-guild predation or IGP) (Chapter 3, 5).

6.1 INNS and trophic interactions

Previous studies have demonstrated that even single INNS introduced into a novel site can lead to dramatic modifications in the ecosystem and in the community interactions (Walsh, Lathrop and Vander Zanden, 2017). Detecting trophic interactions and studying the impact of INNS within ecological communities are not novel fields of research (Ings, Montoya and Bascompte, 2009). However, the resolution and sensitivity that DNA metabarcoding is enabling has the potential to massively improve our understanding not only of the processes shaping the ecological communities but also to better understand how species influence ecosystem health and ecosystem functioning (Pyšek and Richardson, 2010; Landi *et al.*, 2018). This thesis can contribute to the understanding of the trophic interactions of INNS by describing the diet of three generalist invertebrate predators in the UK; including instances of intraguild predation (IGP) which I will describe further below.

The methodology used in this thesis (DNA metabarcoding) is being widely applied for describing species communities and for detecting trophic interactions across a wide variety of systems (De Barba *et al.*, 2014; Kitson *et al.*, 2018; Pansu *et al.*,

2018). However as Thomas *et al.* (2014) demonstrated with their controlled experiment on sea lions diet, the DNA results and prey DNA proportions obtained from the consumers do not directly correlate with the actual consumer diet for biological (e.g. differential digestion of the consumers), and methodological biases (differential prey DNA amplification of the primers) (Thomas *et al.*, 2014). Controlled feeding experiments are a great tool for accounting individual biases avoiding the noise and variation present in wild conditions (Aebi *et al.*, 2011). In Chapter 2 I provide an example of these controlled feeding experiments with the scope of proving the concept of being able to detect prey DNA in gut contents. In particular I wanted to detect DNA from a single prey individual of the size of *Daphnia magna* in the gut contents of a much bigger consumer (*Dikerogammarus villosus*), which I was able to do after 4 h from a predation event, and the probability of detection increases with bigger prey species (like *Asellus aquaticus*), which I was able to detect even after 36 h from a predation event. Digestion time and prey size, are important parameters to understand and interpret the DNA results obtained from field conditions but they are not the only parameters influencing the detection of prey species, e.g. digestion rates and ingested quantity (discussed below in “Challenges and Opportunities of MATI”).

In Chapter 3 I have focussed on field detection of trophic interactions of *D. villosus*, using the UK native *Gammarus zaddachi* as control species, across two seasons. I found that only a small percentage (between 4.3% and 18%) of *D. villosus* individuals analysed were positive for prey DNA, in comparison to the native Amphipod for which between 43% and 75% of the individuals were positive for prey DNA (Chapter 3). These results rise again the question regarding the predatory behaviour of *D. villosus*, which is known to be a facultative omnivore (Platvoet *et al.*, 2009) but is also often considered a voracious predator (Rewicz *et al.*, 2014). Koester *et al.* (2016) described the diet of *D. villosus* (Amphipoda) integrating single-target molecular assay and stable isotopes, and found that this invasive amphipod in field might predate less than expected from feeding experiments (Koester, Bayer and Gergs, 2016). The results from Chapter 3 are indeed representative of only 2 seasons, thus prone to being influenced by natural variation in diet, however the agreement with previous field studies seem to indicate that the diet of *D. villosus* might be more varied than initially expected, with predation being a smaller component than initially thought (Koester, Bayer

and Gergs, 2016, Chapter 3). Therefore we might be missing important non predatory trophic interactions created by *D. villosus*, such as detritivory (Little and Altermatt, 2019), that would require further investigation.

Chapter 4 was focussed on the methodological challenge of the prey DNA recovery from gut contents (Vestheim and Jarman, 2008), and on the predatory activity of *Harmonia axyridis* (Coccinellidae) in the UK across a spatial and temporal transects. I have developed a set of blocking primers that were tested in a paired experiment that demonstrate the increased proportion of prey DNA recovered from gut contents without accidental co-blocking of DNA from prey species. Regarding the field samples I demonstrated that the diet *H. axyridis* seems to be influenced by both the season and the location, with *Euceraphis* (Aphididae) that represented the majority of the interactions detected (~74%). I did not detect any IGP interactions, however I detected interactions from the parasitic *Dinocampus coccinellae* (Hymenoptera) in 13 individuals including one detection confirmed by microscopy. These results are in agreement with the expected diet of *H. axyridis* based mostly on aphids and barkflies (Pell *et al.*, 2008; Roy *et al.*, 2016). The lack of detection of IGP in *H. axyridis* from field conditions is another important element under discussion, since previous studies also detected low levels of IGP (<10%) (Brown *et al.*, 2015) although in contrast with the reported decline in UK native Coccinellidae caused by *H. axyridis* IGP (Brown *et al.*, 2011; Roy *et al.*, 2012).

The last data chapter, Chapter 5, focussed on trophic networks and IGP of two congeneric and cryptic Amphipod species (*Crangonyx pseudogracilis* and *C. floridanus*) that I detected cohabiting in the same lake. The results of this chapter demonstrate the importance of integrating DNA barcoding and morphology to correctly identify the two cryptic *Crangonyx* species, similarly to the work carried out by Mauvisseau *et al.* (2018); but with the added information of the haplotype differences between the two *Crangonyx* (Mauvisseau *et al.*, 2018, Chapter 5). Secondly, I described the trophic interactions of each species across two seasons, detecting also the IGP interaction between the two species which I found being asymmetrical in favour of *C. floridanus* against *C. pseudogracilis*.

6.2 Intraguild predation (IGP)

Detecting and describing IGP was one of the main goals of this thesis, and I successfully managed to describe instances of IGP involving my target species. The role played by IGP in ecological communities has started to interest since the late 1980s with Polis *et al.* (1989) who formalised this interaction and its potential role (Polis, Myers and Holt, 1989). Currently IGP is being investigated both for it role played in natural and invaded communities, with natural communities that seems to benefit from IGP with an improvement in biodiversity and an improvement of ecosystem functioning (Wang, Brose and Gravel, 2019). In invaded communities instead the role of IGP is still debated upon, since there is evidence that IGP from INNS can lead to the elimination or substitution of native species (Kestrup, Dick and Ricciardi, 2011), however there is also evidence that IGP can lead to a coexistence between INNS and native guild members (Henkanaththegedara and Stockwell, 2014).

In this thesis I mainly focussed on detecting IGP from the target INNS in the invaded ecosystems (Chapter 3 and 5), however I also included some data on IGP in native communities (Chapter 3). Based one the results from several authors who pointed at a higher IGP from the INNS against native species (Dick and Platvoet, 2000; Snyder, Clevenger and Eigenbrode, 2004; Hall, 2011), my initial predictions were that my target INNS had a higher IGP rate that would promote their success in the invaded sites. Contrary to my initial predictions, in Chapter 3 I found a low rate of IGP from *D. villosus* in the invaded sites, while I found a higher rate of IGP in the native Amphipod *Gammarus zaddachi* in a non-invaded site. Although initially surprising, the low IGP rate (16%) in *D. villosus* I detected appear in agreement with the results from Koester and Gergs (2014), who detected comparable low rates (16%) of IGP at this Amphipod at the invasion front in Germany (Koester and Gergs, 2014, Chapter 3).

In contrast with these results, and in agreement with my initial predictions that the more recent INNS would have stronger IGP (Hall, 2011), I demonstrated that IGP instead constitutes a large proportion of the diet in two cohabiting species, *Crangonyx floridanus* and *C. pseudogracilis* (Chapter 5). The challenge in interpreting ecologically the results were mainly influenced by the sparse literature existing on *C. floridanus*, and the relative unknown aspect of the cohabitation with the congeneric species (Chapter 5). Interestingly, the IGP in this

case was asymmetric in favour of *C. floridanus*, that we assume being the more recent invader in the UK (Mauvisseau *et al.*, 2018), against *C. pseudogracilis*, resident in the UK since 1960s (Garland, 1980); which seems to find support in the potential invasiveness that *C. floridanus* showed to have in Japan (Nagakubo *et al.*, 2011). Ultimately my results on the IGP from *Harmonia axyridis* appears to be in agreement with the low detected rate of IGP that has been recorded among ladybirds in field conditions (Brown *et al.*, 2015), Chapter 4); although I expected to detect IGP in field conditions especially at the core of *H. axyridis* invasion range in the UK (Thomas *et al.*, 2013; Roy and Brown, 2015).

Regarding the apparently contradicting results on IGP I obtained in this thesis (Chapter 3, 4 and 5), there are several reasons that might explain them. Ontogenetic diet shift might be behind the lack in IGP of *H. axyridis* since Snyder *et al.* (2004), and Ware and Majerus (2008) reported that IGP to be more relevant during the larval stages than in the adults (Snyder, Clevenger and Eigenbrode, 2004; Ware and Majerus, 2008). It would be interesting to compare *H. axyridis* larvae and adults in field mesocosms and in open field conditions to better explore the IGP in ladybirds communities across the larval and adult stages. In *D. villosus* results the IGP rate I detected could be dictated by the low abundance of IG preys in the investigated sites. This aspect might indicate that the community is already deeply modified by the invader; although this element still wouldn't explain Koester and Gergs (2014) results which were obtained from the front of the *D. villosus* invasion (Koester and Gergs, 2014). Hence in *D. villosus*, as demonstrated by the study from MacNeil and Briffa (2019), different non predatory processes might be at play (MacNeil and Briffa, 2019). In Chapter 5 instead the temporal resolution and the data I have collected don't yet allow to better infer a link between the predation rate of *C. floridanus* with the reduced abundance of *C. pseudogracilis* in the site across both seasons.

6.3 Non-IGP interactions

Describing and studying trophic interaction in field conditions poses challenges and should not surprise to lead to unexpected results. Although the focus of this thesis was on IGP interactions between INNS and the native counterparts, the

presence of potential non-IGP processes and interactions (e.g. spillover, competitive exclusion hypothesis) need to be taken into consideration.

In chapter 4 I did not detect IGP interaction between *H. axyridis* and native Coccinellidae from molecular data. The gut contents results however, showed that a large proportion of *H. axyridis* diet was composed by species that were in high proportions in the communities, and that would also have been included in the diet of native aphidophagous ladybirds (Sloggett, 2008). The lack of a comparative analysis in my thesis between the invasive and native ladybirds from the same site over a larger period of time is at the base of my lack of support to the competitive exclusion hypothesis.

Another aspect that worth metnioning is the detection of *D. coccinellae* in the invasive species. Previous study by Comont *et al.* (2014) showed a lack of parasitism of *H. axyridis* by *D. coccinellae* in a comparative study between invasive and native ladyrbids in controlled mesocosms across England; however the incedence of parastism in field conditions is still not fully explored. In my study I detected 13 individuals parasitised by *D. coccinellae* (~4% of the total individual sampled), with one case that was also morphologically confirmed during dissection (See Appendix 4.1). The implications of these detections cannot yet be extrapolated to confirm macro processes in the invasion of *H. axyridis*; but it can provide an initial step in the investigation of it as a spillover event (Roy and Lawson Handley, 2012).

6.4 Challenges and opportunities in MATI

The molecular analysis of trophic interactions (MATI) is massively increasing the resolution at which we can investigate diet and community networks, but it also introduces challenges, both regarding the methodology and the ecological interpretation.

In Chapter 2 I attempted to explore the digestion rate of one of my target species (*Dikerogammarus villosus*) and the detection of three prey species using DNA metabarcoding. While I was able to detect single specimens in a consumer gut contents for all of my three prey species; including species like *Daphnia magna* (Cladocera) and *Crangonyx pseudogracilis* (Amphipoda) that are much smaller than my consumer, it was not possible to estimate the digestion rate of the

consumer through a time series due to high mortality of specimens during the trials (which meant I did not have enough statistical power for the digestion rate analysis). It is not possible to fully represent the complexity of wild conditions within a controlled feeding experiment, however the importance of carrying out this type of experiments still needs to be highlighted (Greenstone *et al.*, 2014). Feeding experiments allow for a better understanding of the variables, in isolation from others, influencing the digestion and molecular detection of consumed items in the consumers diet, and the results can inform the ecological interpretation from the field studies. For example the digestion rate of the consumer can dictate how long prey DNA can still be detected in the consumer from a predation event; hence understanding if a consumer has a slow digestion, like spiders (Waldner *et al.*, 2013), or a faster digestion, like ladybirds (Thomas *et al.*, 2013), defines the temporal resolution of the diet under investigation. Overall in my feeding experiment I could detect the smaller species no more than 4 and 8 hours, while the larger specie could be detected up to 36 hours from a predation event (Chapter 2). These detection times seem to indicate a fast digestion rate set with detection limit counted in hours more than days; however, replicating the feeding experiment with preys of different sizes might provide further information.

In consideration to the results I obtained in this thesis, especially in Chapter 2, ingested eDNA might not be as influential as I might expect, and with already existing contamination control and quality control systems we might be able to remove this potential bias. Although I believe it would still be interesting to set up a controlled experiment to verify this assumption, by exposing a target specie (e.g. an aquatic species) known volume and known concentration of DNA. If set up in a time series, it could provide a better understanding if this potential bias is already removed during current quality control and contamination thresholds processes. Overall however this type of bias need to be framed with in mind the ecology of a species; because in the case of invertebrate facultative omnivores, like *D. villosus*, the detection of large vertebrates DNA like birds, which I detected in the raw data of Chapter 3, might indicate a scavenging behaviour on carcasses, or a potential coprophagy rather than actual interaction (Chapter 3). On the topic of contamination control, the common practice is to have environmental (e.g. sampling blanks), laboratory (e.g. dissection blanks), and procedural (e.g. PCR blanks and PCR positive) control samples to be added to the experiment design. From those samples we can then calculate a fixed or

species-specific threshold to be applied to our dataset in order to filter out not reliable results. This is a conservative method, and it is the system that I have applied in all the data chapters in this thesis (Chapters 2-5). Applying contamination thresholds is assumed to remove the bias from different sources of contamination; however in the case of trophic interactions with high proportion of consumer DNA (>98% as shown in Chapter 4) too stringent thresholds might cause the loss of important interactions.

The important aspect in analysing molecular trophic interactions of minimizing the ratio of consumer DNA to prey DNA. Throughout my thesis, I tried to minimize the number of predators' DNA reads by using only the digestive tract in DNA extractions. However, despite this, the proportion of DNA reads were heavily biased towards the consumers, this was the case for *D. villosus* and *H. axyridis*, for which I obtained respectively 98.97% and 99.82% of mean proportion of consumer DNA compared to 1.03% and 0.18% of mean proportion of prey DNA (Chapter 3, 4). The target species that better performed in field conditions was *G. zaddachi* (Chapter 3) in which the mean proportion of consumer DNA was 90.46% against mean proportion of prey DNA of 10.54% (Chapter 3).

I succeeded in developing a set of blocking primers for *H. axyridis* that resulted in approximately twenty-fold improvement in the number of prey reads (96% consumer DNA against 4% of prey DNA); however the results I obtained are far from the results obtained in other studies that used a similar method which achieved close to 100% of prey DNA (Vestheim and Jarman, 2008). Following Vestheim and Jarman (2008) study, the primers I developed reflected the location of the binding site, and the primer type that appeared to better inhibit consumer DNA; which meant blocking primer binding site to partially overlap with the Leray-Geller binding site (Geller *et al.*, 2013; Leray, Yang, *et al.*, 2013) and that included a 3'-C3 (three carbon chain) spacer to prevent polymerase binding (Vestheim and Jarman, 2008). The results I obtained could be explained with the blocking primer concentration that was too low and wasn't able to fully compete with the universal primers. In general, I found that attempting to find a specie-specific region overlapping with a universal binding region is quite challenging and prone to issues.

Overall I agree with other studies, such as Pinol *et al.* (2014), in that introducing blocking primers might introduce a further bias in the results because of

accidental co-blocking (Piñol *et al.*, 2014); however the field will surely benefit from finding a method to reduce the consumer DNA especially (as in this thesis) when investigating invertebrate-invertebrate interactions with universal primers. The use of restriction enzymes in molecular analysis is long established, and they are extensively used in population genetics approaches such as RADseq (Davey and Blaxter, 2010). The potential application of restriction enzymes to break consumer DNA in specific regions (generally 5-8 bases long) as a method to inhibit amplification of consumer DNA was explored by Juen *et al.* (2012) and by Leray *et al.* (2013), but with mixed success because of the requirements of DNA fragment length needed to recover prey DNA: specifically while shorter DNA fragments allow for a better detection chance in the digestion tract, longer DNA fragment allows for a better chance of species-specific restriction sites (Juen *et al.*, 2012; Leray, Agudelo, *et al.*, 2013). One method that is worth exploring is the use of Crispr/Cas9. The basis of Crispr/Cas9 is that it uses a 20-nucleotide sequence on the guide RNA to target the correct region in the DNA (Jiang and Doudna, 2017). The inclusion of 20-nt sequence makes Crispr/Cas9 a promising candidate for specie-specific inhibition of consumer DNA, through a set of potential interventions such as substituting part of the consumer binding site with a non-binding sequence, or breaking the consumer DNA (which remove the possibility of having both universal primers binding sites). This long sequence to target the correct region allows also for effective work in the short DNA fragments (in my case it was 313bp fragment) required and recommended in molecular trophic interactions analysis.

6.5 Limitations of DNA metabarcoding

Although the use of molecular methods to detect DNA from the environment is improving thank to the changes in the sequencing technology, which leads also to an increasing its applications, there is the need to highlight some of the limitations that I have encountered during the thesis. I will not discuss the limitations in the general field of molecular ecology (Pawlowski *et al.*, 2018), rather I will try and focus on the limitations more pertinent to the use of molecular data for trophic interactions and community analysis.

As demonstrated in Chapter 4, the detection of prey species in the gut contents of a predator is challenging when using universal primers due to the

overamplification of the predator DNA (Piñol *et al.*, 2015). While in my study I attempted to develop and implement consumer-specific blocking primers following the study by Vestheim and Jarman (2008), I found a dramatic change when moving from *in silico* and *in vitro* testing, to the field samples which obtained a much reduced blocking than the tests. This issue highlights one of the main limitations in the molecular analysis of trophic interactions, which is the amount of information that can be lost when using universal primers; and especially in field samples this can reduce the interactions that can be detected. As described by Pinol *et al*. (2015), most of these missed interactions can be expected to be rare; however, depending on the focus of the study they can still be considered valuable and important to be detected and described which could justify the development of blocking primers.

Another ~~One~~ potential limitation of MATI in the field is that it is difficult to distinguish active predation from passive ingestion of environmental DNA, although the ingestion of eDNA can be considered minimal in comparison to the ingestion of tissue material (e.g. active predation), it could lead to overestimating the number of interactions in the consumers. Currently whether DNA metabarcoding is sensitive enough to be able to detect this signal is not fully clear, and to what extent this issue might introduce biases is not quantified.

Overall, the current inability of DNA metabarcoding to describe the temporal frame of a predation event is an element that will need to be improved and developed. The use of qPCR assays is helping in understanding how long prey DNA resides in the digestive systems of specific consumers (e.g. Kamenova *et al.*, 2018); however the application of this information to field conditions cannot yet be achieved which can ultimately influence our ability to detect an interaction. This temporal perspective brings another dimension to the concept just expressed of accepting the exclusion of rare and elusive interactions, because while the decision over whether an interactions is rare happens when interpreting the results from a large number of consumers; we can risk of introducing a further bias caused by the time past from the predation event if a not sufficient number of specimens is collected.

6.6 Further directions

The results I obtained about INNS trophic interactions and IGP highlights important strengths of this field and further paths that could be interesting to investigate in the future. Ecological communities and their networks need to be considered as dynamic and in constant change, following natural variability and fluctuations (Mittelbach and Schemske, 2015); therefore species interactions and community studies need to better include this dynamism with longer temporal transects.

In Chapter 3, 4 and 5, I've found that the short temporal resolution I had (2 seasons) allowed for a good preliminary understanding of the interactions, but it did not allow for a proper assessment of the long term diet breadth and INNS impacts. This was particularly the case in Chapter 4 and Chapter 5. Chapter 4 was planned to include a longer 7-months temporal transect, in parallel to the spatial transect that I presented in this thesis; but due to time constraints and cost limitation, I could not process the extra samples. In Chapter 5 instead, I've demonstrated the trophic interaction of congenus species in cohabitation, but again I've shown that I could detect an asymmetric intraguild predation (IGP) in favour of the most recent INNS *Crangonyx floridanus* against *C. pseudogracilis*, although I was not able to infer an actual impact on the population of this latter based on the data I had.

These snapshots of the trophic analysis are still valuable and massively important if framed with the right goals and objectives. The temporal resolution I planned was a suitable time for the aims and objectives of my thesis, and hopefully will be useful in future studies. The same applies for the communities, for which to understand the impacts and changes of novel species on local community networks and on other guild member species, there is the need of a longer temporal replicates. Longer temporal resolutions are a key element to assess interactions, and broader communities changes (e.g. INNS impact assessment) without the potential noise from natural population dynamics. They can lead to important results, as demonstrated by the long term survey of ladybirds population changes in the UK following *H. axyridis* invasion (Roy and Brown, 2015).

On a smaller scale, it can be essential to include and investigate the ontogenetic diet shift of species (Reum *et al.*, 2019). There are plenty of examples of species

that go through a change in diet during their life stages, including *H. axyridis* which is considered to be a stronger intra guild predator during the larval stage than when adult (Wells *et al.*, 2017); however, whether this process happens to other INNS, like *D. villosus*, is not clear and might open for interesting research. This path of research might improve our understanding of impact potential of INNS by understanding not only the ability of propagules to establish in novel habitats, but also to better establish whether and how different life stages can impact the invaded community and the invaded ecosystem. I see this information potentially better feeding into the work of 'horizon scanning' that has been done for the UK, and recently for Antarctic Region, in the form of prioritising pathways, understanding the life stage with better chances of establishing in a novel environment, by understanding the life stage(s) that might cause more impact on ecosystem functions and local communities, and therefore driving conservation, prevention and eradication planning (Roy *et al.*, 2014; Hughes *et al.*, 2020).

6.7 Final remarks

This thesis attempted and succeeded in describing the diet of target INNS by understanding to what extent INNS uses their communities and what trophic interactions they were creating, including IGP against other guild member species. As I've shown, DNA-based analysis (in this thesis DNA metabarcoding) are massively increasing our ability to investigate large-scale systems, at a reduced cost per sample. This cost is also expected to be further reduced as the read depth of the sequencing platform increases (e.g. MiSeq with 8-25 million reads per run, and the NextSeq with 130-400 million reads per run), and as new platforms are developed and become more accessible (e.g. Nanopore platforms, Ion Torrent sequencer, among others).

In the future I see the field of trophic interactions (including INNS), community analysis and ecological genomics massively increasing in strength and in teasing apart ecological links that are still unclear. The massive strength in ecology is ultimately the temporal resolution, and larger scale processes happening in ecosystems and communities, and on this DNA-based analysis (DNA metabarcoding) can be massively beneficial (Bohan *et al.*, 2017).

Appendices

Chapter 2 - Appendices

Appendix 2.1 - Feeding trials arena

Figure S2.1. Image showing the organisation of the feeding trials arenas with one individual predator. Each arena contained 100 mL of water and one pebble to act as substratum for the predator (visible in the centre).

Appendix 2.2 - Sequencing primers

Table S2.1. PCR 1 sequencing primers (Forward)

Primer name	Tag name	pre-adapter	Sequencing primer sequence	First i7 sequence	heterogeneity spacer	Farward locus primer
het_LeRayF_N701	N701	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCGCCTTA		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	N702	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CTAGTACG	T	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	N703	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TTCTGCCT	GT	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	N704	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GCTCAGGA	CGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	N705	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGGAGTCC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	N706	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CATGCCTA	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	N707	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GTAGAGAG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	N708	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CCTCTCTG	CCTGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	N709	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGCGTAGC		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N710	N710	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CAGCCTCG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	N711	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TGCCTCTT	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	N712	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCCTCTAC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC

Primer name	Combined Forward primer sequence
het_LeRayF_N701	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGCCTTAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTAGTACGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCTGCCTGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTCAGGACGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGAGTCCATGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGCCTATGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGAGAGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTCTCTGCCTGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGTAGCGGWACWGGWTGAACWGTWTAYCCYCC

het_LeRayF_N710	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCCTCGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCCTCTTTGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCTCTACATGAGGWACWGGWTGAACWGTWTAYCCYCC

Table S2.2. PCR 1 sequencing primers (Reverse)

Primer name	Tag name	**pre-adapter**	**Sequencing primer sequence**	**First i5 sequence**	heterogeneity spacer	**Reverse locus primer**
het_LeRayR_N501	N501	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TAGATCGC		TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	N502	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTCTCTAT	T	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	N503	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TATCCTCT	GT	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	N504	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AGAGTAGA	CGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	N505	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	GTAAGGAG	ATGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	N506	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	ACTGCATA	TGCGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	N507	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AAGGAGTA	GAGTGG	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	N508	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTAAGCCT	CCTGTGG	TAIACYTCIGGRTGICCRAARAAYCA

Primer name	Combined Reverse primer sequence
het_LeRayR_N501	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGATCGCTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCTCTATTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCCTCTGTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAGTAGACGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAGGAGATGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTGCATATGCGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGGAGTAGAGTGGTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAAGCCTCCTGTGGTAIACYTCIGGRTGICCRAARAAYCA

Appendix 2.3 - Creation of DNA database

The curated database of COI sequences using for metabarcoding analysis was created from the list of UK invertebrates species that was compiled by the Centre of Ecology and Hydrology (available at: https://www.ceh.ac.uk/services/coded-macroinvertebrates-list).

From this species list, each order was analysed separately but following the same workflow; with the only exclusion of Diptera that could not be included in the database because the issues in the taxonomy and in the number of records.

Each order was processed with Reprophylo pipeline (Szitenberg *et al.*, 2015). This includes using the species list by order to download the COI sequences from GenBank and EMBL, in the eventuality that the sequences were not available then the genus was used to look for available COI sequences. The sequences were first clustered at 100% similarity using VSEARCH v1.1.0 (Rognes *et al.*, 2016), then they were filtered to keep all sequences equal or longer than 500 bp. The retained sequences were then aligned with MUSCLE (Edgar, 2004), and trimmed using trimAl (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The trimmed sequences were controlled using SATIVA (Kozlov *et al.*, 2016) to highlight potential mislabelled records that were then removed from the dataset. Ultimately the sequences were used to construct a Maximum Likelihood tree using RAxML v8 (Stamatakis, 2014).

The final databases created included: 412/423 Coleoptera species (97.3%), 54/59 Odonata species (91.5%), 83/92 species between Ephemertoptera/Plecoptera/Neuroptera and Megaloptera (90.2%), 187/206 species between Trichoptera and Lepidoptera (90.8%), 53/114 species between Hemiptera and Hymenoptera (46.5%), 154/388 Crustacea species (46.3%), 78/111 Mollusca species (70.3%), 333/333 Arachnida species (100%), 129/152 Annelida species (84.9%). All databases have been deposited at the link: https://osf.io/j9adr/ (doi: 10.17605/OSF.IO/J9ADR)

Appendix 2.4 - Prey DNA detection by time

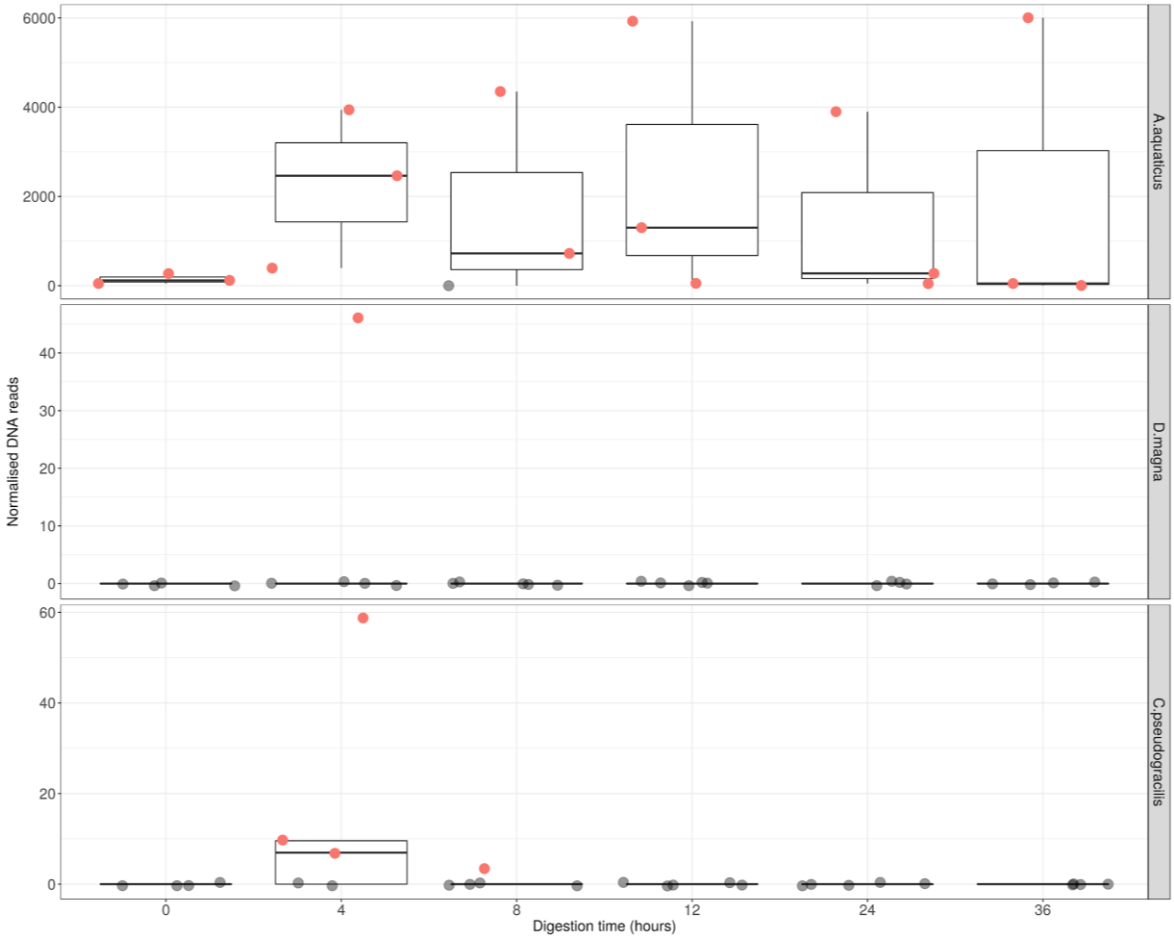


Figure S2.2. Boxplot showing the normalised DNA reads of each known prey in D. villosus gut contents from controlled mesocosms at different digestion times (0h to 36h) after a known predation event.

Appendix 2.5 – Benthic invertebrates community from kick sampling

Sample type	Kick sampling									
Lake	GW				WB					
Site	GW1	GW2	GW3	GW4	WB1	WB2	WB3	WB4	WB5	WB6
Dreissena polymorpha	0	0	0	0	1	1	2	0	0	1
Gammarus tigrinus	0	0	2	4	0	0	0	0	0	0
Chironomidae	134	43	136	101	10	2	4	2	11	10
Dikerogammarus villosus	16	42	7	11	25	26	54	117	73	137
Asellus aquaticus	9	0	0	0	0	0	0	0	0	0
Bithynia tentaculata	0	1	0	0	0	0	0	0	0	0
Caenis robusta	0	1	0	0	0	0	0	0	0	0
Corixidae	0	0	4	0	0	0	0	0	0	0
Crangonyx pseudogracilis	2	0	0	0	0	0	0	0	0	0
Dolichopodidae	0	0	1	0	0	0	0	0	0	0
Dytiscidae	0	0	1	0	0	0	1	0	0	0
Eurycercus sp.	0	0	35	1	0	0	0	0	0	0
Gammarus sp.	0	0	1	0	0	0	0	0	0	0
Helobdella sp.	0	0	1	0	0	0	0	0	0	0
Leptoceridae	0	0	1	0	0	0	0	0	0	0
Lymnaea auricolaria	0	1	0	0	1	0	0	0	0	0
Lymnaea sp.	0	0	1	0	0	0	0	0	0	1
Lymnaea truncatula	0	1	0	0	0	0	0	0	0	0
Nematocera	1	2	3	3	0	0	0	0	0	1
Pisidium sp.	0	2	0	0	0	0	0	0	0	0
Planorbis vortex	0	1	0	0	0	0	0	0	0	0
Tipulidae	1	1	1	0	0	0	0	0	0	0

Tubificidae	0	0	44	10	0	0	0	0	0	0
Valvata piscinalis	0	3	1	0	0	0	0	0	0	0
Brachycera	0	0	0	0	0	0	0	0	1	0
Caenis luctuosa	0	0	0	0	1	0	0	0	0	0
Ceratopogonidae	0	0	0	0	0	0	9	13	3	1
Cloeon dipterum	0	0	0	0	0	0	0	0	0	1
Corixa sp.	0	0	0	0	0	0	0	1	0	0
Corophium curvispinum	0	0	0	0	0	2	0	1	0	0
Dikerogammarus sp.	0	0	0	0	2	0	0	0	0	0
Erythromma najas	0	0	0	0	1	0	0	0	0	0
Micronecta sp.	0	0	0	0	0	0	10	0	10	143
Notonecta sp.	0	0	0	0	0	0	0	0	0	2
Platambus sp.	0	0	0	0	0	0	0	0	0	1
Plea sp.	0	0	0	0	0	0	0	0	0	1
Sphaerium sp.	0	0	0	0	0	0	0	0	0	1
Theodoxus fluviatilis	0	0	0	0	3	1	0	0	0	0
Velliidae	0	0	0	0	0	0	1	0	0	0

Chapter 3 - Appendices

Appendix 3.1 - Sequencing primers

Table S2.1. PCR 1 sequencing primers (Forward)

Primer name	Tag name	pre-adapter	Sequencing primer sequence	First i7 sequence	heterogeneity spacer	Farward locus primer
het_LeRayF_N701	N701	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCGCCTTA		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	N702	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CTAGTACG	T	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	N703	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TTCTGCCT	GT	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	N704	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GCTCAGGA	CGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	N705	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGGAGTCC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	N706	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CATGCCTA	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	N707	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GTAGAGAG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	N708	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CCTCTCTG	CCTGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	N709	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGCGTAGC		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N710	N710	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CAGCCTCG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	N711	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TGCCTCTT	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	N712	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCCTCTAC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC

Primer name	Combined Forward primer sequence
het_LeRayF_N701	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGCCTTAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTAGTACGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCTGCCTGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTCAGGACGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGAGTCCATGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGCCTATGCGAGGWACWGGWTGAACWGTWTAYCCYCC

het_LeRayF_N707	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGAGAGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTCTCTGCCTGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGTAGCGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N710	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCCTCGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCCTCTTTGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCTCTACATGAGGWACWGGWTGAACWGTWTAYCCYCC

Table S2.2. PCR 1 sequencing primers (Reverse)

Primer name	Tag name	**pre-adapter**	**Sequencing primer sequence**	**First i5 sequence**	heterogeneity spacer	**Reverse locus primer**
het_LeRayR_N501	N501	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TAGATCGC		TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	N502	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTCTCTAT	T	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	N503	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TATCCTCT	GT	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	N504	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AGAGTAGA	CGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	N505	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	GTAAGGAG	ATGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	N506	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	ACTGCATA	TGCGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	N507	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AAGGAGTA	GAGTGG	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	N508	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTAAGCCT	CCTGTGG	TAIACYTCIGGRTGICCRAARAAYCA

Primer name	Combined Reverse primer sequence
het_LeRayR_N501	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGATCGCTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCTCTATTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCCTCTGTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAGTAGACGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAGGAGATGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTGCATATGCGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGGAGTAGAGTGGTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAAGCCTCCTGTGGTAIACYTCIGGRTGICCRAARAAYCA

Appendix 3.3 - Environmental parameters used for the envfit.

Table S3.1. Environmental variables measured during October sampling. Three replicates were collected, and analysis was run by averaging the replicates for each sample. D. villosus values were extracted from number of individuals collected in the kick samples plus the number of consumers used for the gut contents analysis.

	Temperature (℃)			pH			Cond. (µS)			D. villosus
Site	Repl.1	Repl.2	Repl.3	Repl.1	Repl.2	Repl.3	Repl.1	Repl.2	Repl.3	N/A
GW1	16	16	16	8.6	8.6	8.6	854	852	852	49
GW2	15.9	16	16	8.3	8.5	8.6	858	855	856	176
GW3	15.6	15.7	15.5	8.3	8.4	8.4	866	867	866	232
GW4	15.7	15.7	15.7	8.5	8.5	8.5	860	862	860	86
GW5	16	16	16	8.5	8.6	8.6	854	853	855	0
GW6	16	15.9	15.9	8.4	8.5	8.5	845	845	849	354
WB1	14.1	14.1	14.2	8.1	8	8	780	780	790	4
WB2	14.1	14.2	14.2	7.1	7.1	7.2	780	770	780	4
WB3	14	14.2	14.1	8	7.9	8.1	780	770	790	30
WB4	14.4	14.3	14.3	8.1	8.3	8.1	760	780	760	17
WB5	14.1	14.1	14.1	8.1	8.1	8.3	780	770	780	17
WB6	14.2	14.2	14.2	8.1	8.1	8.1	780	780	780	114
RB1	14.7	14.6	14.6	7.7	7.6	7.4	310	320	360	0
RB2	15	15	15	7.8	7.8	7.7	890	890	900	0
RB3	15	15	15	7.8	7.9	7.9	910	900	910	0
RB4	13.1	13.1	13.2	8.1	8	7.9	920	910	910	0
RB5	14.3	14.4	14.4	7.8	7.7	7.8	900	910	900	0
RB6	14.7	14.8	14.8	8	7.9	7.9	910	910	900	0

Appendix 3.2 - Creation of DNA database

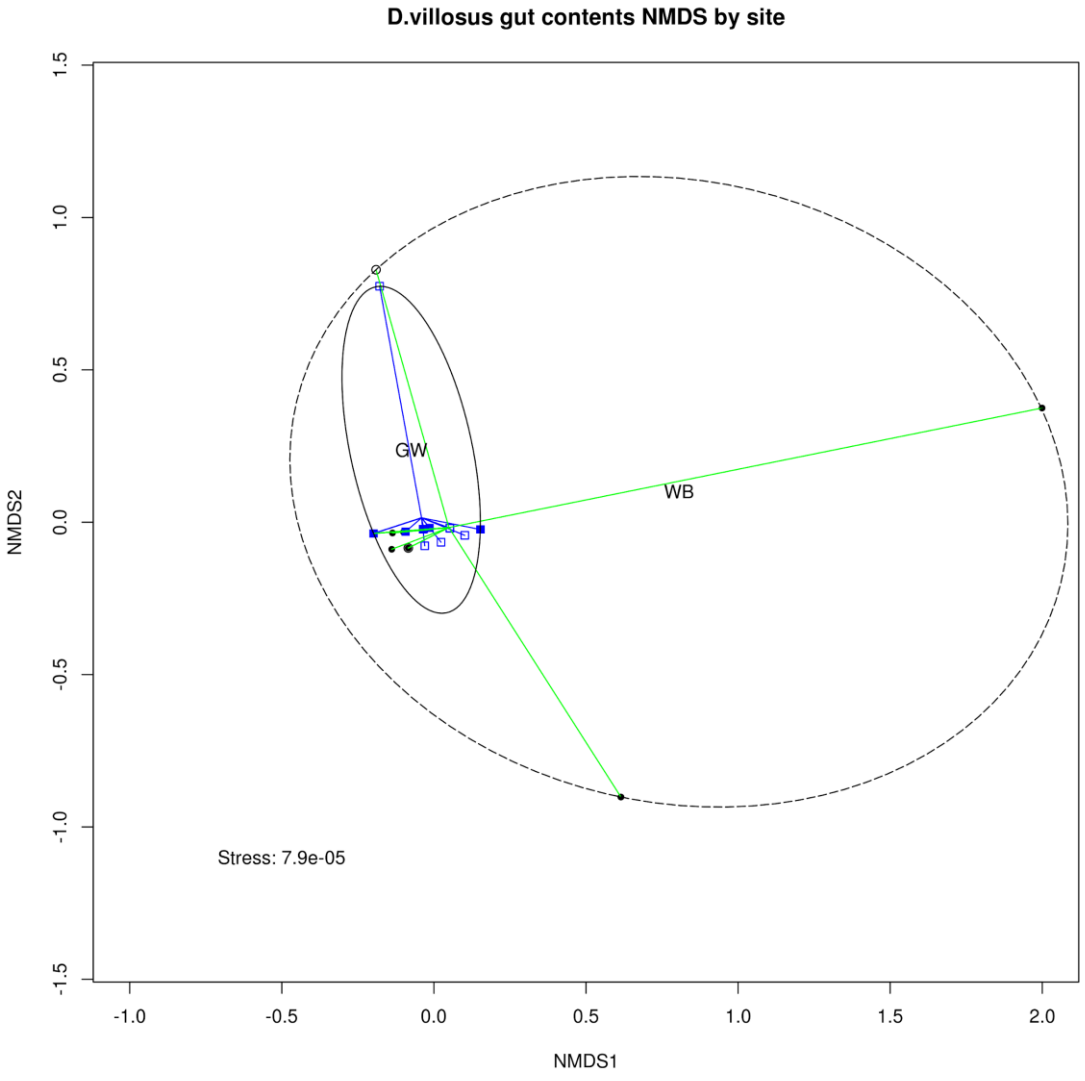
The curated database of COI sequences using for metabarcoding analysis was created from the list of UK invertebrates species that was compiled by the Centre of Ecology and Hydrology (available at: https://www.ceh.ac.uk/services/coded-macroinvertebrates-list).

From this species list, each order was analysed separately but following the same workflow; with the only exclusion of Diptera that could not be included in the database because the issues in the taxonomy and in the number of records. Each order was processed with Reprophylo pipeline (Szitenberg *et al.*, 2015). This includes using the species list by order to download the COI sequences from GenBank and EMBL, in the eventuality that the sequences were not available then the genus was used to look for available COI sequences. The sequences were first clustered at 100% similarity using VSEARCH v1.1.0 (Rognes *et al.*, 2016), then they were filtered to keep all sequences equal or longer than 500 bp. The retained sequences were then aligned with MUSCLE (Edgar, 2004), and trimmed using trimAl (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The trimmed sequences were controlled using SATIVA (Kozlov *et al.*, 2016) to highlight potential mislabelled records that were then removed from the dataset. Ultimately the sequences were used to construct a Maximum Likelihood tree using RAxML v8 (Stamatakis, 2014). The final databases created included: 412/423 Coleoptera species (97.3%), 54/59 Odonata species (91.5%), 83/92 species between Ephemertoptera/Plecoptera/Neuroptera and Megaloptera (90.2%), 187/206 species between Trichoptera and Lepidoptera (90.8%), 53/114 species between Hemiptera and Hymenoptera (46.5%), 154/388 Crustacea species (46.3%), 78/111 Mollusca species (70.3%), 333/333 Arachnida species (100%), 129/152 Annelida species (84.9%). All databases have been deposited at the link: https://osf.io/j9adr/ (doi: 10.17605/OSF.IO/J9ADR)

Appendix 3.4 - PERMANOVA of gut contents of *D. villosus*

The PERMANOVA analysis of *D. villosus* gut contents across these two invaded lakes was influenced by the low number of individuals for which prey DNA could be detected (N total=35). Overall however it appears that seasonality had a stronger influence (ADONIS: Pr(>F) = 0.0008, R2 = 0.10615); than sampling location which was not significant (ADONIS: Pr(>F) = 0.0471, R2 = 0.05406) (Fig. S3.1).

a)



b)

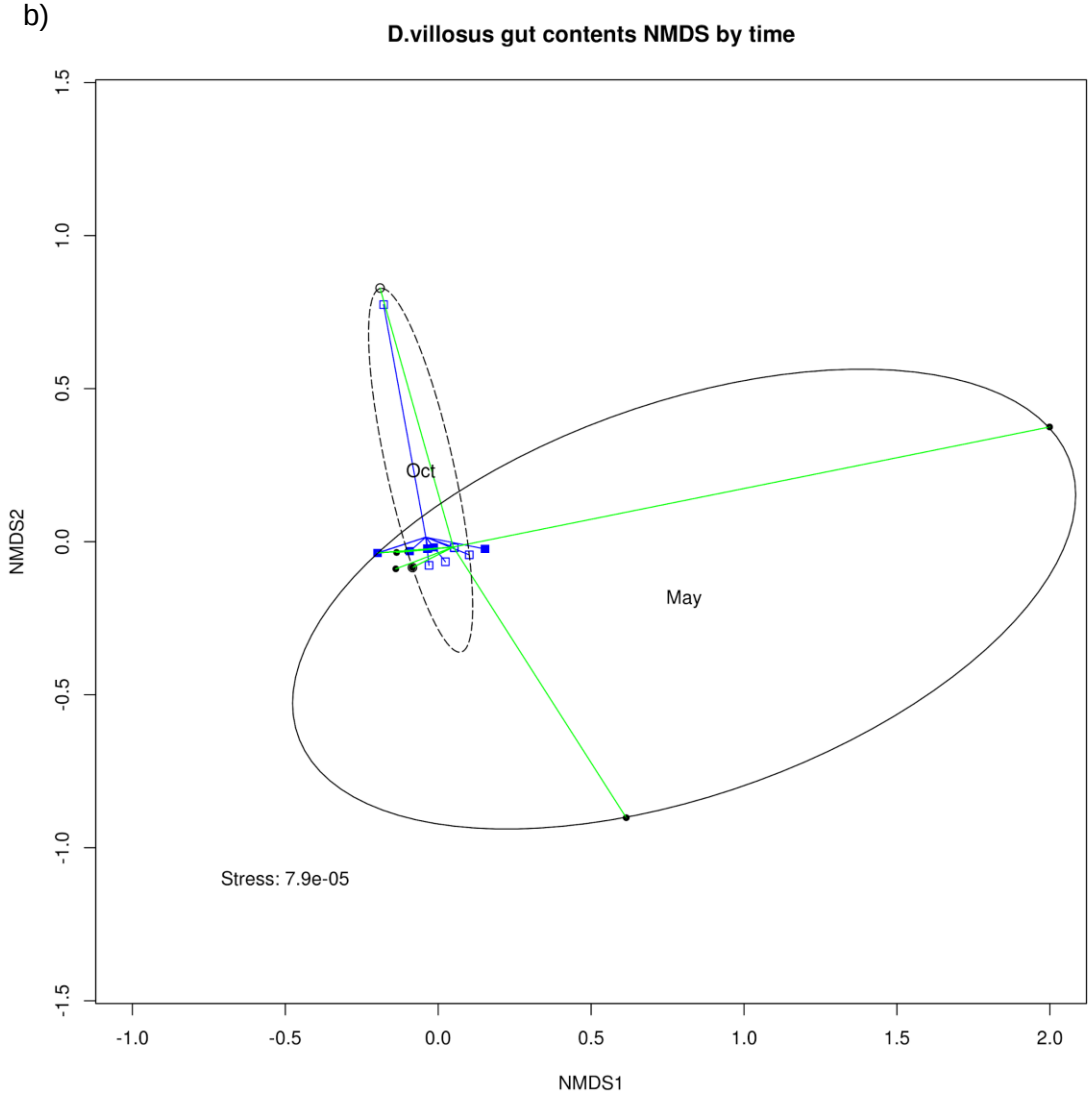


Figure S3.1. D.villosus gut contents NMDS based on Euclidean distance, a) across sites, and b) across seasons. Square symbols indicate Grafham Water, circle symbols indicate Wroxham Broad; solid symbols indicate May, hollow indicate October.

Appendix 3.5 – Benthic invertebrate community from kick sampling

Sample type	Kick sampling					
Ssite	GW		WB		RB	
Time	May	Oct	May	Oct	May	Oct
Limnesia sp.	0	1	0	0	6	2
Chydoridae	0	0	0	0	0	1
Daphniidae	0	0	0	0	0	1
Eurycercus sp.	36	0	0	0	0	0
Dytiscidae	1	0	1	0	0	0
Elmidae	0	0	0	0	6	0
Haliplidae	0	2	0	0	3	0
Limnius sp.	0	0	0	0	1	0
Platambus sp.	0	0	1	0	0	0
Asellus aquaticus	9	8	0	0	8	2
Corophium curvispinum	0	0	3	0	84	0
Corophium sp.	0	0	0	176	3	117
Crangonyx floridanus	0	0	0	0	0	1
Crangonyx pseudogracilis	2	0	0	0	0	11
Crangonyx sp.	0	0	0	1	0	5
Dikerogammarus sp.	0	45	2	0	0	0
Dikerogammarus villosus	76	852	432	186	0	0
Gammaridae	0	0	0	0	0	1
Gammarus sp.	1	68	0	12	4	3
Gammarus tigrinus	6	1	0	0	0	0
Gammarus zaddachi	0	0	0	0	128	81
Brachycera	0	0	1	0	0	0
Ceratopogonidae	0	1	26	0	0	0
Chironomidae	414	16	39	35	385	43
Dolichopodidae	1	0	0	0	0	0
Nematocera	9	0	1	0	0	0
Psychodidae	0	0	0	1	0	0
Tipulidae	3	0	0	0	0	0
Caenis luctuosa	0	0	1	0	0	0
Caenis robusta	1	0	0	0	1	0
Caenis sp.	0	0	0	0	0	2
Cloeon dipterum	0	0	1	0	0	0
Corixa sp.	0	0	1	0	0	0
Corixidae	4	24	0	0	7	2
Micronecta sp.	0	0	163	0	0	0
Nepa cinerea	0	0	0	0	1	0
Notonecta glauca	0	0	0	0	1	0
Notonecta sp.	0	0	2	0	0	0
Plea sp.	0	0	1	0	0	0
Velliidae	0	0	1	0	0	0
Erpobdella sp.	0	1	0	0	0	0

Glossiphonia complanata	0	4	0	1	0	4
Glossiphonia heteroclita	0	0	0	0	1	0
Helobdella sp.	1	0	0	0	0	0
Helobdella stagnalis	0	24	0	1	3	2
Placobdella costata	0	0	0	0	1	0
Sialidae	0	0	0	1	0	0
Bithynia sp.	0	2	0	5	17	1
Bithynia tentaculata	1	8	0	72	15	1
Bithyniidae	0	0	0	0	12	0
Corbicula fluminea	0	0	0	0	0	3
Dreissena polymorpha	0	4	5	154	2	0
Hydrobiidae	0	0	0	0	0	2
Lymnaea auricolaria	1	0	1	1	2	0
Lymnaea sp.	1	7	1	0	0	0
Lymnaea stagnalis	0	0	0	3	10	2
Lymnaea truncatula	1	0	0	22	1	2
Lymnaeidae	0	1	0	1	1	0
Physa fontinalis	0	0	0	15	0	1
Physella acuta	0	47	0	0	0	1
Pisidium sp.	2	0	0	1	24	11
Planorbidae	0	0	0	0	0	1
Planorbis contortus	0	0	0	0	3	9
Planorbis vortex	1	0	0	0	0	0
Potamopyrgus antipodarum	0	23	0	33	7	1
Radix auricularia	0	0	0	0	0	1
Radix balthica	0	4	0	3	0	0
Radix peregra	0	0	0	0	0	2
Segmentina sp.	0	0	0	2	1	0
Sphaerium sp.	0	0	1	0	0	7
Stagnicola	0	0	0	0	0	0
Theodoxus fluviatilis	0	0	4	6	14	0
Unio pictorum	0	0	0	0	1	0
Valvata cristata	0	0	0	1	0	5
Valvata piscinalis	4	1	0	4	2	2
Valvatidae	0	0	0	2	1	0
Tubificidae	54	0	0	0	0	0
Ceraclea senilis	0	0	0	0	1	0
Ecnomus sp.	0	0	0	0	1	0
Ecnomus tenellus	0	0	0	0	2	0
Leptoceridae	1	0	0	0	0	0
Oecetis lacustris	0	0	0	0	1	0
Oecetis sp.	0	0	0	0	1	0
Coenagrionidae	0	2	0	10	7	3
Erythromma najas	0	0	1	0	0	0

Chapter 4 - Appendices

Appendix 4.1 - Microscopy images

Figure S4.1 Microscopy image of *Dinocampus coccinellae* larva detected during dissection of *Harmonia axyridis*.

Appendix 4.2 - Tapestation *in silico* results

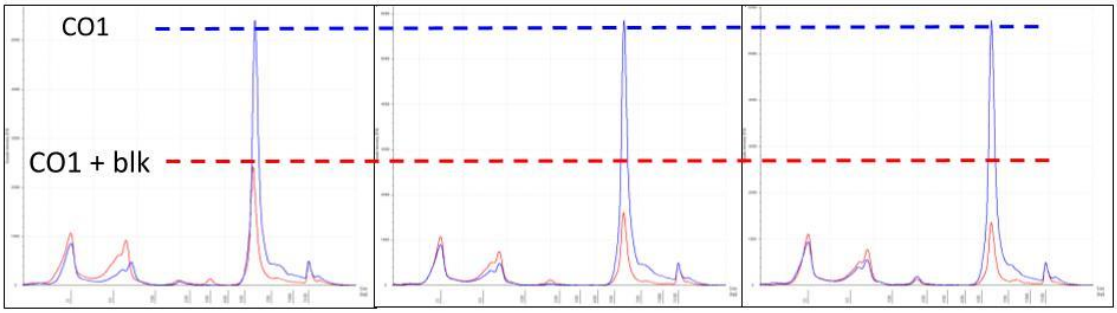
CO1 primers

Sample name	Calibrated conc. (pg/ul)
Ha1-Ler	4620
Ha2-Ler	5310
Ha3-Ler	5060

Reduction in amplified DNA conc.

CO1 + blocking primers

Calibrated conc. (pg/ul)	Sample name
2690	Ha1-Ler-blk
1860	Ha2-Ler-blk
1960	Ha3-Ler-blk



Ha1 Ha2 Ha3

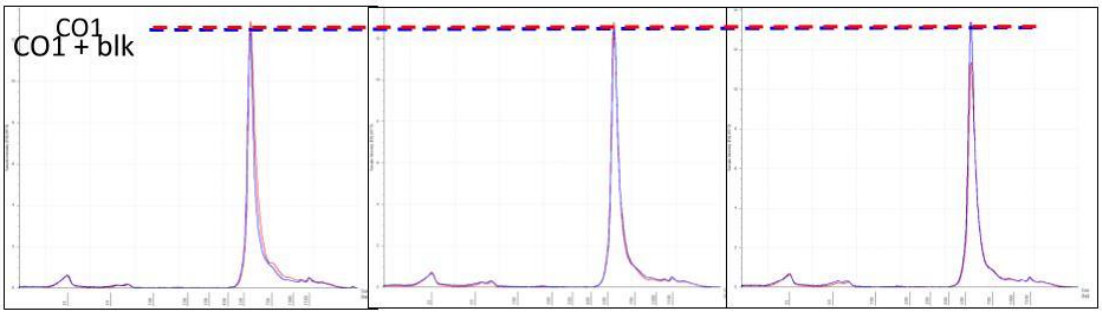
CO1 primers

Sample name	Calibrated conc. (pg/ul)
Ad1-Ler	8280
Ad2-Ler	9460
Ad3-Ler	8490

NO Reduction

CO1 + blocking primers

Calibrated conc. (pg/ul)	Sample name
8990	Ad1-Ler-blk
8590	Ad2-Ler-blk
7930	Ad3-Ler-blk



Ad1 Ad2 Ad3

CO1 primers

CO1 + blocking primers

NO Reduction

Sample name	Calibrated conc. (pg/ul)
Hs1-Ler	6340
Hs2-Ler	5010
Cq1-Ler	3490

Calibrated conc. (pg/ul)	Sample name
6230	Hs1-Ler-blk
5870	Hs2-Ler-blk
4620	Cq1-Ler-blk

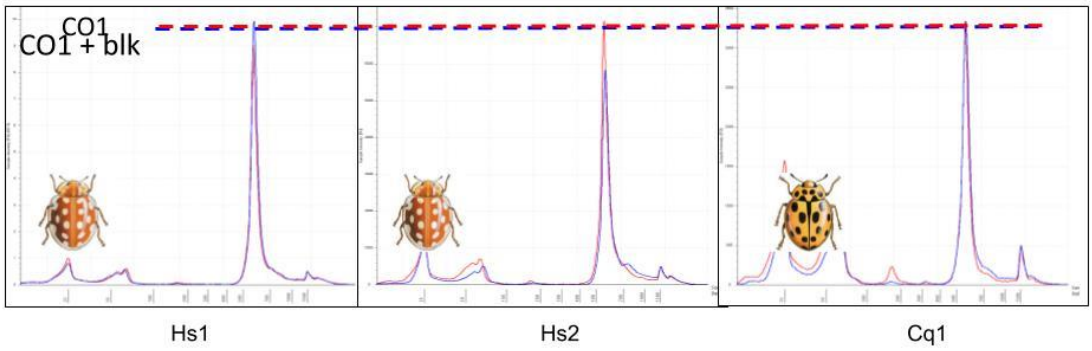


Figure S4.2 Tapestation results of the blocking primers *in silico* test using single-species tissue DNA.

Appendix 4.3 - Sequencing primers

Table S4.1. PCR 1 sequencing primers (Forward)

Primer name	Tag name	pre-adapter	Sequencing primer sequence	First i7 sequence	heterogeneity spacer	Farward locus primer
het_LeRayF_N701	N701	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCGCCTTA		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	N702	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CTAGTACG	T	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	N703	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TTCTGCCT	GT	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	N704	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GCTCAGGA	CGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	N705	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGGAGTCC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	N706	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CATGCCTA	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	N707	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GTAGAGAG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	N708	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CCTCTCTG	CCTGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	N709	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGCGTAGC		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N710	N710	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CAGCCTCG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	N711	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TGCCTCTT	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	N712	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCCTCTAC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC

Primer name	Combined Forward primer sequence
het_LeRayF_N701	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGCCTTAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTAGTACGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCTGCCTGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTCAGGACGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGAGTCCATGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGCCTATGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGAGAGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTCTCTGCCTGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGTAGCGGWACWGGWTGAACWGTWTAYCCYCC

het_LeRayF_N710	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCCTCGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCCTCTTTGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCTCTACATGAGGWACWGGWTGAACWGTWTAYCCYCC

Table S4.2. PCR 1 sequencing primers (Reverse)

Primer name	Tag name	pre-adapter	Sequencing primer sequence	First i5 sequence	heterogeneity spacer	Reverse locus primer
het_LeRayR_N501	N501	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TAGATCGC		TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	N502	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTCTCTAT	T	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	N503	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TATCCTCT	GT	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	N504	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AGAGTAGA	CGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	N505	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	GTAAGGAG	ATGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	N506	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	ACTGCATA	TGCGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	N507	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AAGGAGTA	GAGTGG	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	N508	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTAAGCCT	CCTGTGG	TAIACYTCIGGRTGICCRAARAAYCA

Primer name	Combined Reverse primer sequence
het_LeRayR_N501	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGATCGCTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCTCTATTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCCTCTGTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAGTAGACGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAGGAGATGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTGCATATGCGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGGAGTAGAGTGGTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAAGCCTCCTGTGGTAIACYTCIGGRTGICCRAARAAYCA

Appendix 4.4 - Creation of DNA database

The curated database of COI sequences using for metabarcoding analysis was created from the list of UK invertebrates species available by the Natural History Museum list (available at: https://www.nhm.ac.uk/our-science/data/uk-species/browse-uk-species/index.html).

From the complete list 12 orders were selected because of being species expected to compose arboreal communities, as potential prey of *Harmonia axyridis* and as potential guild member species. Each order was analysed separately but following the same workflow; with the only exclusion of Diptera that could not be included in the database because the issues in the taxonomy and in the number of records. Each order was processed with Reprophylo pipeline (Szitenberg *et al.*, 2015). This includes using the species list by order to download the COI sequences from GenBank and EMBL, in the eventuality that the sequences were not available then the genus was used to look for available COI sequences. The sequences were first clustered at 100% similarity using VSEARCH v1.1.0 (Rognes *et al.*, 2016), then they were filtered to keep all sequences equal or longer than 500 bp. The retained sequences were then aligned with MUSCLE (Edgar, 2004), and trimmed using trimAl (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The trimmed sequences were controlled using SATIVA (Kozlov *et al.*, 2016) to highlight potential mislabelled records that were then removed from the dataset. Ultimately the sequences were used to construct a Maximum Likelihood tree using RAxML v8 (Stamatakis, 2014). The final databases created included: 30/49 Adelgidae-Anthocoridae-Coccidae species (61.2%), 298/521 Aphididae species (55.5%), 70/181 Braconidae species (38.7%), 18/65 Chalcidae-Encyrtidae-Eulophidae species (27.7%), 215/441 Coleoptera (including 46/53 Coccinellidae) species (48.7%), 50/65 Neuroptera species (76.9%), 110/336 Phoridae species (32.7%), 38/98 Psocoptera species (40.9%), 29/73 Psyllidae species (39.7%).

All databases have been deposited at the link: https://osf.io/j9adr/ (doi: 10.17605/OSF.IO/J9ADR)

Chapter 5 - Appendices

Appendix 5.1 - Sample preparation for DNA barcoding

We removed a pereopod (P5, 6 or 7) for DNA extraction from each specimen used in species confirmation. All tools and surfaces were sterilised before and after each specimen with 10 v/v of commercial bleach. The Eppendorf tubes were sterilised with exposure to UV-light for 1h. Pereopods were placed in individual sterile 1.5 mL Eppendorf tubes and air dried to remove any traces of ethanol. DNA extraction was modified from the Mu-DNA tissue extraction protocol as described by Sellers *et al.* (2018), with the additions described below (Sellers *et al.*, 2018). Initial digestion was done in 300 µl of Lysis mix (270 µl lysis solution, 20 µl tissue lysis additive and 10 µl Proteinase-K) and followed by a grinding step with individual sterilised pestels to allow a better digestion. Tissue samples were digested at 56 ^{o}C for 3-5 hours on a ThermoMixer at 650 rpm. After digestion the tubes were centrifuged at x10,000g for 1 min, and the supernatant transferred to a new 1.5 mL Eppendorf tube. 50 µl of flocculant solution was added, the samples were briefly vortex and placed on ice for 10 mins.

1.5x volume of solid phase reversible immobilisation (SPRI) magnetic bead solution (adapted from (Rohland and Reich, 2012)), was added and the samples were placed on a magnetic stand. The supernatant was discarded, and the beads were washed twice with 500 µl of 80% ethanol. Samples were air dried to remove all ethanol before adding 50 µl of elution buffer pre-heated at 55 ^{o}C. The beads were resuspended and mixed for 5 min on a Hulamixer. All tubes were then placed on the magnetic stand and the supernatant transferred to a 1.5 ml Eppendorf tube. PCR amplification targeted 658 bp COI fragment using metazoan universal primers (Folmer *et al.*, 1994). PCRs ran using 0.4 µM of each primer, 1x MyFi™ Mix *Taq* polymerase (Bioline, UK), and 2 µl of DNA template in a 25 µl reaction volume. PCR conditions were 95℃ for 3 mins, followed by 40 cycles of 95 ^{o}C for 90 sec, 53 ^{o}C for 30 sec, 72 ^{o}C for 60 sec, and one cycle at 72 ^{o}C for 5 min. PCR products were visualised on 1.5% Agarose gel in Sodium Boric acid (Brody and Kern, 2004) at 200 V for 20 min, using 2 µl of product. Samples were cleaned from primer dimers following the one step Magnetic beads clean up protocol following Quail and others (2009), with the only modification that T Buffer was used to elute the cleaned products (Quail, Swerdlow and Turner, 2009). Samples were sequenced (Macrogen Europe B.V.) using 5 µl of clean product and 5 µl of sequencing primer (5 µM) diluted in T Buffer.

Appendix 5.2 - Sequencing primers

Table S5.1. PCR 1 sequencing primers (Forward)

Primer name	Tag name	pre-adapter	Sequencing primer sequence	First i7 sequence	heterogeneity spacer	Farward locus primer
het_LeRayF_N701	N701	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCGCCTTA		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	N702	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CTAGTACG	T	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	N703	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TTCTGCCT	GT	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	N704	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GCTCAGGA	CGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	N705	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGGAGTCC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	N706	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CATGCCTA	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	N707	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	GTAGAGAG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	N708	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CCTCTCTG	CCTGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	N709	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	AGCGTAGC		GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N710	N710	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	CAGCCTCG	GAGTGG	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	N711	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TGCCTCTT	TGCGA	GGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	N712	TCGTCGGCAGCGTC	AGATGTGTATAAGAGACAG	TCCTCTAC	ATGA	GGWACWGGWTGAACWGTWTAYCCYCC

Primer name	Combined Forward primer sequence
het_LeRayF_N701	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCGCCTTAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N702	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTAGTACGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N703	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCTGCCTGTGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N704	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTCAGGACGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N705	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGAGTCCATGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N706	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATGCCTATGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N707	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTAGAGAGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N708	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTCTCTGCCTGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N709	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGCGTAGCGGWACWGGWTGAACWGTWTAYCCYCC

het_LeRayF_N710	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGCCTCGGAGTGGGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N711	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGCCTCTTTGCGAGGWACWGGWTGAACWGTWTAYCCYCC
het_LeRayF_N712	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCTCTACATGAGGWACWGGWTGAACWGTWTAYCCYCC

Table S5.2. PCR 1 sequencing primers (Reverse)

Primer name	Tag name	**pre-adapter**	**Sequencing primer sequence**	**First i5 sequence**	heterogeneity spacer	**Reverse locus primer**
het_LeRayR_N501	N501	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TAGATCGC		TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	N502	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTCTCTAT	T	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	N503	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	TATCCTCT	GT	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	N504	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AGAGTAGA	CGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	N505	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	GTAAGGAG	ATGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	N506	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	ACTGCATA	TGCGA	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	N507	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	AAGGAGTA	GAGTGG	TAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	N508	GTCTCGTGGGCTCGG	AGATGTGTATAAGAGACAG	CTAAGCCT	CCTGTGG	TAIACYTCIGGRTGICCRAARAAYCA

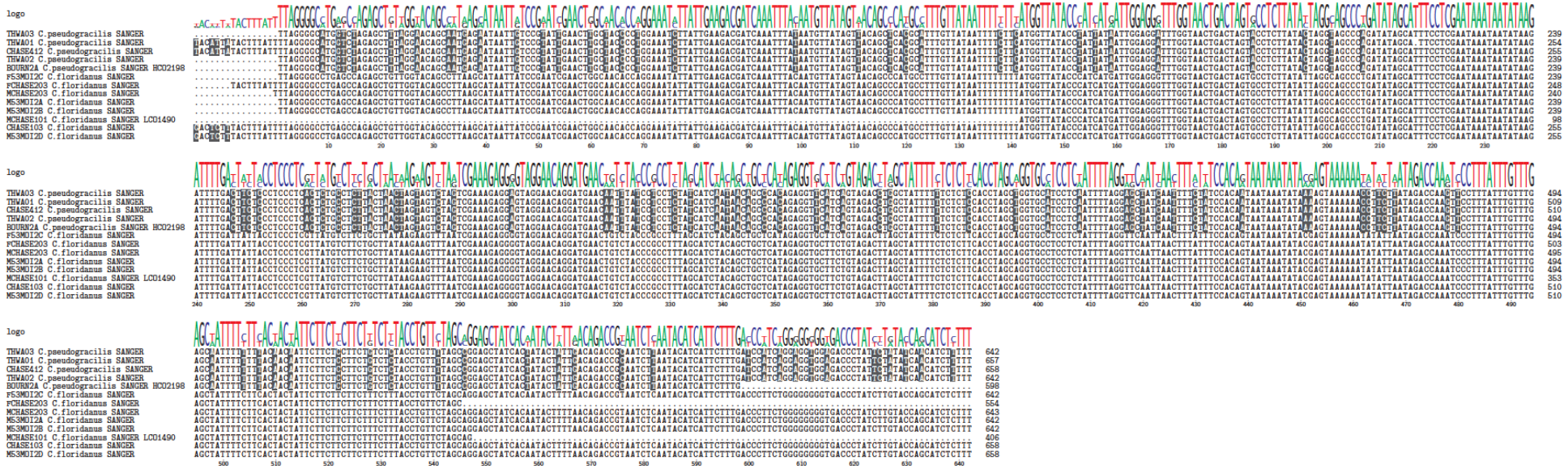
Primer name	Combined Reverse primer sequence
het_LeRayR_N501	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAGATCGCTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N502	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTCTCTATTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N503	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCCTCTGTTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N504	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAGTAGACGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N505	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTAAGGAGATGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N506	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTGCATATGCGATAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N507	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAGGAGTAGAGTGGTAIACYTCIGGRTGICCRAARAAYCA
het_LeRayR_N508	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTAAGCCTCCTGTGGTAIACYTCIGGRTGICCRAARAAYCA

Appendix 5.3 - Creation of DNA database

The curated database of COI sequences using for metabarcoding analysis was created from the list of UK invertebrates species that was compiled by the Centre of Ecology and Hydrology (available at: https://www.ceh.ac.uk/services/coded-macroinvertebrates-list).

From this species list, each order was analysed separately but following the same workflow; with the only exclusion of Diptera that could not be included in the database because the issues in the taxonomy and in the number of records. Each order was processed with Reprophylo pipeline (Szitenberg *et al.*, 2015). This includes using the species list by order to download the COI sequences from GenBank and EMBL, in the eventuality that the sequences were not available then the genus was used to look for available COI sequences. The sequences were first clustered at 100% similarity using VSEARCH v1.1.0 (Rognes *et al.*, 2016), then they were filtered to keep all sequences equal or longer than 500 bp. The retained sequences were then aligned with MUSCLE (Edgar, 2004), and trimmed using trimAl (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The trimmed sequences were controlled using SATIVA (Kozlov *et al.*, 2016) to highlight potential mislabelled records that were then removed from the dataset. Ultimately the sequences were used to construct a Maximum Likelihood tree using RAxML v8 (Stamatakis, 2014). The final databases created included: 412/423 Coleoptera species (97.3%), 54/59 Odonata species (91.5%), 83/92 species between Ephemertoptera/Plecoptera/Neuroptera and Megaloptera (90.2%), 187/206 species between Trichoptera and Lepidoptera (90.8%), 53/114 species between Hemiptera and Hymenoptera (46.5%), 154/388 Crustacea species (46.3%), 78/111 Mollusca species (70.3%), 333/333 Arachnida species (100%), 129/152 Annelida species (84.9%). All databases have been deposited at the link: https://osf.io/j9adr/ (doi: 10.17605/OSF.IO/J9ADR)

Appendix 5.4 - Barcode sequences alignment



Appendix 5.5 - Phylogenetic tree

The complete Phylogenetic tree is available at the Github link: https://github.com/mbenucci/Cohabiting_C.floridanus_and_C.pseudogracilis/tree/master/Crangonyx_confirmation/4-infer_phylogeny/supplementary

Appendix 5.6 – Benthic invertebrate community from kick sampling

Sample type	Kick sampling											
Site	CH1		CH2		CH3		CH4		CH5		CH6	
Time	May	Oct	May	Oct	May	Oct	May	Oct	May	Oct	May	Oct
Corophium multisetosum	0	0	0	0	0	0	0	0	0	0	0	0
Crangonyx	126	31	3	2	4	26	30	10	149	26	183	220
Crangonyx floridanus	65	21	6	0	0	8	4	0	4	8	0	9
Crangonyx pseudogracilis	18	1	5	0	11	2	21	1	5	2	1	1
Corophium	0	0	0	0	0	0	1	0	0	0	0	0
Agabus	0	0	0	0	0	0	0	0	1	0	0	0
Haliplus	1	4	0	0	2	8	1	7	6	6	1	6
Platambus	14	0	3	0	1	0	2	2	5	0	1	8
Dryopidae	0	0	0	0	0	0	0	1	0	0	0	0
Hyphydrus	0	0	0	1	0	0	0	0	0	0	0	0
Chironomidae	14	3	3	2	0	1	3	0	2	7	38	3
Tabanidae	0	0	1	0	0	0	0	0	0	0	0	0
Limoniidae	0	0	0	0	0	0	0	0	0	0	0	2
Stratiomys	0	0	0	0	0	0	0	0	0	1	0	0
Tipulidae	0	0	0	0	0	0	0	0	0	0	0	2
Caenis	0	0	0	0	0	0	0	0	0	0	2	0
Procleon pennulatum	0	0	0	0	0	0	0	0	1	0	0	0
Potamopyrgus antipodarum	2	0	0	6	0	11	0	0	2	3	9	17
Bithynia	0	0	0	0	0	0	0	0	1885	0	0	0
Bithynia tentaculata	4	0	2	0	1	0	1	2	0	5	0	20

Lymnaea stagnalis	0	0	0	0	0	0	0	0	0	0	1	0
Planorbis carinatus	0	0	0	0	0	0	0	0	4	0	0	0
Planorbis	0	0	0	0	0	0	0	6	0	4	8	0
Planorbis vortex	0	3	0	3	0	0	0	0	4	0	0	0
Valvata piscinalis	0	0	0	0	0	0	0	0	0	0	1	0
Planorbis planorbis	0	0	0	0	0	1	0	0	0	0	0	7
Segmentina complanata	0	1	0	1	0	0	0	0	0	2	0	4
Corixidae	0	0	0	0	0	0	0	0	0	0	3	0
Glossiphonia complanata	0	0	0	0	1	1	0	2	0	0	0	0
Haemopis sanguisuga	0	0	0	0	0	0	2	0	0	0	1	0
Bereobdella	0	0	0	3	0	0	0	0	0	0	0	0
Bereobdella verrucata	0	0	0	0	0	0	0	2	0	0	0	0
Erpobdella testacea	0	0	0	0	0	0	0	0	0	0	0	4
Helobdella stagnalis	0	3	0	0	0	2	0	3	0	3	0	7
Hydra oligactis	0	0	0	0	0	0	0	0	0	0	0	0
Asellus aquaticus	11	0	0	0	0	1	59	143	67	16	100	64
Ceraclea	0	0	0	0	0	0	0	0	0	0	1	0
Hydropsychidae	0	0	0	0	0	0	0	0	0	0	1	0
Psychomyia	1	0	0	0	0	0	2	0	0	0	0	0
Psychomiidae	0	0	0	0	0	2	0	0	0	0	0	0
Hydracarina	0	0	0	0	0	0	0	0	0	0	1	0
Dreissena polymorpha	1	1	0	2	0	3	0	7	4	0	11	5
Pisidium	19	0	0	0	0	1	0	0	5	26	56	71
Sphaerium	0	0	0	0	0	0	0	0	11	0	0	0
Coenagrionidae	2	0	0	0	0	0	1	0	4	0	1	0

References

Adriaens, T., San Martin y Gomez, G. and Maes, D. (2008) 'Invasion history, habitat preferences and phenology of the invasive ladybird Harmonia axyridis in Belgium', BioControl, 53, pp. 69-88. Available from: doi: 10.1007/s10526-007-9137-6

Aebi, A. *et al.* (2011) 'Detecting arthropod intraguild predation in the field', *Biocontrol*, 56(4), pp. 429–440. Available from: doi: 10.1007/s10526-011-9378-2

Alberdi, A. *et al.* (2018) 'Promises and pitfalls of using high-throughput sequencing for diet analysis', *Molecular ecology resources*, 19(2), pp. 327-348. Available from: doi: 10.1111/1755-0998.12960

Alexander, J. M. and Edwards, P. J. (2010) 'Limits to the niche and range margins of alien species', *Oikos,* 119(9), pp. 1377–1386. Available from: doi: 10.1111/j.1600-0706.2009.17977.x

Allison, G. (2004) 'The influence of species diversity and stress intensity on community resistance and resilience', *Ecological monographs*, 74(1), pp. 117–134. Available from: doi: 10.1890/02-0681

Altschul, S. F. *et al.* (1990) 'Basic local alignment search tool', *Journal of molecular biology*, 215(3), pp. 403–410. Available from: doi: 10.1016/S0022-2836(05)80360-2

Amplicon, P. C. R., Clean-Up, P. C. R. and Index, P. C. R. (2013) '16s metagenomic sequencing library preparation'. Available at: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

Arim, M. and Marquet, P. A. (2004) 'Intraguild predation: a widespread interaction related to species biology', *Ecology letters,* 7(7), pp. 557–564. Available from: doi: 10.1111/j.1461-0248.2004.00613.x

Bącela, K., Grabowski, M. and Konopacka, A. (2008) 'Dikerogammarus villosus (Sowinsky, 1894) (Crustacea, Amphipoda) enters Vistula – the biggest river in the Baltic basin', *Aquatic Invasions*, 3(1), pp. 95–98. Available from: doi: 10.3391/ai.2008.3.1.16

Bacela-Spychalska, K. and Van Der Velde, G. (2013) 'There is more than one "killer shrimp": trophic positions and predatory abilities of invasive amphipods of Ponto-Caspian origin', *Freshwater biology*, 58(4), pp. 730–741. Available from: doi: 10.1111/fwb.12078

Baird, D. J. and Hajibabaei, M. (2012) 'Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing', *Molecular ecology*, 21(8), pp. 2039–2044. Available from: doi: 10.1111/j.1365-294X.2012.05519.x

Bašić, T. *et al.* (2019) ‘Trophic consequences of an invasive, small-bodied non-native fish, sunbleak Leucaspius delineatus, for native pond fishes’, *Biological invasions*, 21(1), pp. 261–275. Available from: doi: 10.1007/s10530-018-1824-y

Bastos, A. D. *et al.* (2011) ‘Genetic monitoring detects an overlooked cryptic species and reveals the diversity and distribution of three invasive Rattus congeners in South Africa’, *BMC genetics*, 12(26). Available from: doi: 10.1186/1471-2156-12-26

Raak-van den Berg, C. L. *et al.* (2014) ‘Invasive alien species under attack: natural enemies of Harmonia axyridis in the Netherlands’, *Biocontrol*, 59(2), pp. 229–240. Available from: doi: 10.1007/s10526-014-9561-3

Berkvens, N. *et al.* (2010) ‘Dinocampus coccinellae as a parasitoid of the invasive ladybird Harmonia axyridis in Europe’, *Biological control*, 53(1), pp. 92–99. Available from: doi: 10.1016/j.biocontrol.2009.11.001

Berlow, E. L. *et al.* (2004) ‘Interaction strengths in food webs: issues and opportunities’, *The Journal of animal ecology*, 73(3), pp. 585–598. Available from: doi: 10.1111/j.0021-8790.2004.00833.x

Bickford, D. *et al.* (2007) ‘Cryptic species as a window on diversity and conservation’, *Trends in ecology & evolution*, 22(3), pp. 148–155. Available from: doi: 10.1016/j.tree.2006.11.004

Biggs, J. *et al.* (2015/3) ‘Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (Triturus cristatus)’, *Biological conservation*, 183, pp. 19–28. Available from: doi: 10.1016/j.biocon.2014.11.029

Blackman, R. *et al.* (2017) ‘Detection of a new non-native freshwater species by DNA metabarcoding of environmental samples — first record of Gammarus fossarum in the UK’, *Aquatic Invasions*, 12(2), pp. 177–189. Available from: 10.3391/ai.2017.12.2.06

Blackman, R. (2018) ‘The use of environmental DNA as an early warning tool in the detection of new freshwater invasive non-native species’, *CAB Reviews,* 13(010), pp. 1-15. Available from: doi: 10.1079/PAVSNNR201813010.

Blanchet, S. (2012) ‘The use of molecular tools in invasion biology: an emphasis on freshwater ecosystems: Using molecular tools in biological invasions’, *Fisheries management and ecology*, 19(2), pp. 120–132. Available from: doi: 10.1111/j.1365-2400.2011.00832.x

Blüthgen, N. (2010) ‘Why network analysis is often disconnected from community ecology: A critique and an ecologist’s guide’, *Basic and applied ecology*, 11(3), pp. 185–195. Available from: doi: 10.1016/j.baae.2010.01.001

Bohan, D. A. *et al.* (2017) ‘Next-Generation Global Biomonitoring: Large-scale,

Automated Reconstruction of Ecological Networks', *Trends in ecology & evolution*, 32(7), pp. 477-487. Available from: doi: 10.1016/j.tree.2017.03.001.

Bojko, J. *et al.* (2015/6) 'Cucumispora ornata n. sp. (Fungi: Microsporidia) infecting invasive "demon shrimp" (Dikerogammarus haemobaphes) in the United Kingdom', *Journal of invertebrate pathology*, 128, pp. 22–30. Available from: doi: 10.1016/j.jip.2015.04.005

Bojko, J. *et al.* (2013) 'Baseline histopathological survey of a recently invading island population of "killer shrimp", Dikerogammarus villosus', *Diseases of aquatic organisms*, 106(3), pp. 241–253. Available from: doi: 10.3354/dao02658

Bolger, A. M., Lohse, M. and Usadel, B. (2014) 'Trimmomatic: a flexible trimmer for Illumina sequence data', *Bioinformatics,* 30(15), pp. 2114–2120. Available from: 10.1093/bioinformatics/btu170

Bollache, L. *et al.* (2004) 'Rapid range extension of the Ponto-Caspian amphipod Dikerogammarus villosus in France: potential consequences', *Archiv für Hydrobiologie*, 160(1), pp. 57–66. Available from: doi: 10.1127/0003-9136/2004/0160-0057

Booy, O. *et al.* (2017) 'Risk management to prioritise the eradication of new and emerging invasive non-native species', *Biological invasions*, 19(8), pp. 2401–2417. Available from: doi: 10.1007/s10530-017-1451-z

Borrvall, C., Ebenman, B. and Jonsson, T. (2000) 'Biodiversity lessens the risk of cascading extinction in model food webs', *Ecology letters*, 3(2), pp. 131–136. Available from: doi: 10.1046/j.1461-0248.2000.00130.x

Borza, P. *et al.* (2017) 'Current velocity shapes co-existence patterns among invasive Dikerogammarus species', *Freshwater biology*, 62(2), pp. 317–328. Available from: doi: 10.1111/fwb.12869

Britton, J. R. (2013) 'Introduced parasites in food webs: new species, shifting structures?', *Trends in ecology & evolution*, 28(2), pp. 93–99. Available from: doi: 10.1016/j.tree.2012.08.020

Brody, J. R. and Kern, S. E. (2004) 'Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis', *BioTechniques*, 36(2), pp. 214–216. Available from: doi: 10.2144/04362BM02

Brown, P. M. J. *et al.* (2007) 'Harmonia axyridis in Europe: spread and distribution of a non-native coccinellid', *Biocontrol*, 53(1), pp. 5–21. Available from: doi: 10.1007/s10526-007-9132-y

Brown, P. M. J., Frost, R., *et al.* (2011) 'Decline in native ladybirds in response to the arrival of Harmonia axyridis: early evidence from England', *Ecological entomology*, 36(2), pp. 231–240. Available from: doi: 10.1111/j.1365-2311.2011.01264.x

Brown, P. M. J., Thomas, C. E., *et al.* (2011) 'The global spread of Harmonia axyridis (Coleoptera: Coccinellidae): distribution, dispersal and routes of

invasion', *Biocontrol*, 56(4), pp. 623–641. Available from: doi: 10.1007/s10526-011-9379-1

Brown, P. M. J. *et al.* (2015) 'Intraguild predation by Harmonia axyridis (Coleoptera: Coccinellidae) on native insects in Europe: molecular detection from field samples', *Entomological science*, 18(1), pp. 130–133. Available from: doi: 10.1111/ens.12092

Brown, P. M. J. *et al.* (2018) 'Spread of a model invasive alien species, the harlequin ladybird Harmonia axyridis in Britain and Ireland', *Scientific data*, 5(180239). Available from: doi: 10.1038/sdata.2018.239

Brown, P. M. J. and Roy, H. E. (2018) 'Native ladybird decline caused by the invasive harlequin ladybird Harmonia axyridis: evidence from a long-term field study', *Insect conservation and diversity,* 11(3), pp. 230–239. Available from: doi: 10.1111/icad.12266

Buglione, M. *et al.* (2018) 'A pilot study on the application of DNA metabarcoding for non-invasive diet analysis in the Italian hare', *Mammalian biology,* 88, pp. 31–42. Available from: doi: 10.1016/j.mambio.2017.10.010

Burns, J. H. and Strauss, S. Y. (2011) 'More closely related species are more ecologically similar in an experimental test', *Proceedings of the National Academy of Sciences of the United States of America*, 108(13), pp. 5302–5307. Available from: doi: 10.1073/pnas.1013003108

Camacho-Cervantes, M., Ortega-Iturriaga, A. and Del-Val, E. (2017) 'From effective biocontrol agent to successful invader: the harlequin ladybird (Harmonia axyridis) as an example of good ideas that could go wrong', *PeerJ*, 5:e3296. Available from: doi: 10.7717/peerj.3296

Capella-Gutiérrez, S., Silla-Martínez, J. M. and Gabaldón, T. (2009) 'trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses', *Bioinformatics*, 25(15), pp. 1972–1973. Available from: doi: 10.1093/bioinformatics/btp348

Carreon-Martinez, L. and Heath, D. D. (2010) 'Revolution in food web analysis and trophic ecology: diet analysis by DNA and stable isotope analysis', *Molecular ecology*, 19(1), pp. 25–27. Available from: doi: 10.1111/j.1365-294X.2009.04412.x

Carvalheiro, L. G., Buckley, Y. M. and Memmott, J. (2010) 'Diet breadth influences how the impact of invasive plants is propagated through food webs', *Ecology*, 91(4), pp. 1063–1074. Available from: doi: 10.1890/08-2092.1

Casellato, S. *et al.* (2006) 'Dikerogammarus villosus (Sowinsky, 1894) (Crustacea, Amphipoda, Gammaridae) for the first time in Italy', *Italian Journal of Zoology*, 73(1), pp. 97–104. Available from: doi: 10.1080/11250000500502293.

Casellato, S., Visentin, A. and La Piana, G. (2007) 'The predatory impact of Dikerogammarus villosus on fish', in Gherardi F. (eds) Biological invaders

in inland waters: Profiles, distribution, and threats. Invading Nature - Springer Series in Invasion Ecology, vol 2. Springer Dordrecht. pp. 495–506.

de Castro Guedes, C. F., Dios, R. de V. P. and de Almeida, L. M. (2018) 'Parasitism of Harmonia axyridis (Pallas)(Coleoptera: Coccinellidae) by Strongygaster brasiliensis (Towsend)(Diptera: Tachinidae)', *EntomoBrasilis*. Entomologistas do Brasil, 11(1), pp. 49–51. Available from: doi: 10.12741/ebrasilis.v11i1.713

Ceryngier, P. *et al.* (2018) 'Predators and parasitoids of the harlequin ladybird, Harmonia axyridis, in its native range and invaded areas', *Biological invasions*, 20(4), pp. 1009–1031. Available from: doi: 10.1007/s10530-017-1608-9

Chapman, J. W. (2007) 'Amphipoda: Chapter 39 of The Light and Smith Manual: Intertidal Invertebrates from Central California to Oregon. Fourth Edition, Completely Revised and Expanded'. Available at: https://ir.library.oregonstate.edu/concern/defaults/fq977z86c.

Charles, H. and Dukes, J. S. (2007) 'Impacts of Invasive Species on Ecosystem Services', in Nentwig, W. (ed.) *Biological Invasions*. Springer Berlin Heidelberg, pp. 217–237.

Clare, E. L. *et al.* (2009) 'Species on the menu of a generalist predator, the eastern red bat (Lasiurus borealis): using a molecular approach to detect arthropod prey', *Molecular ecology*, 18(11), pp. 2532–2542. Available from: doi: 10.1111/j.1365-294X.2009.04184.x.

Clare, E. L. (2014) 'Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications', *Evolutionary applications*, 7(9), pp. 1144–1157. Available from: doi: 10.1111/eva.12225

Clare, E. L. *et al.* (2014) 'The diet of Myotis lucifugus across Canada: assessing foraging quality and diet variability', *Molecular ecology*, 23(15), pp. 3618–3632. Available from: doi: 10.1111/mec.12542.

Clare, E. L. *et al.* (2019) 'Approaches to integrating genetic data into ecological networks', *Molecular ecology*, 28(2), pp. 503–519. Available from: doi: 10.1111/mec.14941

Coble, A. A. *et al.* (2019) 'eDNA as a tool for identifying freshwater species in sustainable forestry: A critical review and potential future applications', *The Science of the total environment*, 649, pp. 1157–1170. Available from: doi: 10.1016/j.scitotenv.2018.08.370

Colautti, R. I. *et al.* (2004) 'Is invasion success explained by the enemy release hypothesis?', *Ecology letters*, 7(8), pp. 721–733. Available from: doi: 10.1111/j.1461-0248.2004.00616.x

Comont, R. F. *et al.* (2014) 'Escape from parasitism by the invasive alien ladybird, Harmonia axyridis', *Insect conservation and diversity*, 7(4), pp. 334–342. Available from: doi: 10.1111/icad.12060

Constable, D. (2014) *Notes on the importance of sexing Gammaridae for identification: using Dikerogammarus as an example*. Environment Agency. Available at: https://www.fba.org.uk/sites/default/files/Notes%20on%20Gammaridae%20ID%20DC.pdf.

Crawford, G. I. (1937) 'An Amphipod, Eucrangonyx gracilis S. I. Smith, new to Britain', *Nature*, 139(3512), pp. 327–327. Available from: doi: 10.1038/139327b0

Creer, S. *et al.* (2016) 'The ecologist's field guide to sequence-based identification of biodiversity', *Methods in ecology and evolution,* 7(9), pp. 1008–1018. Available from: doi: 10.1111/2041-210X.12574

Davey, J. W. and Blaxter, M. L. (2010) 'RADSeq: next-generation population genetics', *Briefings in functional genomics*, 9(5-6), pp. 416–423. Available from: doi: 10.1093/bfgp/elq031

David, P. *et al.* (2017) 'Chapter One - Impacts of Invasive Species on Food Webs: A Review of Empirical Data', in Bohan, D. A., Dumbrell, A. J., and Massol, F. (eds) *Advances in Ecological Research (Vol 56)*. Academic Press Oxford London Cambridge San Diego, pp. 1–60.

Deagle, B. E. *et al.* (2018) 'Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data?', *Molecular ecology*, 28(2), pp. 391-406. Available from: doi: 10.1111/mec.14734.

De Barba, M. *et al.* (2014) 'DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: application to omnivorous diet', *Molecular ecology resources*, 14(2), pp. 306–323. Available from: doi: 10.1111/1755-0998.12188

Deiner, K. *et al.* (2017) 'Environmental DNA metabarcoding: Transforming how we survey animal and plant communities', *Molecular ecology*, 26(21), pp. 5872–5895. Available from: doi: 10.1111/mec.14350

Dejean, T. *et al.* (2012) 'Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus', *The Journal of applied ecology*, 49(4), pp. 953–959. Available from: doi: 10.1111/j.1365-2664.2012.02171.x

Dick, J. *et al.* (2013) 'Predators vs. alien: differential biotic resistance to an invasive species by two resident predators', *NeoBiota*. 19, p. 1. Available from: doi: 10.3897/neobiota.19.4839

Dick, J. T. A., Alexander, M. E. and MacNeil, C. (2012) 'Natural born killers: an invasive amphipod is predatory throughout its life-history', *Biological invasions*, 15(2), pp. 309–313. Available from: doi: 10.1007/s10530-012-0287-9

Dick, J. T. A., Ian Montgomery, W. and Elwood, R. W. (1999) 'Intraguild predation may explain an amphipod replacement: evidence from laboratory populations', *Journal of zoology*, 249(4), pp. 463–468. Available from: doi: 10.1111/j.1469-7998.1999.tb01216.x

Dick, J. T. A., Platvoet, D. and Kelly, D. W. (2002) 'Predatory impact of the freshwater invader Dikerogammarus villosus (Crustacea: Amphipoda)', *Canadian journal of fisheries and aquatic sciences. Journal canadien des sciences halieutiques et aquatiques*, 59(6), pp. 1078–1084. Available from: doi: 10.1139/f02-074

Dick, J. T. and Platvoet, D. (2000) 'Invading predatory crustacean Dikerogammarus villosus eliminates both native and exotic species', *Proceedings. Biological sciences*, 267(1447), pp. 977–983. Available from: doi: 10.1098/rspb.2000.1099

Dodd, J. A. *et al.* (2014) 'Predicting the ecological impacts of a new freshwater invader: functional responses and prey selectivity of the "killer shrimp", Dikerogammarus villosus, compared to the native Gammarus pulex', *Freshwater biology*, 59(2), pp. 337–352. Available from: doi: 10.1111/fwb.12268

Dormann, C. F., Gruber, B. and Fründ, J. (2008) 'Introducing the bipartite package: analysing ecological networks', R News, 8(2), 8-11.

Dudgeon, D. *et al.* (2006) 'Freshwater biodiversity: importance, threats, status and conservation challenges', *Biological reviews of the Cambridge Philosophical Society*, 81(2), pp. 163–182. Available from: doi: 10.1017/S1464793105006950

Dunne, J. A., Williams, R. J. and Martinez, N. D. (2002) 'Network structure and biodiversity loss in food webs: robustness increases with connectance', *Ecology letters*, 5(4), pp. 558–567. Available from: doi: 10.1046/j.1461-0248.2002.00354.x

Elbrecht, V. *et al.* (2017) 'Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring', *Methods in ecology and evolution / British Ecological Society*, 8(10), pp. 1265–1275. Available from: doi: 10.1111/2041-210X.12789

Elbrecht, V. and Leese, F. (2017) 'Validation and Development of COI Metabarcoding Primers for Freshwater Macroinvertebrate Bioassessment', *Frontiers of Environmental Science & Engineering in China*, 5, p. 11. Available from: doi: 10.3389/fenvs.2017.00011

Elbrecht, V., Peinert, B. and Leese, F. (2017) 'Sorting things out: Assessing effects of unequal specimen biomass on DNA metabarcoding', *Ecology and evolution*, 7(17), pp. 6918–6926. Available from: doi: 10.1002/ece3.3192

Evans, D. M. *et al.* (2016) 'Merging DNA metabarcoding and ecological network analysis to understand and build resilient terrestrial ecosystems', *Functional ecology*, 30(12), pp. 1904–1916. Available from: doi: 10.1111/1365-2435.12659

Evans, N. T. *et al.* (2015) 'Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding', *Molecular ecology resources,* 16(1), pp. 29-41. Available from: doi: 10.1111/1755-0998.12433.

Ficetola, G. F. *et al.* (2008) ‘Species detection using environmental DNA from water samples’, *Biology letters*, 4(4), pp. 423–425. Available from: doi: 10.1098/rsbl.2008.0118 ·

Filipova, L. *et al.* (2013) ‘Prevalence of the crayfish plague pathogen Aphanomyces astaci in populations of the signal crayfish Pacifastacus leniusculus in France: evaluating the threat to native crayfish’, Plos One, 8(7), p. e70157. Available from: doi: 10.1371/journal.pone.0070157

Folmer, O. *et al.* (1994) ‘DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates’, *Molecular marine biology and biotechnology*, 3(5), pp. 294–299.

Free, J. B. (1960) ‘The Behaviour of Honeybees Visiting Flowers of Fruit Trees’, *The Journal of animal ecology*, 29(2), pp. 385–395. Available from: doi: 10.2307/2211

Frossard, V. and Fontvieille, D. (2018) ‘What is the invasiveness of Hemimysis anomala (Crustacea, Mysidae) in the large deep Lake Bourget, France?’, *Hydrobiologia*, 814, pp. 1–14. Available from: doi: 10.1007/s10750-018-3538-7

Furlong, M. J. (2015) ‘Knowing your enemies: Integrating molecular and ecological methods to assess the impact of arthropod predators on crop pests’, *Insect science*, 22(1), pp. 6–19. Available from: doi: 10.1111/1744-7917.12157.

Gagnon, A.-È. *et al.* (2011) ‘Prey DNA detection success following digestion by intraguild predators: influence of prey and predator species’, *Molecular ecology resources*, 11(6), pp. 1022–1032. Available from: doi: 10.1111/j.1755-0998.2011.03047.x

Galiana, N. *et al.* (2014) ‘Invasions cause biodiversity loss and community simplification in vertebrate food webs’, *Oikos,* 123(6), pp. 721–728. Available from: doi: 10.1111/j.1600-0706.2013.00859.x

Gallardo, B. *et al.* (2016) ‘Global ecological impacts of invasive species in aquatic ecosystems’, *Global change biology*, 22(1), pp. 151–163. Available from: doi: 10.1111/gcb.13004

Gallardo, B. and Aldridge, D. C. (2013) ‘The “dirty dozen”: socio-economic factors amplify the invasion potential of 12 high-risk aquatic invasive species in Great Britain and Ireland’, *The Journal of applied ecology*, 50(3), pp. 757–766. Available from: doi: 10.1111/1365-2664.12079

Gallardo, B. and Aldridge, D. C. (2015) ‘Is Great Britain heading for a Ponto–Caspian invasional meltdown?’, *The Journal of applied ecology*, 52(1), pp. 41–49. Available from: doi: 10.1111/1365-2664.12348

Garland, E. M. (1980) ‘The colonization of Windermere by Crangonyx pseudogracilis (Crustacea, Amphipoda) 1961 to 1964’. Freshwater Biological Association Ambleside.

Geist, J. (2011) ‘Integrative freshwater ecology and biodiversity conservation’,

Ecological indicators, 11(6), pp. 1507–1516. Available from: doi: 10.1016/j.ecolind.2011.04.002

Geist, J. and Hawkins, S. J. (2016) 'Habitat recovery and restoration in aquatic ecosystems: current progress and future challenges: Aquatic restoration', *Aquatic conservation: marine and freshwater ecosystems*, 26(5), pp. 942–962. Available from: doi: 10.1002/aqc.2702

Geller, J. *et al.* (2013) 'Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys', *Molecular ecology resources*, 13(5), pp. 851–861. Available from: doi: 10.1111/1755-0998

Geller, J. B. (1999) 'Decline of a native mussel masked by sibling species invasion', Conservation biology*,* 13(3), pp. 661 – 664. Available from: doi: 10.1046/j.1523-1739.1999.97470.x

Gergs, R. and Rothhaupt, K.-O. (2014) 'Invasive species as driving factors for the structure of benthic communities in Lake Constance, Germany', *Hydrobiologia*, 746(1), pp. 245–254. Available from: doi: 10.1007/s10750-014-1931-4

Gorički, Š. *et al.* (2017) 'Environmental DNA in subterranean biology: range extension and taxonomic implications for Proteus', *Scientific reports*, 7, p. 45054. Available from: doi: 10.1038/srep45054

Grason, E. W. and Buhle, E. R. (2016/6) 'Comparing the influence of native and invasive intraguild predators on a rare native oyster', *Journal of experimental marine biology and ecology*, 479, pp. 1–8. Available from: doi: 10.1016/j.jembe.2016.02.012

Green, P. T. *et al.* (2011) 'Invasional meltdown: invader-invader mutualism facilitates a secondary invasion', *Ecology*, 92(9), pp. 1758–1768. Available from: doi: 10.1890/11-0050.1

Greenstone, M. H. *et al.* (2014) 'The detectability half-life in arthropod predator-prey research: what it is, why we need it, how to measure it, and how to use it', *Molecular ecology*, 23(15), pp. 3799–3813. Available from: doi: 10.1111/mec.12552.

Grez, A. A. *et al.* (2016) 'Rapid spread of Harmonia axyridis in Chile and its effects on local coccinellid biodiversity', *Diversity & distributions*, 22(9), pp. 982–994. Available from: doi: 10.1111/ddi.12455

Grez, A. A. *et al.* (2017) 'In the shadow of the condor: invasive Harmonia axyridis found at very high altitude in the Chilean Andes', *Insect conservation and diversity*, 10(6), pp. 483–487. Available from: doi: 10.1111/icad.12258

Halbritter, A. H. *et al.* (2012) 'Testing assumptions of the enemy release hypothesis: generalist versus specialist enemies of the grass Brachypodium sylvaticum', *Mycologia*, 104(1), pp. 34–44. Available from: doi: 10.3852/11-071

Hall, R. J. (2011) ‘Eating the competition speeds up invasions’, *Biology letters*, 7(2), pp. 307–311. Available from: doi: 10.1098/rsbl.2010.0766

Hänfling, B. *et al.* (2016) ‘Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods’, *Molecular ecology*, 25(13), pp. 3101–3119. Available from: doi: 10.1111/mec.13660

Hänfling, B., Edwards, F. and Gherardi, F. (2011) ‘Invasive alien Crustacea: dispersal, establishment, impact and control’, *Biocontrol*, 56(4), p. 573. Available from: doi: 10.1007/s10526-011-9380-8

Harms-Tuohy, C. A., Schizas, N. V. and Appeldoorn, R. S. (2016) ‘Use of DNA metabarcoding for stomach content analysis in the invasive lionfish Pterois volitans in Puerto Rico’, *Marine ecology progress series*, 558, pp. 181–191. Available from: doi: 10.3354/meps11738

Harper, G. L. *et al.* (2005) ‘Rapid screening of invertebrate predators for multiple prey DNA targets’, *Molecular ecology*, 14(3), pp. 819–827. Available from: doi: 10.1111/j.1365-294X.2005.02442.x

Hartigan, A. *et al.* (2011) ‘A suspected parasite spill-back of two novel Myxidium spp. (Myxosporea) causing disease in Australian endemic frogs found in the invasive Cane toad’, *PloS one*. journals.plos.org, 6(4), p. e18871. Available from: doi: 10.1371/journal.pone.0018871

Harwood, J. D. *et al.* (2007) ‘An order-specific monoclonal antibody to Diptera reveals the impact of alternative prey on spider feeding behavior in a complex food web’, *Biological control,* 41(3), pp. 397–407. Available from: doi: 10.1016/j.biocontrol.2007.02.008

Hebert, P. D. N. *et al.* (2003) ‘Biological identifications through DNA barcodes’, *Proceedings of the Royal Society of London,* 270(1512), pp. 313–321. Available from: doi: 10.1098/rspb.2002.2218

Hebert, P. D. N. *et al.* (2004) ‘Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator’, *Proceedings of the National Academy of Sciences of the United States of America*. 101(41), pp. 14812–14817. Available from: doi: 10.1073/pnas.0406166101

Hellmann, C. *et al.* (2016) ‘River-specific effects of the invasive amphipod Dikerogammarus villosus (Crustacea: Amphipoda) on benthic communities’, *Biological invasions*, 19, pp. 1–18. Available from: doi: 10.1007/s10530-016-1286-z

Henkanaththegedara, S. M. and Stockwell, C. A. (2014) ‘Intraguild predation may facilitate coexistence of native and non-native fish’, *The Journal of applied ecology*, 51(4), pp. 1057–1065. Available from: doi: 10.1111/1365-2664.12285

Hester, S. M. and Cacho, O. J. (2017) ‘The contribution of passive surveillance to invasive species management’, *Biological invasions*, 19(3), pp. 737–748. Available from: doi: 10.1007/s10530-016-1362-4

Higgins, S. N. and Zanden, M. J. V. (2010) ‘What a difference a species makes: a meta–analysis of dreissenid mussel impacts on freshwater ecosystems’, *Ecological monographs*, 80(2), pp. 179–196. Available from: doi: 10.1890/09-1249.1

Hohenadler, M. A. A. *et al.* (2019) ‘How Ponto-Caspian invaders affect local parasite communities of native fish’, *Parasitology research,* 118(9), pp. 2543–2555. Available from: doi: 10.1007/s00436-019-06399-3.

Holmes, J. M. C. (1975) ‘Crangonyx pseudogracilis Bousfield a freshwater amphipod new to Ireland’, *The Irish naturalists’ journal,* 26(7/8), pp. 225–226.

Honek, A. *et al.* (2014) ‘Long-term trends in the composition of aphidophagous coccinellid communities in Central Europe’, *Insect conservation and diversity*, 7(1), pp. 55–63. Available from: doi: 10.1111/icad.12032

Honek, A. *et al.* (2017) ‘Life cycle of Harmonia axyridis in central Europe’, *Biocontrol*, 63, pp. 1–12. Available from: doi: 10.1007/s10526-017-9864-2

Hooper, R. *et al.* (2019) ‘Host-derived population genomics data provides insights into bacterial and diatom composition of the killer whale skin’, *Molecular ecology*, 28(2), pp. 484–502. Available from: doi: 10.1111/mec.14860.

Howe, A. G. *et al.* (2016) ‘Potential for exploitative competition, not intraguild predation, between invasive harlequin ladybirds and flowerbugs in urban parks’, *Biological invasions*, 18(2), pp. 517–532. Available from: doi: 10.1007/s10530-015-1024-y

Hughes, K. A. *et al.* (2020) ‘Invasive non-native species likely to threaten biodiversity and ecosystems in the Antarctic Peninsula region’, *Global change biology*. Available from: doi: 10.1111/gcb.14938.

Hulme, P. E. (2009) *Handbook of Alien Species in Europe*. Springer Dordrecht.

Hunter, M. E. *et al.* (2015) ‘Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive burmese pythons’, *PloS one*, 10(4), p. e0121655. Available from: doi: 10.1371/journal.pone.0121655

Ingels, B. *et al.* (2013) ‘Molecular analysis of the gut contents of Harmonia axyridis (Coleoptera: Coccinellidae) as a method for detecting intra-guild predation by this species on aphidophagous predators other than coccinellids’, *European journal of entomology*, 110(4), pp. 567–576. Available from: doi: 10.14411/eje.2013.077

Ingels, B. *et al.* (2015) ‘Feeding History Affects Intraguild Interactions between Harmonia axyridis (Coleoptera: Coccinellidae) and Episyrphus balteatus (Diptera: Syrphidae)’, *PloS one*, 10(6), p. e0128518. Available from: doi: 10.1371/journal.pone.0128518

Ings, T. C., Montoya, J. M. and Bascompte, J. (2009) ‘Ecological networks–beyond food webs’, *Journal of Animal Ecology,* 73(1), pp. 253-269.

Available from: doi: 10.1111/j.1365-2656.2008.01460.x.

Jackson, M. C., Ruiz-Navarro, A. and Britton, J. R. (2015) 'Population density modifies the ecological impacts of invasive species', *Oikos* . Wiley Online Library, 124(7), pp. 880–887.

Jackson, M. *et al.* (2017) 'Novel and Disrupted Trophic Links Following Invasion in Freshwater Ecosystems', in Bohan, D. A., Dumbrell, A. J., and Massol, F. (eds) *Advances in Ecological Research (Vol 56)*. *Academic Press Oxford London Cambridge San Diego,* pp. 55-97.

Jakubavičiūtė, E. *et al.* (2017) 'DNA metabarcoding reveals diverse diet of the three-spined stickleback in a coastal ecosystem', *PloS one*, 12(10), p. e0186929. Available from: doi: 10.1371/journal.pone.0186929

Jerde, C. L. *et al.* (2011) '"Sight-unseen" detection of rare aquatic species using environmental DNA', *Conservation Letters*, 4(2), pp. 150–157. Available from: doi: 10.1111/j.1755-263X.2010.00158.x

Jeschke, J. M. (2014) 'General hypotheses in invasion ecology', *Diversity & distributions*, 20(11), pp. 1229–1234. Available from: doi: 10.1111/ddi.12258

Jiang, F. and Doudna, J. A. (2017) 'CRISPR-Cas9 Structures and Mechanisms', *Annual review of biophysics*, 46, pp. 505–529. Available from: doi: 10.1146/annurev-biophys-062215-010822

Jombart, T. (2008) 'adegenet: a R package for the multivariate analysis of genetic markers', *Bioinformatics*, 24(11), pp. 1403–1405. Available from: doi: 10.1093/bioinformatics/btn129

Juen, A. *et al.* (2012) 'Analysing the diets of invertebrate predators using terminal restriction fragments', *Journal of Pest Science*. Springer-Verlag, 85(1), pp. 89–100. Available from: doi: 10.1007/s10340-011-0406-x

Juen, A. and Traugott, M. (2005) 'Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system', *Oecologia*, 142(3), pp. 344–352. Available from: doi: 10.1007/s00442-004-1736-7

Juen, A. and Traugott, M. (2007) 'Revealing species-specific trophic links in soil food webs: molecular identification of scarab predators', *Molecular ecology*, 16(7), pp. 1545–1557. Avaiable from: doi: 10.1111/j.1365-294X.2007.03238.x

Kamenova, S. *et al.* (2018) 'Comparing three types of dietary samples for prey DNA decay in an insect generalist predator', *Molecular ecology resources,* 18(1), pp. 966–973. doi: 10.1111/1755-0998.12775.

Kanada S. *et al.* (2007) 'Distribution of an alien species, Crangonyx floridanus Bousfield (Crustacea: Amphipoda: Crangonyctidae) in Japan', *Japanese Journal of Limnology*, 68(3), pp. 449-460. Available from: doi: 10.3739/rikusui.68.449

Kartzinel, T. R. *et al.* (2015) 'DNA metabarcoding illuminates dietary niche

partitioning by African large herbivores', *Proceedings of the National Academy of Sciences of the United States of America*, 112(26), pp. 8019–8024. Available from: doi: 10.1073/pnas.1503283112

Katoh, K. *et al.* (2002) 'MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform', *Nucleic acids research*, 30(14), pp. 3059–3066. Available from: doi: 10.1093/nar/gkf436

Katsanis, A. *et al.* (2013) 'Intraguild predation between the invasive ladybird Harmonia axyridis and non-target European coccinellid species', *Biocontrol,* 58(1), pp. 73–83. Available from: doi: 10.1007/s10526-012-9470-2

Kaunisto, K. M. *et al.* (2017) 'Pellets of proof: First glimpse of the dietary composition of adult odonates as revealed by metabarcoding of feces', *Ecology and evolution*, 7(20), pp. 8588–8598. Available from: doi: 10.1002/ece3.3404

Keller, R. P. *et al.* (2011) 'Linking environmental conditions and ship movements to estimate invasive species transport across the global shipping network', *Diversity and Distributions,* 17(1), pp. 93–102. Available from: doi: 10.1111/j.1472-4642.2010.00696.x

Kelly, D. W. *et al.* (2009) 'Parasite spillback: a neglected concept in invasion ecology?', *Ecology*, 90(8), pp. 2047–2056. Available from: doi: 10.1890/08-1085.1

Kenna, D. *et al.* (2016) 'Antagonistic effects of biological invasion and environmental warming on detritus processing in freshwater ecosystems', *Oecologia, 183, pp. 875–886*. Available from: doi: 10.1007/s00442-016-3796-x.

Kestrup, Å. M., Dick, J. T. A. and Ricciardi, A. (2011) 'Interactions between invasive and native crustaceans: differential functional responses of intraguild predators towards juvenile hetero-specifics', *Biological invasions*, 13(3), pp. 731–737. Available from: doi: 10.1371/journal.pone.0147017

King, R. A. *et al.* (2008) 'Molecular analysis of predation: a review of best practice for DNA-based approaches', *Molecular ecology*, 17(4), pp. 947–963. Available from: doi: 10.1111/j.1365-294X.2007.03613.x

Kinzler, W. *et al.* (2008) 'Mutual predation between and cannibalism within several freshwater gammarids: Dikerogammarus villosus versus one native and three invasives', *Aquatic ecology*, 43(2), pp. 457–464. Available from: doi: 10.1007/s10452-008-9206-7

Kinzler, W. and Maier, G. (2003) 'Asymmetry in mutual predation: possible reason for the replacement of native gammarids by invasives', *Archiv für Hydrobiologie*, 157(4), pp. 473–481. Available from: doi: 10.1127/0003-9136/2003/0157-0473

Kitson, J. J. N. *et al.* (2018) 'Detecting host-parasitoid interactions in an invasive Lepidopteran using nested tagging DNA metabarcoding', *Molecular ecology,* 28(2), pp. 471-483. Available from: doi: 10.1111/mec.14518.

Klimaszewski, J. *et al.* (2013) ‘Molecular and microscopic analysis of the gut contents of abundant rove beetle species (Coleoptera, Staphylinidae) in the boreal balsam fir forest of Quebec, Canada’, *ZooKeys*, (353), pp. 1–24. Available from: doi: 10.3897/zookeys.353.5991

Kobak, J. *et al.* (2016) ‘Impact of predators and competitors on the depth selection by two invasive gammarids’, *Journal of zoology,* 301(3), pp. 174-183. Available from: doi: 10.1111/jzo.12409.

Koch, R. L. *et al.* (2003) ‘Susceptibility of immature monarch butterfly, Danaus plexippus (Lepidoptera: Nymphalidae: Danainae), to predation by Harmonia axyridis (Coleoptera: Coccinellidae)’, *Biological control*, 28(2), pp. 265–270. Available from: doi: 10.1016/S1049-9644(03)00102-6

Koch, R. L. (2003) ‘The multicolored Asian lady beetle, Harmonia axyridis: a review of its biology, uses in biological control, and non-target impacts’, *Journal of insect science* , 3, p. 32. Available from: doi: 10.1093/jis/3.1.32

Koester, M. (2016) ‘Impact of the invasive amphipod Dikerogammarus villosus on trophic interactions under field conditions’. kola.opus.hbz-nrw.de. Available at: https://kola.opus.hbz-nrw.de/opus45-kola/frontdoor/deliver/index/docId/1280/file/Dissertation_Koester.pdf.

Koester, M. *et al.* (2018) ‘Is The invasive amphipod Dikerogammarus villosus the main factor structuring the benthic community across different types of water bodies in the River Rhine system?’, *Limnologica*, 71, pp. 44–50. Available from: doi: 10.1016/j.limno.2018.06.001.

Koester, M., Bayer, B. and Gergs, R. (2016) ‘Is Dikerogammarus villosus (Crustacea, Gammaridae) a “killer shrimp”in the River Rhine system?’, *Hydrobiologia*, 768(1), pp. 299–313. Available from: doi: 10.1007/s10750-015-2558-9

Koester, M., Claßen, S. and Gergs, R. (2013) ‘Establishment of group-specific PCR primers for the identification of freshwater macroinvertebrates’, *Conservation genetics resources*, 5(4), pp. 1091–1093. Available from: doi: 10.1007/s12686-013-9998-1

Koester, M. and Gergs, R. (2014) ‘No evidence for intraguild predation of Dikerogammarus villosus (Sowinsky, 1894) at an invasion front in the Untere Lorze, Switzerland’, *Aquatic Invasions*, 9(4), pp. 489–497. Available from: doi: 10.3391/ai.2014.9.4.07

Kornobis, E. *et al.* (2011) ‘Molecular taxonomy and phylogenetic affinities of two groundwater amphipods, Crangonyx islandicus and Crymostygius thingvallensis, endemic to Iceland’, *Molecular phylogenetics and evolution*, 58(3), pp. 527–539. Available from: doi: 10.1016/j.ympev.2010.12.010

Kozlov, A. M. *et al.* (2016) ‘Phylogeny-aware identification and correction of taxonomically mislabeled sequences’, *Nucleic acids research*, 44(11), pp. 5022–5033. Available from: doi: 10.1093/nar/gkw396

Krebs, C. J. and Others (1989) *Ecological methodology*. Harper & Row New York.

Krehenwinkel, H. *et al.* (2017) ‘A cost-efficient and simple protocol to enrich prey DNA from extractions of predatory arthropods for large-scale gut content analysis by Illumina sequencing’, *Methods in ecology and evolution*, 8(1), pp. 126–134. Available from: doi: 10.1111/2041-210X.12647

Krüger, F. *et al.* (2014) ‘Diet of the insectivorous bat Pipistrellus nathusii during autumn migration and summer residence’, *Molecular ecology*, 23(15), pp. 3672–3683. Available from: doi: 10.1111/mec.12547

Kuhlmann, U. (1993) ‘Techniques for rearing tachinid parasitoids of the European Earwig Forficula auricularia’, *Biocontrol science and technology*, 3(4), pp. 475–480. Available from: doi: 10.1080/09583159309355302

Kuhlmann, U. (1994) ‘Ocytata pallipes (Fallén) (Dipt., Tachinidae), a potential agent for the biological control of the European earwig’, *Journal of applied entomology*, 117(1-5), pp. 262–267. Available from: doi: 10.1111/j.1439-0418.1994.tb00734.x

Kuiper, J. J. *et al.* (2015) ‘Food-web stability signals critical transitions in temperate shallow lakes’, *Nature communications*, 6, p. 7727. Available from: doi: 10.1038/ncomms8727

Labrie, G., Coderre, D. and Lucas, É. (2008) ‘Overwintering Strategy of Multicolored Asian Lady Beetle (Coleoptera: Coccinellidae): Cold-Free Space as a Factor of Invasive Success’, *Annals of the Entomological Society of America*, 101(5), pp. 860–866. Available from: doi: 10.1093/aesa/101.5.860

Landi, P. *et al.* (2018) ‘Complexity and stability of ecological networks: a review of the theory’, *Population Ecology*, 60(4), pp. 319–345. Available from: doi: 10.1007/s10144-018-0628-3

Lawson Handley, L. (2015) ‘How will the “molecular revolution” contribute to biological recording?’, *Biological journal of the Linnean Society*, 115(3), pp. 750–766. Available from: doi: 10.1111/bij.12516

Layman, C. A. *et al.* (2015) ‘A primer on the history of food web ecology: Fundamental contributions of fourteen researchers’, *Food Webs*, 4, pp. 14–24. Available from: doi: 10.1016/j.fooweb.2015.07.001

Leal, M. C. *et al.* (2014) ‘Coral feeding on microalgae assessed with molecular trophic markers’, *Molecular ecology*, 23(15), pp. 3870–3876. Available from: doi: 10.1111/mec.12486

Leal, M. C. *et al.* (2014) ‘Molecular assessment of heterotrophy and prey digestion in zooxanthellate cnidarians’, *Molecular ecology*, 23(15), pp. 3838–3848. Available from: doi: 10.1111/mec.12496

Leray, M., Yang, J. Y., *et al.* (2013) ‘A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents’, *Frontiers in zoology*, 10, p. 34. Available from: doi: 10.1186/1742-9994-10-34

Leray, M., Agudelo, N., *et al.* (2013) 'Effectiveness of annealing blocking primers versus restriction enzymes for characterization of generalist diets: unexpected prey revealed in the gut contents of two coral reef fish species', *PloS one,* 8(4), p. e58076. Available from: doi: 10.1371/journal.pone.0058076

Leung, B. *et al.* (2002) 'An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species', *Proceedings Biological sciences*, 269(1508), pp. 2407–2413. Available from: doi: 10.1098/rspb.2002.2179

Li, J. *et al.* (2019) 'Ground-truthing of a fish-based environmental DNA metabarcoding method for assessing the quality of lakes', *The Journal of applied ecology*, 704, p. 193. Available from: doi: 10.1111/1365-2664.13352

Li, S.-P. *et al.* (2015) 'The effects of phylogenetic relatedness on invasion success and impact: deconstructing Darwin's naturalisation conundrum', *Ecology letters*, 18(12), pp. 1285–1292. Available from: doi: 10.1111/ele.12522

Little, C. J. and Altermatt, F. (2018) 'Species turnover and invasion of dominant freshwater invertebrates alter biodiversity-ecosystem-function relationship', *Ecological monographs*, 26, p. 15. Available from: doi: 10.1002/ecm.1299

Little, C. J. and Altermatt, F. (2019) 'Differential resource consumption in leaf litter mixtures by native and non-native amphipods', *Aquatic ecology*, 53, pp. 151–162. Available from: doi: 10.1007/s10452-019-09679-3.

Little, C. J., Fronhofer, E. A. and Altermatt, F. (2019) 'Dispersal syndromes can impact ecosystem functioning in spatially structured freshwater populations', *Biology letters*, 15(3), p. 20180865. Available from: doi: 10.1098/rsbl.2018.0865

Littlefair, J. E. *et al.* (2018) 'DNA metabarcoding reveals changes in the contents of carnivorous plants along an elevation gradient', *Molecular ecology*, 28(1). Available from: doi: 10.1111/mec.14832.

Liu, H. and Stiling, P. (2006) 'Testing the enemy release hypothesis: a review and meta-analysis', *Biological invasions,* 8(7), pp. 1535–1545. Available from: doi: 10.1007/s10530-005-5845-y

Lobo, J. *et al.* (2017) 'DNA metabarcoding for high-throughput monitoring of estuarine macrobenthic communities', *Scientific reports*, 7(1), p. 15618. Available from: doi: 10.1038/s41598-017-15823-6

Lods-Crozet, B. and Reymond, O. (2006) 'Bathymetric expansion of an invasive gammarid (Dikerogammarus villosus, Crustacea, Amphipoda) in Lake Léman', *Journal of limnology*, 65(2), pp. 141–144. Available from: doi: 10.4081/jlimnol.2006.141

Lyngdoh, S. *et al.* (2014) 'Prey preferences of the snow leopard (Panthera uncia): regional diet specificity holds global significance for conservation', *PloS one*, 9(2), p. e88349. Available from: doi: 10.1371/journal.pone.0088349

MacNeil, C. *et al.* (2010) ‘The Ponto-Caspian “killer shrimp”, Dikerogammarus villosus (Sowinsky, 1894), invades the British Isles’, *Aquatic Invasions*, 5(4), pp. 441–445. Available from: doi: 10.3391/ai.2010.5.4.15

MacNeil, C. *et al.* (2011) ‘Direct and indirect effects of species displacements: an invading freshwater amphipod can disrupt leaf-litter processing and shredder efficiency’, *Journal of the North American Benthological Society*, 30(1), pp. 38–48. Available from: doi: 10.1899/10-056.1

MacNeil, C. and Briffa, M. (2019) ‘Fear alone reduces energy processing by resident “keystone”prey threatened by an invader; a non-consumptive effect of “killer shrimp”invasion of freshwater ecosystems is revealed’, *Acta Oecologica*, 98, pp. 1–5. Available from: doi: 10.1016/j.actao.2019.05.001

MacNeil, C. and Dick, J. T. A. (2014) ‘The enemy of my enemy is my friend: intraguild predation between invaders and natives facilitates coexistence with shared invasive prey’, *Biology letters*. royalsocietypublishing.org, 10(8). Available from: doi: 10.1098/rsbl.2014.0398.

Macneil, C., Dick, J. T. A. and Elwood, R. W. (1999) ‘The dynamics of predation on Gammarus spp. (Crustacea: Amphipoda)’, *Biological reviews of the Cambridge Philosophical Society*, 74(4), pp. 375–395. Available from: doi: 10.1017/S0006323199005368

MacNeil, C., Elwood, R. W. and Dick, J. T. A. (1999) ‘Differential microdistributions and interspecific interactions in coexisting Gammarus and Crangonyx amphipods’, *Ecography*, 22(4), pp. 415–423. Available from: doi: 10.1111/j.1600-0587.1999.tb00578.x

MacNeil, C. and Platvoet, D. (2005) ‘The predatory impact of the freshwater invader Dikerogammarus villosus on native Gammarus pulex (Crustacea: Amphipoda); influences of differential microdistribution and food resources’, *Journal of zoology*, 267(1), pp. 31–38. Available from: doi: 10.1017/S0952836905007351

MacNeil, C., Platvoet, D. and Dick, J. T. A. (2008) ‘Potential roles for differential body size and microhabitat complexity in mediating biotic interactions within invasive freshwater amphipod assemblages’, *Fundamental and Applied Limnology*, 172(3), pp. 175-182. Available from: doi: 10.1127/1863-9135/2008/0172-0175.

Maczey, N. *et al.* (2016) ‘Biology and host range testing of Triarthria setipennis and Ocytata pallipes (Diptera: Tachinidae) for the control of the European earwig (Forficula auricularia)’, *Biocontrol science and technology,* 26(4), pp. 447–461. Available from: doi: 10.1080/09583157.2015.1123675

Magoč, T. and Salzberg, S. L. (2011) ‘FLASH: fast length adjustment of short reads to improve genome assemblies’, *Bioinformatics*, 27(21), pp. 2957–2963. Available from: doi: 10.1093/bioinformatics/btr507

Majerus, M., Strawson, V. and Roy, H. (2006) ‘The potential impacts of the arrival of the harlequin ladybird, Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae), in Britain’, *Ecological entomology*, 31(3). Available from: doi: 10.1111/j.1365-2311.2006.00734.x

Mariani, S. *et al.* (2019) ‘Sponges as natural environmental DNA samplers’, Current biology: CB, 29(11), pp. R401–R402. Available from: doi: 10.1016/j.cub.2019.04.031

Mastitsky, S. E. and Veres, J. K. (2010) ‘Field evidence for a parasite spillback caused by exotic mollusc Dreissena polymorpha in an invaded lake’, *Parasitology research*, 106(3), pp. 667–675. Available from: doi: 10.1007/s00436-010-1730-4

Mauvisseau, Q. and Davy-Bowker, J. (2018) ‘First detection of a highly invasive freshwater amphipod Crangonyx floridanus (Bousfield, 1963) in the United Kingdom’, *Bioinvasions records,* 8(1), pp. 1-7. Available from: doi: 10.3391/bir.2019.8.1.01

Mayer, G. *et al.* (2008) ‘Mouthparts of the Ponto-Caspian Invader Dikerogammarus Villosus (Amphipoda: Pontogammaridae)’, *Journal of Crustacean Biology*, 28(1) pp. 1–15. Available from: doi: 10.1651/07-2867r.1.

May, R. M. (1972) ‘Will a large complex system be stable?’, *Nature*, 238(5364), pp. 413–414. Available from: doi: 10.1038/238413a0

McClenaghan, B. *et al.* (2015) ‘Discrimination of grasshopper (Orthoptera: Acrididae) diet and niche overlap using next-generation sequencing of gut contents’, *Ecology and evolution*, 5(15), pp. 3046–3055. Available from: doi: 10.1002/ece3.1585

McGeoch, M. A. *et al.* (2010) ‘Global indicators of biological invasion: species numbers, biodiversity impact and policy responses’, *Diversity and Distributions*. 16(1), pp. 95–108. Available from: doi: 10.1111/j.1472-4642.2009.00633.x

McIntosh, R. R., Page, B. and Goldsworthy, S. D. (2006) ‘Dietary analysis of regurgitates and stomach samples from free-living Australian sea lions’, *Wildlife Research*, 33, pp. 661-669. Available from: doi: 10.1071/wr06025.

Meyerson, L. A. and Mooney, H. A. (2007) ‘Invasive Alien Species in an Era of Globalization’, *Frontiers in ecology and the environment*. Ecological Society of America, 5(4), pp. 199–208. Available from: doi: 10.1890/aesa/20440622

Michaud, J. P. (2002) ‘Invasion of the Florida Citrus Ecosystem by Harmonia axyridis (Coleoptera: Coccinellidae) and Asymmetric Competition with a Native Species, Cycloneda sanguinea’, *Environmental entomology*, 31(5), pp. 827–835. Available from: doi: 10.1603/0046-225X-31.5.827

Michaud, J. P. and Grant, A. K. (2003) ‘Intraguild predation among ladybeetles and a green lacewing: do the larval spines of Curinus coeruleus(Coleoptera: Coccinellidae) serve a defensive function?’, *Bulletin of entomological research*, 93(6), pp. 499–505. Available from: doi: 10.1079/BER2003269

Miller, M. A. *et al.* (2018) ‘Parasite spillover: indirect effects of invasive Burmese pythons’, *Ecology and evolution*, 8(2), pp. 830–840. Available from: doi:

10.1002/ece3.3557

Mittelbach, G. G. and Schemske, D. W. (2015) ‘Ecological and evolutionary perspectives on community assembly’, *Trends in ecology & evolution*, 30(5), pp. 241–247. Available from: doi: 10.1016/j.tree.2015.02.008

Mlynarek, J. J. (2015) ‘Testing the enemy release hypothesis in a native insect species with an expanding range’, *PeerJ*, 3, p. e1415. Available from: doi: 10.7717/peerj.1415

Moorhouse, T. P. and Macdonald, D. W. (2015) ‘Are invasives worse in freshwater than terrestrial ecosystems?: Freshwater and terrestrial invasive species’, *Wiley Interdisciplinary Reviews: Water*, 2(1), pp. 1–8. Available from: doi: 10.1002/wat2.1059

Morales-Hojas, R. (2017) ‘Molecular ecology of insect pests of agricultural importance: the case of aphids’, *Ecological entomology*, 42, pp. 18–27. Available from: doi: 10.1111/een.12445

Nagakubo, A. *et al.* (2011) ‘Rapid expansion of the distributional range and the population genetic structure of the freshwater amphipod Crangonyx floridanus in Japan’, *Limnology*, 12(1), pp. 75–82. Available from: doi: 10.1007/s10201-010-0323-3

Nesemann, H. and Pöckl, M. (1995) ‘Distribution of epigean Malacostraca in the middle and upper Danube (Hungary, Austria, Germany)’, *Miscellanea zoologica*, 10, pp. 49-68.

Nielsen, C. *et al.* (2005) ‘The Giant Hogweed Best Practice Manual. Guidelines for the management and control of an invasive weed in Europe’, *Forest and Landscape Denmark, Hoersholm*. neobiota.lu. Available at: https://neobiota.lu/wp/wp-content/uploads/2013/07/Heracleum_mantegazzianum_brochure-UK.pdf.

Nielsen, J. M. *et al.* (2018) ‘Diet tracing in ecology: Method comparison and selection’, *Methods in ecology and evolution*, 9(2), pp. 278–291. Available from: doi: 10.1111/2041-210X.12869

Oksanen, J. *et al.* (2018) ‘vegan: Community Ecology Package. R package version 2.5-2. 2018’.

Olds, B. P. *et al.* (2016) ‘Estimating species richness using environmental DNA’, *Ecology and evolution*, 6(12), pp. 4214–4226. Available from: doi: 10.1002/ece3.2186

Olson, L. J. (2006) ‘The economics of terrestrial invasive species: a review of the literature’, *Agricultural and Resource Economics Review*, 35(1), pp. 178-194. Available from: doi: 10.1017/S1068280500010145

O’Rorke, R. *et al.* (2012) ‘Determining the diet of larvae of western rock lobster (Panulirus cygnus) using high-throughput DNA sequencing techniques’, *PloS one*, 7(8), p. e42757. Available from: doi: 10.1371/journal.pone.0042757

Ovcharenko, M. O. *et al.* (2010) ‘Cucumispora dikerogammari n. gen. (Fungi:

Microsporidia) infecting the invasive amphipod Dikerogammarus villosus: a potential emerging disease in European rivers', *Parasitology*, 137(2), pp. 191–204. Available from: doi: 10.1017/S0031182009991119

Palmer, M. W. and White, P. S. (1994) 'On the existence of ecological communities', *Journal of vegetation science*, 5(2), pp. 279–282. Available from: doi: 10.2307/3236162

Pansu, J. *et al.* (2018) 'Trophic ecology of large herbivores in a reassembling African ecosystem', *The Journal of ecology,* 107(3), pp. 1-22. Available from: doi: 10.1111/1365-2745.13113.

Paradis, E. (2010) 'pegas: an R package for population genetics with an integrated-modular approach', *Bioinformatics* , 26(3), pp. 419–420. Available from: doi: 10.1093/bioinformatics/btp696

Paradis, E. and Schliep, K. (2018) 'ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R', *Bioinformatics*, 35(3), pp. 526–528. Available from: doi: 10.1093/bioinformatics/bty633

Paterson, R. A. *et al.* (2015) 'Predicting invasive species impacts: a community module functional response approach reveals context dependencies', *The Journal of animal ecology*, 84(2), pp. 453–463. Available from: doi: 10.1111/1365-2656.12292

Pell, J. K. *et al.* (2008) 'Intraguild predation involving Harmonia axyridis: a review of current knowledge and future perspectives', *Biocontrol*, 53(1), pp. 147–168. Available from: doi: 10.1007/978-1-4020-6939-0_10

Penk, M. *et al.* (2017) 'A trophic interaction framework for identifying the invasive capacity of novel organisms', *Methods in ecology and evolution*, 8(12), pp. 1786–1794. Available from: doi: 10.1111/2041-210X.12817

Pilosof, S. *et al.* (2017) 'The multilayer nature of ecological networks', *Nature ecology & evolution*. nature.com, 1(4), p. 101. Available from: doi: 10.1038/s41559-017-0101

Pimentel, D., Zuniga, R. and Morrison, D. (2005) 'Update on the environmental and economic costs associated with alien-invasive species in the United States', *Ecological economics*, 52(3), pp. 273–288. Available from: doi: 10.1016/j.ecolecon.2004.10.002

Piñol, J. *et al.* (2014) 'A pragmatic approach to the analysis of diets of generalist predators: the use of next-generation sequencing with no blocking probes', *Molecular ecology resources,* 14(1), pp. 18–26. Available from: doi: 10.1111/1755-0998.12156

Piñol, J. *et al.* (2015) 'Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods', *Molecular ecology resources*, 15(4), pp. 819–830. Available from: doi: 10.1111/1755-0998.12355

Piñol, J., Senar, M. A. and Symondson, W. O. C. (2018) 'The choice of universal primers and the characteristics of the species mixture determine

when DNA metabarcoding can be quantitative', *Molecular ecology*, 28 (2), pp. 407-419. Available from: doi: 10.1111/mec.14776.

Piscart, C. *et al.* (2009) 'Are amphipod invaders a threat to regional biodiversity?', Biological invasions, 12(4), pp. 853–863. Available from: doi: 10.1007/s10530-009-9506-4

Piscart, C., Roussel, J.-M., *et al.* (2011) 'Effects of coexistence on habitat use and trophic ecology of interacting native and invasive amphipods', *Freshwater Biology*, 56(2), pp. 325–334. Available from: doi: 10.1111/j.1365-2427.2010.02500.x.

Piscart, C., Mermillod-Blondin, F., *et al.* (2011) 'Potential impact of invasive amphipods on leaf litter recycling in aquatic ecosystems', *Biological invasions*, 13(12), pp. 2861–2868. Available from: doi: 10.1111/fwb.12158

Platvoet, D. *et al.* (2009) 'Flexible Omnivory in Dikerogammarus villosus (Sowinsky, 1894) (Amphipoda) - Amphipod Pilot Species Project (AMPIS) Report 5', *Crustaceana*, 82(6), pp. 703–720. Available from: doi: 10.1163/156854009X423201

Pöckl, M. (2007) 'Strategies of a successful new invader in European fresh waters: fecundity and reproductive potential of the Ponto-Caspian amphipod Dikerogammarus villosus in the Austrian Danube, compared with the indigenous Gammarus fossarum and G. roeseli', *Freshwater biology*, 52(1), pp. 50–63. Available from: doi: 10.1111/j.1365-2427.2006.01671.x

Pocock, M. J. O. *et al.* (2014) *Choosing and using citizen science: a guide to when and how to use citizen science to monitor biodiversity and the environment*. Wallingford: NERC/Centre for Ecology & Hydrology, p. 24.

Polis, G. A., Myers, C. A. and Holt, R. D. (1989) 'The Ecology and Evolution of Intraguild Predation: Potential Competitors That Eat Each Other', *Annual review of ecology and systematics*, 20(1), pp. 297–330. Available from: doi: 10.1146/annurev.es.20.110189.001501

Pompanon, F. *et al.* (2012) 'Who is eating what: diet assessment using next generation sequencing', *Molecular ecology*, 21(8), pp. 1931–1950. Available from: doi: 10.1111/j.1365-294X.2011.05403.x

Porter, T. M. and Hajibabaei, M. (2018) 'Scaling up: A guide to high-throughput genomic approaches for biodiversity analysis', *Molecular ecology*, 27(2), pp. 313–338. Available from: doi: 10.1111/mec.14478.

Post, D. M. *et al.* (2008) 'Intraspecific variation in a predator affects community structure and cascading trophic interactions', *Ecology*, 89(7), pp. 2019–2032. Available from: doi: 10.1890/07-1216.1

Purse, B. V. *et al.* (2015) 'Landscape and climate determine patterns of spread for all colour morphs of the alien ladybird Harmonia axyridis', *Journal of biogeography*, 42(3), pp. 575–588. Available from: doi: 10.1111/jbi.12423

Pyšek, P. and Richardson, D. M. (2010) 'Invasive Species, Environmental

Change and Management, and Health', *Annual review of environment and resources*, 35(1), pp. 25–55. Available from: doi: 10.1146/annurev-environ-033009-095548

Quail, M. A., Swerdlow, H. and Turner, D. J. (2009) 'Improved protocols for the illumina genome analyzer sequencing system', *Current protocols in human genetics*, Chapter 18, p. Unit 18.2.

Rafferty, N. E. and Ives, A. R. (2013) 'Phylogenetic trait-based analyses of ecological networks', *Ecology*, 94(10), pp. 2321–2333. Available from: doi: 10.1890/12-1948.1

Raso, L. *et al.* (2014) 'Intraguild predation in pioneer predator communities of alpine glacier forelands', *Molecular ecology*, 23(15), pp. 3744–3754. Available from: doi: 10.1111/mec.12649

Razgour, O. *et al.* (2011) 'High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species', *Ecology and evolution*, 1(4), pp. 556–570. Available from: doi: 10.1002/ece3.49

Reum, J. C. P. *et al.* (2019) 'Species-specific ontogenetic diet shifts attenuate trophic cascades and lengthen food chains in exploited ecosystems', *Oikos,* 128(7), pp. 1051–1064. Available from: doi: 10.1111/oik.05630

Rewicz, T. *et al.* (2014) 'The profile of a "perfect" invader - the case of killer shrimp, Dikerogammarus villosus', *Aquatic invasions,* 9(3), pp. 267-288. Available from: doi: 10.3391/ai.2014.9.3.04

Rewicz, T. *et al.* (2015) 'Out of the Black Sea: phylogeography of the invasive killer shrimp Dikerogammarus villosus across Europe', *PloS one*, 10(2), p. e0118121. Available from: doi: 10.1371/journal.pone.0118121

Ribaudo, C. *et al.* (2018) 'Invasive Aquatic Plants as Ecosystem Engineers in an Oligo-Mesotrophic Shallow Lake', *Frontiers in plant science*, 9, p. 1781. Available from: doi: 10.3389/fpls.2018.01781

Ricciardi, A. (2001) 'Facilitative interactions among aquatic invaders: is an" invasional meltdown" occurring in the Great Lakes?', *Canadian journal of fisheries and aquatic sciences. Journal canadien des sciences halieutiques et aquatiques*. 58(12), pp. 2513–2525. Available from: doi: 10.1139/cjfas-58-12-2513

Ricciardi, A. and Atkinson, S. K. (2004) 'Distinctiveness magnifies the impact of biological invaders in aquatic ecosystems', *Ecology letters*, 7(9), pp. 781–784. Available from: doi: 10.1111/j.1461-0248.2004.00642.x

Ricciardi, A. and Cohen, J. (2006) 'The invasiveness of an introduced species does not predict its impact', *Biological invasions*, 9(3), pp. 309–315. Available from: doi: 10.1007/s10530-006-9034-4

van Riel, M. C. *et al.* (2006) 'Trophic Relationships in the Rhine Food Web during Invasion and after Establishment of the Ponto-Caspian Invader Dikerogammarus villosus', *Hydrobiologia,* 565(1), pp. 39–58. Available from: doi: 10.1007/s10750-005-1904-8

van Riel, M. C. *et al.* (2007) 'Interference competition among native and invader amphipods', *Acta Oecologica*, 31(3), pp. 282–289. Available from: doi: 10.1016/j.actao.2006.12.006

Rognes, T. *et al.* (2016) 'VSEARCH: a versatile open source tool for metagenomics', *PeerJ*, 4, p. e2584. Available from: doi: 10.7717/peerj.2584

Rondoni, G. *et al.* (2015) 'Development and application of molecular gut-content analysis to detect aphid and coccinellid predation by Harmonia axyridis (Coleoptera: Coccinellidae) in Italy', *Insect science*, 22(6), pp. 719–730. Available from: doi: 10.1111/1744-7917.12165

Rondoni, G. *et al.* (2018) 'Molecular detection of field predation among larvae of two ladybird beetles is partially predicted from laboratory experiments', *Scientific reports*, 8(1), p. 2594. Available from: doi: 10.1038/s41598-018-20830-2

Rothhaupt, K.-O., Hanselmann, A. J. and Yohannes, E. (2014) 'Niche differentiation between sympatric alien aquatic crustaceans: An isotopic evidence', *Basic and applied ecology*, 15(5), pp. 453–463. Available from: doi: 10.1016/j.baae.2014.07.002

Roy, H. E. *et al.* (2012) 'Invasive alien predator causes rapid declines of native European ladybirds', *Diversity and Distributions*, 18(7), pp. 717–725. Available from: doi: 10.1111/j.1472-4642.2012.00883.x

Roy, H. E. *et al.* (2014) 'Horizon scanning for invasive alien species with the potential to threaten biodiversity in Great Britain', *Global change biology*, 20(12), pp. 3859–3871. Available from: doi: 10.1111/gcb.12603

Roy, H. E. *et al.* (2016) 'The harlequin ladybird, Harmonia axyridis: global perspectives on invasion history and ecology', *Biological invasions*, 18(4), pp. 997-1044. Available from: doi: 10.1007/s10530-016-1077-6.

Roy, H. E. and Brown, P. M. J. (2015) 'Ten years of invasion: Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae) in Britain', *Ecological entomology*, 40(4), pp. 336–348. Available from: doi: 10.1111/een.12203

Roy, H. E. and Lawson Handley, L.-J. (2012) 'Networking: a community approach to invaders and their parasites', *Functional ecology*, 26(6), pp. 1238–1248. Available from: doi: 10.2307/23326820

Ruppert, K. M., Kline, R. J. and Rahman, M. S. (2019) 'Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA', *Global Ecology and Conservation*, 17, p. e00547. Available from: doi: 10.1016/j.gecco.2019.e00547

Sala, O. E. *et al.* (2000) 'Global biodiversity scenarios for the year 2100', *Science*, 287(5459), pp. 1770–1774. Available from: doi: 10.1126/science.287.5459.1770

Schneider, F. D. *et al.* (2016) 'Animal diversity and ecosystem functioning in

dynamic food webs', *Nature communications*, 7, p. 12718. Available from: doi: 10.1038/ncomms12718

Schnell, I. B. *et al.* (2018) 'Debugging diversity--a pan-continental exploration of the potential of terrestrial blood-feeding leeches as a vertebrate monitoring tool', *Molecular ecology resources*,18(6), pp. 1282–1298. Available from: doi: 10.1111/1755-0998.12912

Schönrogge, K. and Crawley, M. J. (2000) 'Quantitative webs as a means of assessing the impact of alien insects', *The journal of animal ecology*, 69(5), pp. 841–868. Available from: doi: 10.1046/j.1365-2656.2000.00443.x

Sebesvari, Z., Ettwig, K. F. and Emons, H. (2005) 'Biomonitoring of tin and arsenic in different compartments of a limnic ecosystem with emphasis on Corbicula fluminea and Dikerogammarus villosus', *Journal of environmental monitoring,* 7(3), pp. 203–207. Available from: doi: 10.1039/b410717a

Secretariat of the Convention on Biological Diversity (2010) *Global Diversity Outlook 3*. Secretariat of the Convention on Biological Diversity. Available at: https://www.cbd.int/doc/publications/gbo/gbo3-final-en.pdf.

Sellers, G. S. *et al.* (2018) 'Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types', *Metabarcoding and Metagenomics*, 2, p. e24556. Available from: doi: 10.3897/mbmg.2.24556

Shaw, J. L. A. *et al.* (2019) 'Retrospective eDNA assessment of potentially harmful algae in historical ship ballast tank and marine port sediments', *Molecular ecology*, 28(10), pp. 2476–2485. Available from: doi: 10.1111/mec.15055

Sheppard, C. S. *et al.* (2018) 'It takes one to know one: Similarity to resident alien species increases establishment success of new invaders', *Diversity and Distributions*, 24(5), pp. 680–691. Available from: doi: 10.1111/ddi.12708

Sheppard, S. K. and Harwood, J. D. (2005) 'Advances in molecular ecology: tracking trophic links through predator–prey food-webs', *Functional ecology*, 19(5), pp. 751–762. Available from: doi: 10.1111/j.1365-2435.2005.01041.x

Shiels, A. B. et al. (2013) 'Dietary niche differentiation among three species of invasive rodents (Rattus rattus, R. exulans, Mus musculus)', *Biological invasions*, 15(5), pp. 1037–1048. Available from: doi: 10.1007/s10530-012-0348-0.

Šidagytė, E. *et al.* (2016) 'The killer shrimp Dikerogammarus villosus (Crustacea, Amphipoda) invades Lithuanian waters, South-Eastern Baltic Sea', *Oceanologia*, 59(1), pp. 85-91. Available from: doi: 10.1016/j.oceano.2016.08.004.

Siegenthaler, A., Wangensteen, O. S., Benvenuto, C., *et al.* (2018) 'DNA metabarcoding unveils multi-scale trophic variation in a widespread

coastal opportunist', *Molecular ecology*, 28(2), pp. 232-249. Available from: doi: 10.1111/mec.14886.

Siegenthaler, A., Wangensteen, O. S., Soto, A. Z., *et al.* (2018) 'Metabarcoding of shrimp stomach content: harnessing a natural sampler for fish biodiversity monitoring', *Molecular ecology resources*, 19(1), pp. 206-220. Available from: doi: 10.1111/1755-0998.12956.

Simmons, M. *et al.* (2016) 'Active and passive environmental DNA surveillance of aquatic invasive species', *Canadian journal of fisheries and aquatic sciences. Journal canadien des sciences halieutiques et aquatiques*, 73(1), pp. 76–83. Available from : doi : 10.1139/cjfas-2015-0262

Sint, D. *et al.* (2019) 'Resolving the predator first paradox: Arthropod predator food webs in pioneer sites of glacier forelands', *Molecular ecology*, 28(2), pp. 336–347. Available from : doi : 10.1111/mec.14839

Sloggett, J. J. (2008) 'Weighty matters: body size, diet and specialization in aphidophagous ladybird beetles (Coleoptera: Coccinellidae)', *European journal of entomology,* 105(3), pp. 381-389. Available from : doi : 10.14411/eje.2008.048

Sloggett, J. J. and Majerus, M. E. N. (2000) 'Habitat preferences and diet in the predatory Coccinellidae (Coleoptera): an evolutionary perspective', *Biological journal of the Linnean Society,* 70(1), pp. 63–88. Available from: doi: 10.1006/bijl.1999.0380

Slothouber Galbreath, J. G. M. *et al.* (2010) 'Reduction in post-invasion genetic diversity in Crangonyx pseudogracilis (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites?', *Biological invasions*, 12(1), pp. 191–209. Available from: doi: 10.1007/s10530-009-9442-3

Smith, C. A. and Gardiner, M. M. (2013) 'Biodiversity loss following the introduction of exotic competitors: does intraguild predation explain the decline of native lady beetles?', *PloS one*, 8(12), p. e84448. Available from: doi: 10.1371/journal.pone.0084448

Snow, N. P. and Witmer, G. (2010) 'American Bullfrogs as Invasive Species: A Review of the Introduction, Subsequent Problems, Management Options, and Future Directions', *Proceedings of the Vertebrate Pest Conference*, 24(24). Available from: doi: 10.5070/V424110490.

Snyder, W. E., Clevenger, G. M. and Eigenbrode, S. D. (2004a) 'Intraguild Predation and Successful Invasion by Introduced Ladybird Beetles', *Oecologia*. Springer, 140(4), pp. 559–565. Available from: doi: 10.1007/s00442-004-1612-5

Snyder, W. E., Clevenger, G. M. and Eigenbrode, S. D. (2004b) 'Intraguild predation and successful invasion by introduced ladybird beetles', *Oecologia*, 140(4), pp. 559–565. Available from: doi: 10.1007/s00442-004-1612-5

Soares, A. O., Coderre, D. and Schanderl, H. (2004) 'Dietary self-selection

behaviour by the adults of the aphidophagous ladybeetle Harmonia axyridis (Coleoptera: Coccinellidae)', *The Journal of animal ecology*, 73(3), pp. 478–486. Available from: doi: 10.1111/j.0021-8790.2004.00822.x

Soininen, E. M. *et al.* (2014) 'Sources of variation in small rodent trophic niche: new insights from DNA metabarcoding and stable isotope analysis', *Isotopes in environmental and health studies*, 50(3), pp. 361–381. Available from: doi: 10.1080/10256016.2014.915824

Sousa, R., Pilotto, F. and Aldridge, D. C. (2011) 'Fouling of European freshwater bivalves (Unionidae) by the invasive zebra mussel (Dreissena polymorpha)', *Freshwater biology*, 56(5), pp. 867–876. Available from: doi: 10.1111/j.1365-2427.2010.02532.x

Stamatakis, A. (2014) 'RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies', *BioinformaticsS*. academic.oup.com, 30(9), pp. 1312–1313. Available from: doi: 10.1093/bioinformatics/btu033

Stat, M. *et al.* (2017) 'Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment', *Scientific reports*, 7(1), p. 12240. Available from: doi: 10.1038/s41598-017-12501-5

Staudacher, K. *et al.* (2011) 'Detecting ingested plant DNA in soil-living insect larvae', *Soil biology & biochemistry*, 43(2), pp. 346–350. Available from: doi: 10.1016/j.soilbio.2010.10.022

Strauss, A., White, A. and Boots, M. (2012) 'Invading with biological weapons: the importance of disease-mediated invasions', *Functional ecology*, 26(6), pp. 1249–1261. Available from: doi: 10.1111/1365-2435.12011

Strauss, S. Y., Webb, C. O. and Salamin, N. (2006) 'Exotic taxa less related to native species are more invasive', *Proceedings of the National Academy of Sciences of the United States of America*, 103(15), pp. 5841–5845. Available from: doi: 10.1073/pnas.0508073103

Suding, K. N., Gross, K. L. and Houseman, G. R. (2004) 'Alternative states and positive feedbacks in restoration ecology', *Trends in ecology & evolution*, 19(1), pp. 46–53. Available from: doi: 10.1016/j.tree.2003.10.005

Su, M. *et al.* (2018) 'Dietary Analysis of Marine Fish Species: Enhancing the Detection of Prey-Specific DNA Sequences via High-Throughput Sequencing Using Blocking Primers', *Estuaries and Coasts*, 41(2), pp. 560–571. Available from: doi: 10.1007%2Fs12237-017-0279-1

Svavarsson, J. and Kristjansson, B. K. (2006) 'Crangonyx islandicus sp. nov., a subterranean freshwater amphipod (Crustacea, Amphipoda, Crangonyctidae) from springs in lava fields in Iceland', *Zootaxa*, 1365(1), pp. 1–17. Available from: doi: 10.5281/zenodo.174801

Symondson, W. (2002) 'Molecular identification of prey in predator diets', *Molecular ecology*, 11(4), pp. 627–641. Available from: doi: 10.1046/j.1365-294x.2002.01471.x

Symondson, W. O. C. and Harwood, J. D. (2014) 'Special issue on molecular

detection of trophic interactions: unpicking the tangled bank. Introduction', *Molecular ecology*, 23(15), pp. 3601–3604. Available from: doi: 10.1111/mec.12831

Szitenberg, A. *et al.* (2015) 'ReproPhylo: An Environment for Reproducible Phylogenomics', *PLoS computational biology*, 11(9), p. e1004447. Available from: doi: 10.1371/journal.pcbi.1004447

Taberlet, P. *et al.* (2012) 'Environmental DNA', *Molecular ecology*, 21(8), pp. 1789–1793. Available from: doi: 10.1111/j.1365-294X.2012.05542.x

Taylor, N. G. and Dunn, A. M. (2016) 'Size matters: predation of fish eggs and larvae by native and invasive amphipods', *Biological invasions*, 19(1), pp. 1–19. Available from: doi: 10.1007/s10530-016-1265-4

Teske, P. R. *et al.* (2011) '"Nested" cryptic diversity in a widespread marine ecosystem engineer: a challenge for detecting biological invasions', *BMC evolutionary biology*, 11(1), p. 176. Available from: doi: 10.1186/1471-2148-11-176

Thébault, E. and Fontaine, C. (2010) 'Stability of Ecological Communities and the Architecture of Mutualistic and Trophic Networks', *Science*, 329(5993), pp. 853–856. Available from: doi: 10.1126/science.1188321

Thiele, J. *et al.* (2010) 'Impact assessment revisited: improving the theoretical basis for management of invasive alien species', *Biological invasions*, 12(7), pp. 2025–2035. Available from: doi: 10.1007/s10530-009-9605-2

Thomas, A. C. *et al.* (2014) 'Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias', *Molecular ecology*, 23(15), pp. 3706–3718. Available from: 10.1111/mec.12523

Thomas, A. P. *et al.* (2013) 'Predation of native coccinellids by the invasive alien Harmonia axyridis (Coleoptera: Coccinellidae): detection in Britain by PCR-based gut analysis', *Insect conservation and diversity,* 6(1), pp. 20–27. Available from: doi: 10.1111/een.12203

Thuiller, W. *et al.* (2010) 'Resolving Darwin's naturalization conundrum: a quest for evidence', *Diversity and Distributions*, 16(3), pp. 461–475. Available from: doi: 10.1111/j.1472-4642.2010.00645.x

Toft, J. D., Cordell, J. R. and Fields, W. C. (2002) 'New Records of Crustaceans (Amphipoda, Isopoda) in the Sacramento/San Joaquin Delta, California, and Application of Criteria for Introduced Species', *Journal of crustacean biology: a quarterly of the Crustacean Society for the publication of research on any aspect of the biology of crustacea,* 22(1), pp. 190–200. Available from: doi: 10.1163/20021975-99990222

Tojo, K. *et al.* (2010) 'Reproductive biology and adaptability of the invasive alien freshwater Amphipod Crangonyx floridanus (Crustacea: Amphipoda, Crangonyctidae)', *Zoological science*, 27(6), pp. 522–527. Available from: doi: 10.2108/zsj.27.522

Toju, H. and Baba, Y. G. (2018) ‘DNA metabarcoding of spiders, insects, and springtails for exploring potential linkage between above- and below-ground food webs’, *Zoological letters,* 4, p. 4. Available from: doi: 10.1186/s40851-018-0088-9

Torchin, M. E. *et al.* (2003) ‘Introduced species and their missing parasites’, *Nature*, 421(6923), pp. 628–630. Available from: doi: 10.1038/nature01346

Torsvik, V. L. (1980) ‘Isolation of bacterial DNA from soil’, *Soil biology & biochemistry*, 12(1), pp. 15–21. Available from: doi: 10.1016/0038-0717(80)90097-8

Traugott, M. *et al.* (2013) ‘Chapter Three - Empirically Characterising Trophic Networks: What Emerging DNA-Based Methods, Stable Isotope and Fatty Acid Analyses Can Offer’, in Woodward, G. and Bohan, D. A. (eds) *Advances in Ecological Research. Academic Press Oxford London Cambridge San Diego,* pp. 177–224.

Tricarico, E. *et al.* (2010) ‘The killer shrimp, Dikerogammarus villosus (Sowinsky, 1894), is spreading in Italy’, *Aquatic Invasions*, 5(2), pp. 211–214. Available from: doi: 10.3391/ai.2010.5.2.14

Tringe, S. G. and Rubin, E. M. (2005) ‘Metagenomics: DNA sequencing of environmental samples’, *Nature reviews. Genetics*, 6(11), pp. 805–814. Available from: doi: 10.1038/nrg1709

Väinölä, R. *et al.* (2008) ‘Global diversity of amphipods (Amphipoda; Crustacea) in freshwater’, in Balian, E. V. et al. (eds) *Freshwater Animal Diversity Assessment*. Springer Dordrecht, pp. 241–255.

Valiente-Banuet, A. and Verdú, M. (2008) ‘Temporal shifts from facilitation to competition occur between closely related taxa: Facilitation-competition balance’, *The Journal of ecology*, 96(3), pp. 489–494. Available from: doi: 10.1111/j.1365-2745.2008.01357.x

Van der Putten, W. H., Macel, M. and Visser, M. E. (2010) ‘Predicting species distribution and abundance responses to climate change: why it is essential to include biotic interactions across trophic levels’, *Philosophical transactions of the Royal Society of London*, 365(1549), pp. 2025–2034. Available from: doi: 10.1098/rstb.2010.0037

Van der Velde, G. *et al.* (2000) ‘Ecological impact of crustacean invaders: general considerations and examples from the Rhine River’, *Crustacean issues*. 12, pp. 3–34.

Vestheim, H. and Jarman, S. N. (2008) ‘Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs’, *Frontiers in zoology*, 5, p. 12. Available from: doi: 10.1186/1742-9994-5-12

Violle, C. *et al.* (2011) ‘Phylogenetic limiting similarity and competitive exclusion’, *Ecology letters*, 14(8), pp. 782–787. Available from: doi: 10.1111/j.1461-0248.2011.01644.x

Waldner, T. *et al.* (2013) 'The effect of predator identity on post-feeding prey DNA detection success in soil-dwelling macro-invertebrates', *Soil biology & biochemistry*, 63, pp. 116–123. Available from: doi: 10.1016/j.soilbio.2013.03.030

Walsh, J. R., Carpenter, S. R. and Vander Zanden, M. J. (2016) 'Invasive species triggers a massive loss of ecosystem services through a trophic cascade', *Proceedings of the National Academy of Sciences of the United States of America*, 113(15), pp. 4081–4085. Available from: doi: 10.1073/pnas.1600366113

Walsh, J. R., Lathrop, R. C. and Vander Zanden, M. J. (2017) 'Invasive invertebrate predator, Bythotrephes longimanus, reverses trophic cascade in a north-temperate lake: Invasive invertebrate reverses cascade', *Limnology and oceanography*, 62(6), pp. 2498–2509. Available from: doi: 10.1002/lno.10582

Wang, S., Brose, U. and Gravel, D. (2019) 'Intraguild predation enhances biodiversity and functioning in complex food webs', *Ecology*, 100(3), p. e02616. Available from: doi: 10.1002/ecy.2616

Ware, R. L. and Majerus, M. E. N. (2008) 'Intraguild predation of immature stages of British and Japanese coccinellids by the invasive ladybird Harmonia axyridis', in Roy, H. E. and Wajnberg, E. (eds) *From Biological Control to Invasion: the Ladybird Harmonia axyridis as a Model Species*. Springer Dordrecht, pp. 169–188.

Ware, R., Yguel, B. and Majerus, M. (2009) 'Effects of competition, cannibalism and intra-guild predation on larval development of the European coccinellid Adalia bipunctata and the invasive species Harmonia axyridis', *Ecological entomology*, 34(1), pp. 12–19. Available from: doi: 10.1111/j.1365-2311.2008.01036.x

Weber, D. C. and Lundgren, J. G. (2009) 'Detection of predation using qPCR: effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA', *Journal of insect science,* 9(41). Available from: doi: 10.1673/031.009.4101.

Wells, P. M. *et al.* (2017) 'Determining the effects of life stage, shared prey density and host plant on intraguild predation of a native lacewing (Chrysoperla carnea) by an invasive coccinellid (Harmonia axyridis)', *Biocontrol*. Springer Netherlands, 62(3), pp. 373–384. Available from: doi: 10.1007/s10530-017-1608-9

Westphal, M. I. *et al.* (2007) 'The link between international trade and the global distribution of invasive alien species', *Biological invasions*. Springer Netherlands, 10(4), pp. 391–398. Available from: doi: 10.1007/s10530-007-9138-5

Whitney, E. R. (2016) 'Do native mites suppress invasive herbivores on eastern hemlock?', *2016 International Congress of Entomology*. doi: 10.1603/ice.2016.113920.

Wickham, H. (2011) 'ggplot2', *Wiley Interdisciplinary Reviews: Computational*

Statistics, 3(2), pp. 180–185. Available from: doi: doi.org/10.1002/wics.147

Wirta, H. K. *et al.* (2014) 'Complementary molecular information changes our perception of food web structure', *Proceedings of the National Academy of Sciences of the United States of America*, 111(5), pp. 1885–1890. Available from: doi: 10.1073/pnas.1316990111

Wirta, H. K. *et al.* (2015) 'Extensive niche overlap among the dominant arthropod predators of the High Arctic', *Basic and applied ecology*, 16(1), pp. 86–92. Available from: doi: 10.1016/j.baae.2014.11.003

Yang, F. *et al.* (2016) 'Intraguild Predation Among Three Common Coccinellids (Coleoptera: Coccinellidae) in China: Detection Using DNA-Based Gut-Content Analysis', *Environmental entomology*, 46(1), pp. 1-10. Available from: doi: 10.1093/ee/nvw154.

Ye, J. *et al.* (2012) 'Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction', *BMC bioinformatics*, 13(134). Available from: doi: 10.1186/1471-2105-13-134

Zarzoso-Lacoste, D. *et al.* (2016) 'Improving morphological diet studies with molecular ecology: An application for invasive mammal predation on island birds', *Biological conservation*, 193, pp. 134–142. Available from: doi: 10.1016/j.biocon.2015.11.018

Zeale, M. R. K. *et al.* (2011) 'Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces', *Molecular ecology resources*, 11(2), pp. 236–244. Available from: doi: 10.1111/j.1755-0998.2010.02920.x

Zhang, J. (2016) 'Species Association Analysis "spaa". R package'.

Zhang, J. and Holsinger, J. R. (2003) *Systematics of the Freshwater Amphipod Genus Crangonyx (Crangonyctidae) in North America*. Virginia Museum of Natural History.

Zilnik, G. and Hagler, J. R. (2013) 'An immunological approach to distinguish arthropod viviphagy from necrophagy', *Biocontrol*, 58(6), pp. 807–814. Available from: doi: 10.1007/s10526-013-9539-6

Zou, K. *et al.* (2016) 'Interactions between the green and brown food web determine ecosystem functioning', *Functional ecology*, 30(8), pp. 1454–1465. Available from: doi: 10.1111/1365-2435.12626