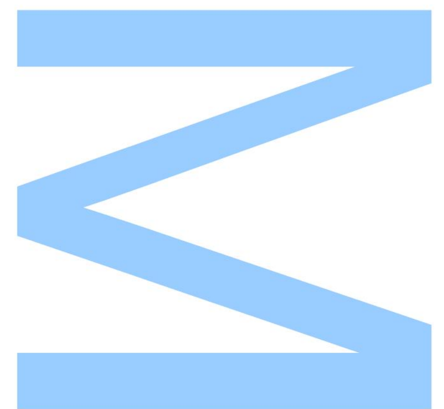
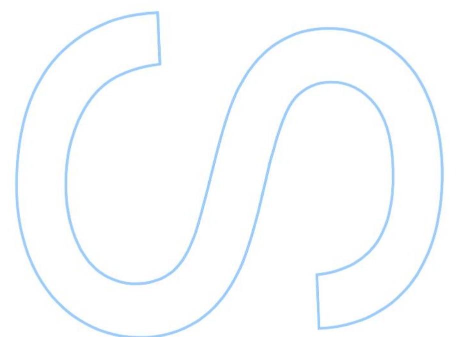
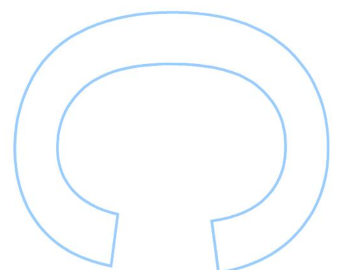
Trophic ecology of two aquatic invaders, the red swamp (*Procambarus clarkii*) and the signal crayfish (*Pacifastacus leniusculus*), in North-eastern Portugal







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O Presidente do Júri,

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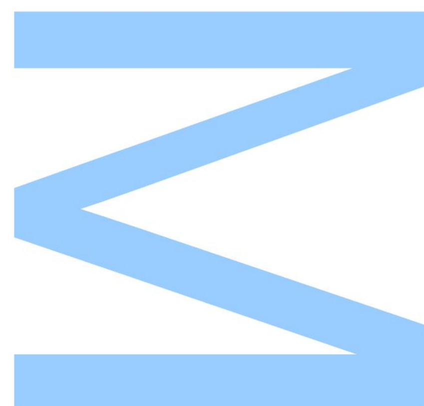

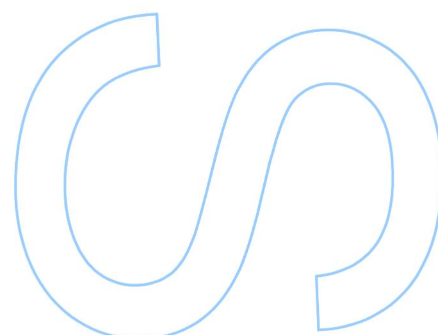

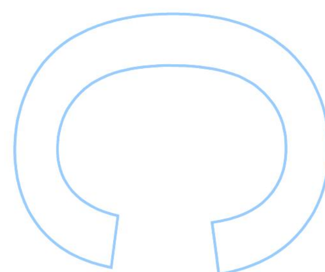

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Abstract

The invasion of stream ecosystems by non-native species is a cause of concern worldwide due to its negative environmental and economic impacts. Crayfish are among the most problematic invaders, with the red swamp crayfish *Procambarus clarkii* and the signal crayfish *Pacifastacus leniusculus* being two of the most widely distributed species worldwide. Their predatory nature, along with the potential trophic overlap with native, threatened fauna, calls for immediate risk assessments. Knowledge of the feeding ecology of these two species is, thus, essential for their control and management, yet, presently it is still limited.

Both the red swamp and the signal crayfish are thought to be generalist consumers, feeding on a broad range of small aquatic vertebrates, invertebrates and plant material. As such, traditional morphological analysis of gut contents is unlikely to reveal the full composition of their diet. However, DNA-based diet analysis has the potential to provide a complementary means to study the trophic ecology of such omnivorous, opportunistic feeders.

Through electrofishing, samples of the two crayfish species were collected from the Sabor watershed, both in sympatry and allopatry. In the laboratory, individuals were divided into three size classes and had their gut contents extracted. A pilot study was performed to optimize DNA extraction, and amplification, while experimental steps were taken to reduce external sources of contamination. A comparison of DNA extraction kits resulted in the selection of *Quick-DNA Fecal/Soil Microbe Miniprep* from ZYMO. In turn, COI (macroinvertebrates), trnL (green plants) and 18S (eukaryotes) proved to be the most reliable amplification markers, and were thus used on the final diet assessment. Additionally, crayfish blocking primers were designed and employed in an attempt to reduce the number of reads coming from predator DNA.

Frequency of occurrence was used to estimate diet composition and diversity. A total of 368 taxa were identified and divided into three main functional groups - Animals, Plants and Algae –, in an effort to closely analyze the trophic role of crayfish. Taxa such as insects (72.6%), Salicaceae (58.2%) and chlorophytes (85.6%) occurred prevalently in each of the functional groups respectively.

Statistical comparisons between crayfish species and size classes were performed using permutational multivariate analyses of variance (PERMANOVA), generalized linear mixed models (GLM) and analyses of similarities (ANOSIM). Diet composition of the two crayfish species and the three size classes exhibited few differences in the context of the functional groups, but certain specific taxa presented strong effects. Examples include the higher occurrence in the guts of *P. clarkii* of taxa such as Diptera (278%) or the annelid

Eiseniella tetraedra (266%), in comparison to *P. leniusculus*. Animal prey contribution to differences between species was assessed with a similarity percentage analysis (SIMPER), showing insects as the main contributors.

By themselves, the procedures employed present some drawbacks, such as the lacking cannibalistic component or the inability to separate the consumption of living prey and detritus. Nonetheless, the results help portraying the vast taxonomic range on which the two invaders feed, finding its best application as an assessment complementary to the traditional techniques.

Finally, the present findings suggest that the two crayfish species should be treated similarly by conservation management strategies. Particular attention must be given to uninvaded ecosystems with delicate balances in the trophic network, as the invasion by *P. clarkii* or *P. leniusculus* will certainly prove disruptive.

Keywords

DNA metabarcoding, diet assessment, freshwater invasive species, *Procambarus clarkii, Pacifastacus leniusculus*, gut contents, COI, 18S, trnL

Resumo

A invasão de ecossistemas dulçaquícolas por espécies exóticas é uma causa de preocupação global, devido aos seus negativos impactos económicos e ambientais. Os lagostins estão entre os invasores mais problemáticos, sendo o Lagostim-vermelho-do-Louisiana *Procambarus clarkii* e o Lagostim-sinal *Pacifastacus leniusculus* as duas espécies mais amplamente distribuídas pelo globo. A sua natureza predatória, juntamente com a potencial sobreposição de nicho trófico em relação a espécies nativas e ameaçadas, exige análises de risco imediatas. O conhecimento acerca da ecologia trófica destas duas espécies é, portanto, essencial para a sua gestão e controlo, porém, este permanece escasso.

Tanto o Lagostim-vermelho-do-Louisiana como o Lagostim-sinal são considerados consumidores generalistas, alimentando-se de um vasto leque de pequenos vertebrados aquáticos, invertebrados e material vegetal. Desta forma, é pouco provável que a tradicional análise morfológica de conteúdos estomacais revele a composição completa das suas dietas. No entanto, a análise da dieta através de técnicas de DNA e *metabarcoding* pode providenciar um método complementar no estudo da ecologia trófica de espécies omnívoras e oportunistas como estas.

Através de pesca-elétrica, recolheram-se amostras das duas espécies de lagostim na bacia hidrográfica do Sabor, tanto em simpatria como em alopatria. Em laboratório, os indivíduos foram divididos por três categorias de tamanho, seguindo-se a extração dos seus conteúdos estomacais. Um estudo piloto foi desenvolvido de maneira a otimizar os processos de extração e amplificação de DNA, ao passo que medidas experimentais foram incluídas de modo a reduzir fontes de contaminação externas. Uma comparação entre kits de extração de DNA resultou na seleção de *Quick-DNA Fecal/Soil Microbe Miniprep*® da ZYMO®. Por sua vez, os fragmentos COI (insetos), trnL (plantas verdes) e 18S (eucariotas) concederam as amplificações de DNA com mais sucesso, sendo utilizados na análise final das dietas. Adicionalmente, *primers* bloqueadores de DNA de lagostim foram desenhados e aplicados visando reduzir o número de leituras provenientes dos próprios predadores.

Utilizou-se a frequência de ocorrência para estimar a composição e diversidade da dieta. Identificaram-se 368 taxa que seguidamente se dividiram por três principais grupos funcionais – Animais, Plantas e Algas -, numa tentative de analisar de perto o papel trófico desempenhado pelos lagostins. Grupos taxonómicos como os insetos (72.6%), as Salicaceae (58.2%) e os clorófitos (85.6%) ocorreram de forma prevalente em cada um dos respetivos grupos funcionais.

Realizaram-se comparações estatísticas entre espécies e categorias de tamanho de lagostim, usando análises de variância permutacionais multivariadas (PERMANOVA), modelos mistos lineares generalizados (GLM) e análises de similaridades (ANOSIM). A composição da dieta das duas espécies e três categorias de tamanho de lagostim mostrou poucas diferenças quando enquadrada nos grupos funcionais, porém certos taxa exibiram efeitos significativos. Exemplos incluem a ocorrência mais elevada nos estômagos de *P. clarkii* por parte de taxa como Diptera (278%) ou o anelídeo *Eiseniella tetraedra* (266%), em comparação com *P. leniusculus*. A contribuição de presas animais para a diferenciação entre espécies foi aferida através de uma análise de percentagem de semelhança (SIMPER), revelando os insetos como principais contribuidores.

Por si só, os procedimentos aqui aplicados apresentam certas limitações, como a ausência da componente de canibalismo ou a incapacidade de separar o consumo entre presas vivas ou detritos. Apesar disto, os resultados ajudam a retratar o largo espectro taxonómico do qual os dois invasores se alimentam, encontrando a sua melhor aplicação como análise complementar às técnicas tradicionais.

Por fim, as presentes observações sugerem que as duas espécies de lagostim devem ser vistas de modo semelhante por estratégias de conservação e gestão natural. Particular atenção é deve ser prestada a ecossistemas não invadidos e com equilíbrios delicados na rede trófica, uma vez que a invasão por *P. clarkii* ou *P. leniusculus* certamente se irá provar perturbadora.

Palavras-chave

DNA metabarcoding, análise de dieta, espécies invasoras dulçaquícolas, *Procambarus clarkii, Pacifastacus leniusculus*, conteúdo estomacal, COI, 18S, trnL

Table of Contents

ACKNOWLEDGEMENTS .... **V**

ABSTRACT .... **VI**

KEYWORDS .... **VII**

RESUMO .... **VIII**

PALAVRAS-CHAVE .... **IX**

TABLE INDEX .... **XII**

FIGURES INDEX .... **XIV**

LIST OF ABBREVIATIONS .... **XVI**

CHAPTER 1: GENERAL INTRODUCTION **1**

1.1. Invasion crisis 2
1.2. Invasive crayfish 3
1.2.1. Invasive crayfish in Portugal 4
1.2.2. Trophic ecology of *Procambarus clarkii* and *Pacifastacus leniusculus* 5
1.3. Diet research 8
1.3.1. Traditional methods 9
1.4. DNA metabarcoding for diet assessment 10
1.4.1. Selecting a barcode 10
1.4.2. Experimental design 11
1.4.3. Data validation 12
1.4.4. The issue of content quantification 13
1.5. Aims and objectives 14

CHAPTER 2: PILOT STUDY **16**

2.1. Introduction 17
2.2. Material & Methods 18
2.2.1. Sample decontamination, dissection and homogenization 18
2.2.2. DNA extraction kits and amplification markers 18
2.2.3. Library preparation 20
2.2.4. Bioinformatic processing 21

2.3. Results & Discussion ... 21
2.3.1. DNA extraction kits ... 21
2.3.2. Marker comparison and selection ... 22
2.3.3. Blocking primers ... 23
CHAPTER 3: DIET ASSESSMENT ... 25
3.1. Introduction ... 26
3.2. Material & Methods ... 27
3.2.1. Study area and sampling technique ... 27
3.2.2. Sample processing ... 28
3.2.3. Bioinformatic processing ... 28
3.2.4. Data analysis ... 30
3.3. Results ... 31
3.3.1. Sampling results ... 31
3.3.2. Diet assessment results ... 32
3.4. Discussion ... 42
3.4.1. Diet composition ... 42
3.4.2. Species comparison ... 44
3.4.3. Ontogenetic shift ... 46
3.4.4. Sex comparison ... 47
3.4.5. Sampling effort ... 47
3.4.6. Shortcomings of the approach ... 48
3.4.7. Final remarks ... 49
CHAPTER 4: REFERENCES ... 50
CHAPTER 5: SUPPLEMENTARY MATERIAL ... 61
5.1. Supplemetary Tables ... 62
5.2. Supplementary Figures ... 68

Table index

Main tables

Table 1. Sets of primers used in the Pilot Study *pg. 20*

Table 2. The two COI blocking primers used in this experiment. *pg. 23*

Table 3. Read count after each bioinformatics processing step after sequencing. Step 1: demultiplexed paired reads; Step 2: paired-end alignment; Step 3: filtering by size according to expected amplicon lengths; Step 4: dereplication and removal of singletons; Step 5: BLAST; Step 6: removal of sequences with low query cover and evalue; Step 7: removal of low resolution hits. *pg. 29*

Table 4. Distribution of CL (cephalothorax length) for the final set of 208 crayfish samples. (n refers to the number of samples, while Min., Max, Avg. and S. E. indicate the minimum, maximum, average and standard error for the measurements in each category. *pg. 31*

Table 5. Number and percentage of taxa detected per marker and taxonomic level. *pg. 32*

Table 6. To the left, number of detected taxa (n) per functional group, crayfish species, size class and sex. On the right, number of samples (N) containing taxa per functional group, crayfish species, size class and sex. Left and right proportions (%) were respectively calculated in relation to the total number of taxa for each row or total samples per category. *pg. 33*

Table 7. Top animal families, plant families and algae classes detected. Preceding taxonomic levels were included for context. Each term consists in the class, order and family for Animals and Plants, or phylum and class for Algae. Number of occurring samples (N) and frequency (%) on the two columns to the left *pg. 36*

Table 8. Taxa experiencing significant effects from the factors and interactions tested using GLM. *pg. 40*

Table 9. Taxa experiencing significant species-related effects according to the individual GLM tests. *pg. 41*

Table 10. Taxa experiencing significant size-related effects according to the individual GLM tests. *pg. 41*

Table 11. Taxa experiencing significant sex-related effects according to the individual GLM tests. *pg. 41*

Table 12. Cumulative contributions of the most influential animal taxa to the differentiation between the two crayfish species. *pg. 41*

Supplementary tables

Table S1. Summary of agarose gel results after PCR, for the marker comparison and selection, in the context of the Pilot Study. *pg. 62*

Table S2. Taxonomic resolution displayed by the primer sets tested during the Pilot Study. *pg. 63*

Table S3. List of sites visited for the collection of crayfish samples. *pg. 63*

Table S4. List of all taxa detected in gut samples. On the left, number of samples in which the taxa occurred (N) and frequency of occurrence (%). *pg. 64*

Table S5. PERMANOVA table, assessing the effects of the interaction between species and size class, within the Plants functional group. *pg. 67*

Figures index

Main figures

Figure 1. The red swamp (Procambarus clarkii) and the signal crayfish (Pacifastacus leniusculus), to the left and right, respectively. *pg. 4*

Figure 2. Simplified representation of the alterations in energy flow through the trophic chain brought upon by the introduction *Procambarus clarkii* and *Pacifastacus leniusculus*. Green and grey arrows indicate energy flow prior to invasion, while orange and red display the post-invasion pattern. Arrow thickness is used to represent resource importance. Adapted from Geiger et al. (2005). *pg. 8*

Figure 3. Steps comprised in crayfish dissection. a) Cutting from telson to rostrum; b) Open crayfish, stomach has already been removed (empty space in the right), intestine remaining; c) isolation of the intestine; d) final sample in an Eppendorf tube. *pg. 19*

Figure 4. Comparison between EZN and ZYM PCR results, here using the SSU3' F/R primer set (18S). Triangles indicate EZN extractions while circles represent ZYM extractions. NC stands for negative control and refers to the last three rows on the left. *pg. 21*

Figure 5. A: *P. clarkii* blocking test. B: *P. leniusculus* blocking test Blocking primer concentrations are displayed on top, relative to the COI primer pair (CF = Crayfish DNA, COW = Cow DNA, NC= Negative Control). *pg. 24*

Figure 6. Map of the Sabor river basin, North-east Portugal. Sampling sites are displayed and coloured according to the detected presence/absence of the two crayfish species. *pg. 27*

Figure 7. Measurement of the cephalothorax length. *pg. 28*

Figure 8. Venn diagram displaying the number of crayfish samples (n=208) containing taxa from each of the three functional groups. *pg. 34*

Figure 9. Euler diagram displaying the number of taxa from the three functional groups detected within samples of both crayfish species. *pg. 34*

Figure 10. Euler diagram displaying the number of taxa from the three functional groups detected within crayfish samples collected in sympatry and allopatry. *pg. 34*

Figure 11. Representation of the taxonomic diversity detected within functional groups. Animals and Plants are divided by Class, Order and Family, with the exception of Zooplankton, which are illustrated through Phylum and Class. Likewise, Algae are also separated by Phylum and Class, to provide recognisability. A – Animals; B – Plants; C – Algae. *pg. 35*

Figure 12. Principal coordinate analysis (PCoA) of animal taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C) *pg. 37*

Figure 13. Principal coordinate analysis (PCoA) of plant taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C) *pg. 38*

Figure 14. Principal coordinate analysis (PCoA) of algae taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C) *pg. 38*

Figure 15. Boxplot representation of the results from the analysis of similarities investigating the effect of crayfish species and performed using the animal matrix after the removal of zooplanktonic taxa. *pg. 39*

Supplementary figures

Figure S1. Sequencing results from amplification of crayfish gut contents using the primer set Leray-XT (COI), during the Pilot Study. *pg. 66*

Figure S2. Sequencing results from amplification of crayfish gut contents using the primer set SSU3’ F/R (18S), during the Pilot Study. *pg. 67*

Figure S3. Sequencing results from amplification of crayfish gut contents using the primer set 12SV5.1 (12S), during the Pilot Study. *pg. 68*

List of Abbreviations

ANOSIM	Analysis of similarities
Bp	base pairs
BLAST	Basic Local Alignment Search Tool
BoLD	Barcode of Life Database
CL	Cephalothorax length
COI	Cytochrome c oxidase subunit I
DNA	Deoxyribonucleic Acid
eDNA	Environmental DNA
ESV	Exact Sequence Variants
GLM	Generalized Linear Mixed Model
IAS	Invasive Alien Species
LCA	Lowest common ancestor
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
nt	NCBI nucleotide database
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
qPCR	Quantitative Polymerase Chain Reaction
SIMPER	Similarity percentage analysis

Chapter 1: General Introduction

1.1. Invasion crisis

Although current anthropogenic impacts on the environment are numerous, the introduction of invasive alien species is one of the most irreversible and uncontrolled causes of ecosystem deterioration (Vitousek et al., 1997; Wilcove et al., 1998). According to international convention (CBD, 2008), the term “invasive alien species” (IAS) refers to species whose introduction or spread beyond their natural range has been found to adversely impact biodiversity and related ecosystem services. Thus, of all species translocated by humans, broadly referred to in the literature as “non-native”, “exotic”, “non-indigenous” or “introduced”, among other terms (Colautti & MacIsaac, 2004), IAS comprise those that have proved harmful to native diversity. While biological invasions have been common through time (Gillson et al., 2008), translocations are currently occurring at a rate that is alarmingly high (Ricciardi, 2007).

Besides the direct harm inflicted on native biodiversity, economic activities can also suffer dramatically from the alterations caused by invasive species (Bradshaw et al., 2016; Walsh et al., 2016). In general, economic losses tend to derive from damages to ecosystem services (Pejchar & Mooney, 2009). Examples include the disturbance of native food resources (e.g. Lupi et al., 2003), the drainage of water reserves (e.g. Gerlach, 2004), the disruption of pollination (e.g. Vanbergen et al., 2018) or the devaluation of touristic landscapes (e.g. Gutrich et al., 2007). Still, invaders might represent important sources of income (Copp et al., 2005), leading to conflict between workers and conservationists.

Among the most threatened ecosystems worldwide (Dudgeon et al., 2006; Vörösmarty et al., 2010), freshwaters have long been recognized as particularly vulnerable targets to biological invasion (Sala et al., 2000). Economic activities are thought to be the main contributor for this condition. Significant actions facilitating biological invasions include the intentional stocking of non-native species for fisheries and aquaculture, along with accidental or naive releases from ballast waters and bait buckets (Havel et al., 2015).

Consequently, freshwater invaders have become numerous (Strayer, 2010), creating an unbalance on trophic networks. The aptitude of these organisms to alter habitat composition (e.g. Pulzatto et al., 2018) and nutrient levels (e.g. Carlsson et al., 2004), may ultimately lead to eutrophication (Caraco et al., 2006). Such disturbances are expected to favor new invasions (Hobbs & Huenneke, 1992), an effect that might be aggravated by other anthropogenic issues like climate change (Rahel & Olden, 2008) or overexploitation (Strayer, 2010). Facing these concerns, research-supported management measures (Bajer et al., 2019) are vital to protect freshwater systems from progressing into further stages of invasion and deterioration.

1.2. Invasive crayfish

Freshwater crayfish (Crustacea, Decapoda) have been commonly recognized as key members of the systems in which they occur (Lodge et al., 2012). Multiple trophic levels are affected by their omnivorous feeding habits and high population densities, allowing crayfish to canalize the flow of biomass and control the biodiversity of communities (Geiger et al., 2005).

Around 20 species of crayfish have presently spread beyond their natural range (Hobbs et al., 1989; Lodge et al., 2012), as a result of stocking actions and improper handling. Ordinarily used as fishing bait, live crayfish are frequently translocated and discarded by fisherman (DiStefano et al., 2009), resulting in range expansions and colonization. Facing the decline of native crayfish populations, new stocks of this food delicacy were established in Europe through the, often illegal, translocation of alien species (Reynolds, 2011). With a considerable propensity for self-dispersal, crayfish are not only able to travel against water flow, but also to move overland around dams and waterfalls (Ramalho & Anastácio, 2014; Thomas et al., 2019), or attaching to water birds (Anastácio et al., 2014). Considering their high population density (Guan & Wiles, 1996) and ability to survive desiccation by burrowing into the ground (Correia & Ferreira, 1995), invasive crayfish display a resilience that makes them difficult pests to manage.

Once introduced to non-native systems, alien crayfish species can lead to significant ecological disturbances. Effects such as the rise in zooplankton and depletion of oxygen, increased algal production due to bioturbation, shifted behavior in fish or loss of macrophytes cover (Stenroth & Nyström, 2003; Dorn & Wojdak, 2004; Light, 2005) have all been associated with the presence of crayfish invaders. Thus, ecological impacts by alien crayfish include not only direct predation, but also habitat alteration. The disturbance of refuge structures such as macrophytes or the sediment bed has been linked to the decline of native species of amphibians, fish and macroinvertebrates (Usio & Townsend, 2004; Rodríguez et al., 2005; Cruz et al., 2008; Peay et al., 2009).

The introduction of alien crayfish species is linked to the spread of diseases and symbionts (Hunt et al., 2018; Oficialdegui et al., 2019), with the oomycete *Aphanomyces astaci* (Schikora, 1906) being a well-documented case (Svoboda et al., 2017). Responsible for the infection known as crayfish plague (aphanomycosis), this North-American fungus has successfully spread worldwide (Alderman, 1996). First reported in Europe in the 19th century (Edgerton et al., 2004), it is described as the main cause of decline for the native crayfish populations, to whom the infection is lethal (Strayer, 2010). Besides being vectors of *A. astaci*, North-American crayfish are resistant to its effects, which favors invasion of infected sites.

1.2.1. Invasive crayfish in Portugal

Two invasive species of crayfish are present in Portugal, the red swamp crayfish *Procambarus clarkii* (Girard, 1852) and the signal crayfish *Pacifastacus leniusculus* (Dana, 1852). Native to North America, these have become two of the most widely distributed invasive crustaceans (Vaeßen & Hollert, 2015; Souty-Grosset et al., 2016). Acting as vectors of the crayfish plague, their arrival is thought to have contributed to the local extinction of the native white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) (Bernardo et al., 1997).

Figure 1 – The red swamp (*Procambarus clarkii*) and the signal crayfish (*Pacifastacus leniusculus*), to the left and right, respectively.

Originally from the southcentral United States and northeastern Mexico, *Procambarus clarkii* was first recorded in Portugal in 1979 (Ramos & Pereira, 1981), after spreading from aquaculture stocks established in Badajoz, Spain, around 1973 (cited in Gutiérrez-Yurrita et al., 1998). Although today it covers the whole territory, *P. clarkii* tends to occur in wide, low-elevation rivers, where flow is slower and aquatic vegetation is abundant (Cruz & Rebelo, 2007; Filipe et al., 2017). While the lower water temperatures of elevated streams may make it less competitive, (Bernardo et al., 2011) the red swamp crayfish is still able to succeed in cooler climates, as seen throughout Europe (e.g. Chucholl, 2013). As a pest in Portuguese rice fields, the red swamp crayfish is estimated to represent losses of one million euros per year (Anastácio et al., 2019). Decreased production results not only from destruction of rice seedlings (Anastácio et al., 2005a), but also from burrowing or attracting predators that may trample the crops (Correia, 2001). Several ecological impacts have been linked to the presence of *P. clarkii* in Portugal, with negative effects reported on algae (Barradas et al., 2006), invertebrates (Banha & Anastácio, 2011), amphibians (Cruz et al., 2006b), water quality (Anastácio et al., 2005b), while also disrupting decomposition rates (Carvalho et al., 2016). Nonetheless, *Procambarus clarkii* has become a reliable resource to native fauna (Correia, 2001; Beja, 2006), as well as for diverse commercial purposes (Souty-Grosset et al., 2016).

Native to the northwest of North America, *Pacifastacus leniusculus* appears to be better adapted to upstream sections at higher elevations, with riffle habitats and plentiful riparian vegetation (Anastácio et al., 2015; Filipe et al., 2017). First introduced in Spain in the mid 1970s for aquaculture (cited in Alonso et al., 2000), the signal crayfish later expanded its range through successive translocations by Spanish authorities, intended to increase fishery stocks (Vedia & Miranda, 2013). *P. leniusculus* was first recorded in Portugal in 1997, in the Sabor watershed, remaining in the northeast region as of this date (Bernardo et al., 2011; Filipe et al., 2017). Few studies on the impacts of signal crayfish have been performed in Portugal, but the reported predation on native mussel species is worrying (Sousa et al., 2019). Nonetheless, general crayfish impacts are expected, as in other invaded locations (Vaeßen & Hollert, 2015).

Currently, the two crayfish invaders coexist in North-eastern Portugal, in a few sections of the Tua and Sabor watersheds (Bernardo et al., 2011; Filipe et al., 2017), where their ranges are expanding. Although it might be unviable for species with such similar resource requirements (Vedia & Miranda, 2013) to coexist, it is hypothesized that the situation will persist in areas where both sides are facing environmental stress (e.g. temperature) (Bernardo et al., 2011). Thus, an obstacle for further range overlap might reside in the fact that optimal conditions are not met for either species. An alternative hypothesis might give more weight to interspecific competition, with each species being more efficient using different resources, specific to the areas where they dominate. Besides helping to answer these questions, assessments to eventual trophic niche overlaps can also provide information on the threats the two predators pose to native fauna. Overall further research is needed to evaluate the joint impact of these invaders and develop informed management measures.

1.2.2. Trophic ecology of *Procambarus clarkii* and *Pacifastacus leniusculus*

In the past, *P. clarkii* and *P. leniusculus* were thought to be almost exclusively herbivores and detritivores, since analyses of gut contents were dominated by plant fragments and detritus (cited in Geiger et al., 2005). Indeed, most assessments agree that macrophytes are the main item in the diet of the two crayfish species (e.g. Guan & Wiles, 1998; Alcorlo et al., 2004). However, the method is biased towards hard, undigested material, as is usually the case with plant-derived content. Weaker in structure, animal tissue is rapidly digested and can end up underrepresented, particularly in the case of soft-bodied organisms, such as molluscs and oligochaetes (e.g. Alcorlo et al., 2004; Chucholl, 2013). Thus, when corrected for assimilation efficiency, crayfish diets seem to reveal a larger dependence on animal sources than otherwise described (e.g. Whitledge & Rabeni,

1997). Today, *Procambarus clarkii* and *Pacifastacus leniusculus* are considered adaptable, opportunistic, omnivores. Besides macrophytes and detritus, the two crayfish species have been found to feed on algae, plankton, bacteria, fungi, crayfish, insects and larvae, bivalves, snails, and vertebrates such as fish and amphibians, as well as their eggs (Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998; Correia, 2002; Alcorlo et al., 2004; Dorn & Wojdak, 2004; Pérez-Bote, 2004; Rebelo & Cruz, 2005; Peay et al., 2009; Grey & Jackson, 2012; Chucholl, 2013; Meira et al., 2019). Diet diversity is, thus, significant, with a large variety of consumed taxa confirmed for groups like insects (e.g. Chironomidae, Ephemeroptera, Odonata, Plecoptera, Tricoptera, among others; see Guan & Wiles, 1998; Pérez-Bote, 2004) or amphibians (see Rebelo & Cruz, 2005).

As opportunistic feeders, local habitat traits have a marked influence on how crayfish diet varies in composition (e.g. Rudnick & Resh, 2005). Although prey selection leans towards the most energy-rich elements, high crayfish densities can quickly exhaust the preferred resources (Alcorlo et al., 2004). Thus, abundant food items tend to be the most consumed, as the higher availability compensates for their lower quality. This mechanism is thought to be the reason for the dominance of plant material found in crayfish diet assessments (cited in Chucholl, 2013). In rice fields, for instance, where diversity of animal prey is lower than in natural marshes, the importance of macrophytes and cannibalism rises (Alcorlo et al., 2004). In turn, lower-energy plant detritus may be consumed in greater quantities, for instance, when interspecific competition for insects prey increases (Jackson et al., 2012). Still, the nutritional contribution of detritus cannot be undervalued, as the accumulation of living bacteria and fungi is thought to provide an important source of protein (cited in Alcorlo et al., 2004). Additionally, when seasonality causes variation in the availability of resources, crayfish are able to adapt their diet composition. Correia (2002) reported that consumption of aquatic macroinvertebrates accompanied the oscillations of that prey's abundance throughout the year. In the same note, while herbivory can be high in winter (Guan & Wiles, 1998), it may decrease during spring (Alcorlo et al., 2004), when insect prey are more diverse and available. Events such as the reduction of water levels are also related to diet shifts, as observed by Grey & Jackson (2012), with red swamp crayfish reportedly moving overland to feed on terrestrial plants.

Since crayfish continuously grow in size as they age, the existence of an ontogenetic shift in their diet has for long been discussed (Guan & Wiles, 1996; Gutiérrez-Yurrita et al., 1998; Correia, 2002; Alcorlo et al., 2004; Stenroth et al., 2006; Usio et al., 2009; Alcorlo & Baltanás, 2013; Chucholl, 2013). The general consensus is that the diet of younger and smaller crayfish relies more on prey with higher mobility. Those comprise animal prey such as the larvae of insects, fish and amphibians, as well as their adult counterparts. Proposed explanations include the hypothesis that younger crayfish require a higher energy input to

meet their metabolic needs, or simply that they are more agile, and thus better suited to hunt those prey (Correia, 2002). Some diet analyses have detected, indeed, a wider range of animal prey in the guts of smaller crayfish (e.g. Correia, 2002; Chucholl, 2013). Other authors suggest the type of animal prey may also vary with crayfish size, with smaller crayfish relying on zooplankton and large adults feeding on small fish (Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998; Pérez-Bote, 2004). Still, some isotopic assessments did not detect a significant ontogenetic difference in crayfish diet (Stenroth et al., 2006; Alcorlo & Baltanás, 2013), implying adult crayfish may be as carnivorous as the young.

One challenge faced by past diet assessments of *P. clarkii* and *P. leniusculus* is the difficulty to categorize the smallest consumed items, a task often performed with low taxonomic resolution. The resulting lack of representation of groups such as plankton, bacteria and fungi might lead to undervalued disruptions of invaded ecosystems. Additionally, morphological interpretation of digested fragments is a laborious and time-consuming task, requiring researchers to have extensive knowledge of local biodiversity. The bait used in the traps needed to capture crayfish might also interfere with the analysis of ingested content. Crayfish collection by electrofishing is a valid alternative, although researchers must consider that the sampling effort is difficult to quantify (Alonso, 2001; Bernardo et al., 2011).

From this knowledge, it is clear that *P. clarkii* and *P. leniusculus* interact with multiple trophic levels, having the potential to disrupt entire food webs (Figure 2). By feeding on freshwater macrophytes, crayfish alter and eliminate microhabitats important for macroinvertebrates and fish (Alcorlo et al., 2004). The decrease in such species is further aggravated by direct predation, which combined with the nutrient release from litter breakdown leads to an increase of phytoplankton biomass and water turbidity (Guan & Wiles, 1998). The efficient consumption of detritus by crayfish is also responsible for alterations in the patterns of energy flow through the trophic chain (Rudnick & Resh, 2005). Small detritivore fauna like insects and oligochaetes tend to slowly use that resource, establishing trophic links to other low-level consumers (Geiger et al., 2005). Crayfish invaders, however, link the detritus energy pool directly to top-predators like mammals and birds (Correia, 2001), eliminating various steps in the food web. The disappearance of native freshwater fauna is, thus, a distinctive consequence of crayfish invasions, exacerbated by predation or competition with vulnerable species.

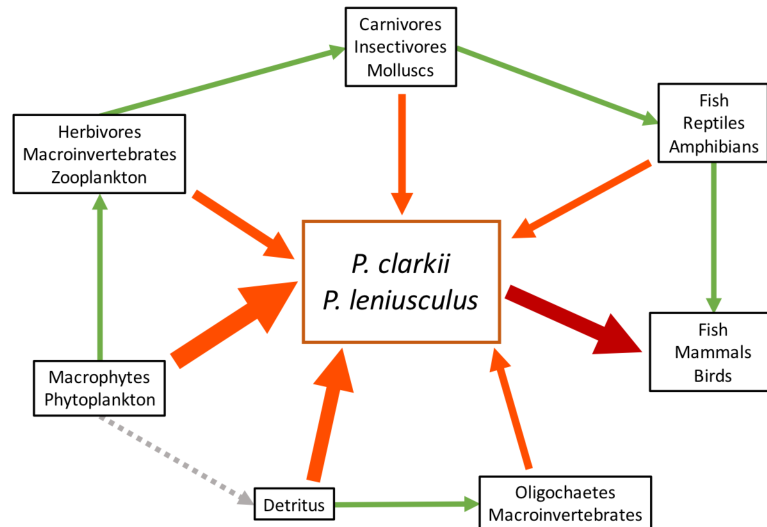

Figure 2 – Simplified representation of the alterations in energy flow through the trophic chain brought upon by the introduction *Procambarus clarkii* and *Pacifastacus leniusculus*. Green and grey arrows indicate energy flow prior to invasion, while orange and red display the post-invasion pattern. Arrow thickness is used to represent resource importance. Adapted from Geiger et al. (2005).

1.3. Diet research

The nature of trophic webs has always been a fundamental topic to ecologists. In these interactions lies the flow of energy and nutrients which structure ecological communities (McCann, 2007). Although complex and intertwined, unveiling trophic links is essential for the comprehension of biogeographic patterns (Betzholtz et al., 2012), evolutionary pressures (Grant & Grant, 2006), behaviour (Januchowski-Hartley et al., 2011) or the particular vulnerabilities (Cook & Blumstein, 2013) of organisms. Moreover, the underestimation of the structuring resulting from bottom-up and top-down regulation has already proved damaging (Estes et al., 2011), while living pictures of ecosystem health are painted by these mechanisms.

Hence, diet assessments supply valuable information for the management of natural systems. In a time when biodiversity management is an urgent necessity worldwide (Reid et al., 2019), dietary research is relevant in a broad range of areas. Progress has been achieved in agroecology, where wild, native, predators are being shown as effective means of pest-control (Aizpurua et al., 2018). In turn, studies on invasive species as consumers are supporting impact evaluations with data on predation (Meira et al., 2019), trophic competition (Brown et al., 2014) and habitat alteration (Pulzatto et al., 2018). Finally, endangered species are benefiting from more detailed vulnerability assessments, thanks to the information collected on their trophic ecology (Gebremedhin et al., 2016; Pinho et al., 2018).

Predator-prey links may be one of the most basic elements in ecological communities, but only by understanding these basal bricks can we build upon with further knowledge and act swiftly on biodiversity's needs.

1.3.1. Traditional methods

Early diet descriptions relied on visual analyses of either direct feeding behaviour or digestion remains (e.g., Sussman & Tattersall, 1976; Putman, 1984). While behavioral observations provide the most faithful evidence of trophic links, the approach is unviable for generalist, elusive or aquatic organisms. In such cases, morphological examination of digestion remains like scats, regurgitated pellets or gut contents can aid in the description of diets (Hyslop, 1980). The information contained in these remnants is usually recorded as the presence/absence of particular prey items, which can be used in quantification measures. Relative abundance by frequency, biomass, or visual estimation of percentage are some of the typical calculations used for diet quantification after content examination (Nielsen et al., 2018). Still, significant biases are present and must be noted, such as the tendency for softer material to go undetected, since a lower resistance to digestion will lead to fewer diagnosable fragments (Symondson, 2002). As a consequence, unidentified fragments can cause a sub-representation of important food resources. Additionally, the judgement of an experienced researcher might be needed for a thorough taxonomic identification of contents.

As technology advanced, sophisticated techniques became accessible and cost-effective, ranging from biomarker analyses like fatty acid (Napolitano, 1999), proteic (e.g. Walrant & Loreau, 1995) or immunological (Boreham & Ohiagu, 1978) profiling, to readings of isotopic signatures (e.g. Alcorlo & Baltanás, 2013). Before the rise of DNA-based methods, stable isotope ratios were the main indicator used in trophic ecology. Having been observed that the isotopic ratio of a consumer reflected that of its consumed item (Smith & Epstein, 1970; Minson et al., 1975), trophic links can be inferred. In fact, stable isotope analyses have the ability of portraying diet information over a long temporal scale, depending on the type of tissue examined (Vander Zanden et al., 2015). However, its discrimination power is limited to only a few food items (Phillips et al., 2014), being more suited for interpretations about the trophic position of consumers. Other difficulties include the fact that not all ingested contents are equally digested and assimilated (Mitra & Flynn, 2007), leading to biased interpretations.

DNA techniques are currently seen as the most successful molecular techniques for assessing the diversity of consumed items (Pompanon et al., 2012). Pioneering practices such as DNA profiling of stomach contents through temperature gradient gel electrophoresis first allowed researchers to glimpse into diet diversity at the molecular level

(Harper et al., 2006). Taxonomic resolution was, however, limited by band superimposition or haplotypic variation (Lessa & Applebaum, 1993). Since then, amplification of specific prey DNA has provided more detailed results, while discrimination power was further increased by taxon-specific fluorescence-labelled primers (Harper et al., 2005). Yet, for comprehensive diet assessments, where extensive sampling is necessary to infer ecologically significant results, such techniques tend to get expensive and laborious.

More recently, the incentive to sequence whole genomes has led to the development of parallel DNA sequencing platforms – next-generation sequencing (NGS) -, able to produce millions of sequences at continually decreasing costs (Shendure & Ji, 2008). This allows for entirely new approaches, such as DNA metabarcoding, representing yet another turning point for diet research.

Although not without its particular challenges, molecular tools have brought important progress for trophic ecology studies. Non-invasive sampling has, for instance, gained a new esteem in the community (Taberlet & Luikart, 1999), while ongoing issues such as the impact of introduced species on native fauna are being further explored (Blanchet, 2012).

1.4. DNA metabarcoding for diet assessment

DNA barcodes are short portions of a genome, standardized as identification markers for certain taxa levels (Kress et al., 2015). First applied by Hebert et al. (2003), where a fragment of cytochrome c oxidase subunit I (COI) was used to distinguish 200 species of lepidopterans, DNA barcodes have since become a common tool for documenting diversity (Joly et al., 2014). Current tools allow for multiple species in complex mixtures like water, scats or gut contents to be identified simultaneously, which is referred to as DNA metabarcoding (Taberlet et al., 2012). Besides the high rates of throughput, dietary analyses benefit from the ability to separate the obtained DNA sequences by sample, through the use of indexed primer tags (Coissac, 2012). These represent fundamental tools for studies investigating the effect that factors such as sex, age, location or season can have on trophic interactions.

1.4.1. Selecting a barcode

Depending on the range of an organism's diet, DNA metabarcoding assessments may require multiple sets of barcodes to adequately cover the main groups of prey (Silva et al., 2019). Thus, it is essential to determine which barcodes will be necessary to answer the main research question. To make this decision, features such as taxonomic coverage and resolution must be taken into account (Pompanon et al., 2012). The taxonomic coverage

of a barcode can be defined as the range of taxa it amplifies, i.e. the number of different genomes to which the primer set can bind. Taxonomic resolution is the maximum level of taxonomic specificity that the barcode can achieve, or in other words, how particular to each taxa is the portion of amplified DNA. A high coverage is needed, for instance, if researchers are looking to describe the whole dietary spectrum of a generalist species, but it can be lower when searching for the presence of just a few particular groups. Resolution, on the other hand, should ideally be high, which is especially hard when there are many taxonomic groups to cover. The trade-off between coverage and resolution lies in finding very conserved primer-binding sites that surround a highly variable amplified region. One solution is to use different barcodes simultaneously, or even in a hierarchical manner (Deagle et al., 2009), by first amplifying a large taxonomic spectrum, and then adding group-specific primers, able to resolve taxa to species level.

Considering that diet-related DNA undergoes digestion, sequence fragments tend to be short and degraded (Deagle et al., 2006; Symondson, 2002). Therefore, barcode regions must also be short (100-250 bp) to increase detection rates, although often at a cost of taxonomic resolution. Additionally, different barcodes should be similarly sized if used simultaneously, to avoid Polymerase Chain Reaction (PCR) biases towards smaller and more numerous fragments (Pompanon et al., 2005). Another factor worthy of attention is the number of DNA molecules present per cell, particularly when comparing nuclear and mitochondrial markers. Given the fact that the latter occur in much larger quantities, their amplification rates will be consequently higher (Pompanon et al., 2012).

The availability of relevant barcodes in public databases is also an important aspect when choosing markers. Although libraries compiling a remarkable variety of barcodes keep on growing (Joly et al., 2014), the development of custom databases is an alternative approach when the taxa of interest are not barcoded (e.g. Rayé et al., 2011). While laborious to assemble, personal barcode libraries come with their own advantages, as discussed further below.

1.4.2. Experimental design

Although employed in diverse ways, investigation of trophic links using NGS has a few common particularities in regards to experimental design. As a starting point, a large number of samples is required in order to obtain a comprehensive list of preys consumed by a population (Pompanon et al., 2012). Then, depending on the source from where the dietary DNA is extracted, subsampling may need to be considered. That can be the case with mammals who produce heterogeneous excrements, to assure the entire diversity of digested content is detected (Deagle et al., 2005). Similar circumstances to account for are gut contents collected under different stages of digestion (and thus, DNA degradation),

or predators that produce many small pellets. These factors may lead to a biased amplification of DNA belonging only to a few of the ingested taxa. Pooling, followed by homogenisation and resampling before DNA extraction, is sometimes a valid method to maximize the variety of prey detected from a limited number of samples (Deagle et al., 2010). Nonetheless, it must be noted that the risk of degradation and contamination is increased, leading to potential loss of diversity (Mata et al., 2019). To counteract the effect of PCR drift and raise the number of detected entities, multiple PCRs per sample or subsample are recommended (Ficetola et al., 2015). PCR replication may also be important to reduce the effect of other types of bias, such as differential amplification by index tag (O'Donnell et al., 2016).

Another common inconvenience among diet assessments using DNA metabarcoding is the amplification of non-target organisms. Unwanted sequences may be abundant and derive from sample contamination, occur as DNA reads from the predator, or even from its parasites and symbionts (Srivathsan et al., 2015). Different tools can be used to prevent amplification of these organisms and improve the ecological significance of the results. Examples include digesting undesirable DNA through endonuclease activity (Green & Minz, 2005) or blocking its amplification with the use of PCR clamps (Vestheim et al., 2011). The latter requires the addition of a target-specific sequence, designed to bind to the same region as the primer set, inhibiting amplification. On the other hand, investigations focused on detecting rare species can also benefit from such tools by excluding DNA from more common prey.

1.4.3. Data validation

Once the dietary DNA has been successfully amplified and sequenced, researchers are left with a considerable amount of data that need to be analysed and validated. Checking for potential errors is crucial before interpreting the results, since a range of inaccuracies can be expected. For instance, erroneous readings of primer tags ("tag jumps", Schnell et al., 2015) can lead to incorrect assignments of sequences to sample classes. Although hard to detect, those can be minimized by using sufficiently distinct tags, able to maintain their identity if misreading occurs (Coissac et al., 2012). A different approach is to include DNA from an exotic taxon as positive control, allowing to measure tag jumping rates from the amount of improper detections and set a filtering threshold (De Barba et al., 2014).

Alterations inside the barcoding region, arising during PCR or sequencing, may lead to incorrect assignments of consumed taxa. If these changes happen during sequencing, low quality scores can allow for the detection of unreliable reads. On the other hand, errors tracing back from degraded or contaminated templates, or resulting from nucleotide

misincorporation during PCR, will not reflect low quality scores. In such cases, more conservative measures are recommended, such as the removal of sequences which do not reach a certain threshold of read count (Huse et al., 2007). Still, it must not be assumed that all errors are rare and occur on later stages of PCR. Hence, more careful approaches involve discarding sequences frequent only in one sample from a population, or including technical replicates when focusing on individual consumers (Pompanon et al., 2012). Nonetheless, an unfortunate consequence of discarding infrequent sequences is the potential loss of rare food items.

A third type of error, prone to happen when amplifying from degraded templates, is the formation of DNA chimeras during PCR (Acinas et al., 2005). Although these sequences contain DNA from different organisms, assignment to higher taxonomic ranks might still be possible if their composing units are not too taxonomically distant (e.g. Soininen et al., 2009).

After going through these necessary verification steps, taxa may finally begin to be assigned. This is done by comparing the obtained sequence data to reference libraries such as National Center for Biotechnology Information (NCBI) and Barcode of Life Database (BoLD), through algorithms like Basic Local Alignment Search Tool (BLAST). Given the amount of data generated, faster processing can be achieved using custom databases compiled with publicly available information and/or sequences produced for the research in question. Since they can be focused on local traits, personally tailored libraries have the benefit of giving more precise and significant results (Corse et al., 2010; Rayé et al., 2011). On the other hand, public data might be essential when a diet spectrum is too broad or unknown to compile. Although fidelity can be a problem when using public information (Harris, 2003), such data can help to identify primarily unassigned sequences, which may, perhaps, represent locally unexpected or even undocumented taxa. Once taxonomic diversity is listed for each sample, metrics such as frequency of occurrence can provide a summary of the diet across samples.

1.4.4. The issue of content quantification

Perhaps the biggest disadvantage of DNA metabarcoding as a tool for diet assessment, in comparison with its traditional counterparts, is the lack of real quantitative information obtained from a typical sequencing run (Deagle et al., 2013). An essential requirement for diet quantification to be viable would be the existence of proportionality between the consumed biomass of a food item and the number of reads it generates. In other words, quantitative signatures must remain unaltered during all steps, from sampling to data analysis (Pompanon et al., 2012). That represents a major constraint for this technique, as too many factors can influence the final amount of DNA obtained (as reviewed by Clare,

2014): organic aspects such as variance in i) cell density, ii) marker abundance in the cells, iii) tissue resistance to digestion, iv) stage of digestion; or simply technical biases like PCR drift and long-fragment dropout. Nonetheless, ingenious solutions have been experimented, mostly using animals under captivity (e.g. Deagle et al., 2010; Willerslev et al., 2014). Some success was achieved by Thomas et al. (2014, 2016), by sequencing materials of known composition along with faecal samples from captive feeding. This allowed to establish correction factors accounting for marker density, digestion bias, and interactions between prey items. The approach, however, is unpractical when there is little knowledge about the diet of an organism, or the range of food items is too broad. Diet quantification is further compromised when using multiple markers sharing target groups (as read count will become unfair) or when only a few taxa are being amplified (Deagle et al., 2019). Comparing the results obtained through metabarcoding with those of traditional methods is a possible solution, although results are not always concordant (Soininen et al., 2009; Jarman et al., 2013; Kartzinel et al., 2015). Overall, diet quantification by DNA metabarcoding is affected by factors that call for further exploration (Lamb et al., 2019).

Even with these constraints in mind, DNA metabarcoding has established itself as a valuable dietary diversity assessment tool. Its cost-efficient representation of trophic interactions is encouraging for integration in management programs. Examples of conservation-oriented uses of this technology include assessing the dietary needs of threatened species (Boyer et al., 2013; Biffi et al., 2017; Sullins et al., 2018), sampling of bioindicators (Siegenthaler et al., 2019) or evaluating the impacts of non-native predators (Brown et al., 2014; Moran et al., 2015; Harms-Tuohy et al., 2016).

1.5. Aims and objectives

The central aim of the present study was to examine the diet composition of two invasive species of crayfish, *Procambarus clarkii* and *Pacifastacus leniusculus*, through the use of DNA metabarcoding, in order to assess the primary threats posed by these invasive species to native Portuguese flora and fauna.

Within the central aim, there were two further objectives:

1. To assess whether there are dietary differences between the two crayfish species, in order to ascertain which, if any, species represents a greater threat to native biodiversity
2. To assess whether there are dietary differences between size classes of each species, in order to ascertain whether certain size classes may represent greater threats to native biodiversity, as this may influence the choice of management control measures

To achieve these goals, a pilot study was first performed in order to develop a cost-efficient laboratory protocol that would allow to obtain and inspect any dietary DNA present in the gut contents of crayfish.

The optimized procedures were then applied to a final set of samples collected from the Sabor basin (North-eastern Portugal), allowing for trophic analyses to be implemented. Such analyses are intended to aid in the assessment of the threats posed to native biodiversity through predation, trophic competition and habitat alteration.

Chapter 2: Pilot Study

2.1. Introduction

DNA metabarcoding is a rising tool in aquatic systems, emerging in diverse applications such as biodiversity monitoring through environmental DNA (eDNA) (e.g. Valentini et al., 2015; Bracken et al., 2019), detection of bioindicators (e.g. Siegenthaler et al., 2019), analysis of diet composition (e.g. Guillerault et al., 2017; Jakubavičiute et al., 2017), as well as aiding in the assessment of impacts by alien invaders (e.g. Darling & Mahon, 2011; Harms-Tuohy et al., 2016; Strand et al., 2019).

Few studies have assessed the diet of crustaceans using DNA metabarcoding (e.g. O'Rorke et al., 2012; Siegenthaler et al., 2019). Even though their impacts as omnivorous invaders are severe, freshwater crayfish have yet to be the targets of diet assessments through next-generation sequencing. This modern approach has the potential to solve problems whose resolution was limited by the technology of previous methods. Examples include assessing the diversity of fungi and bacteria comprised in the diets of crayfish, as well as identifying the actual taxa of food items traditionally tossed into broad categories. A high-resolution taxonomic assessment of the wide dietary spectrum of crayfish invaders can be of particular interest for the improvement of ecosystem conservation strategies. To achieve that, a cost-efficient metabarcoding protocol must first be specifically developed for crayfish-derived dietary content. Optimal DNA extraction, amplification and sequencing need to take place in order to obtain a solid reading of prey DNA ingested by crayfish.

As such, a small set of crayfish samples was used to determine the most appropriate technical procedures for the metabarcoding of crayfish diet. This pilot study begins with testing an eDNA decontamination step, applied prior to dissection. Homogenization of gut samples is then performed and followed by a comparison test between two DNA extraction kits, to ensure enough DNA is isolated for the following steps. As generalist feeders, a wide taxonomic range must be covered, which requires multiple DNA amplification markers in order for resolution to be high (Silva et al., 2019). A total of five sets of primer pairs are tested, targeting a definite number of specific groups. Illumina sequencing is performed and the obtained results are used to adapt and improve the protocol. Improvements include alterations to the gut content isolation and homogenization techniques, the development of blocking primers for crayfish DNA, the selection of the most consistent extraction kit and the exclusion of unreliable amplification markers. A final DNA metabarcoding protocol for crayfish-derived dietary content is, thus, established to be used in a larger set of samples, with the results described in Chapter 3.

2.2. Material & Methods

As the Pilot Study only comprises the development of the sample processing protocol, descriptions of sampling location and technique can be found in Section 2 of Chapter 3.

2.2.1. Sample decontamination, dissection and homogenization

A total of 20 crayfish samples (10 of each species) was used for the pilot study. To reduce the likelihood of detecting environmental DNA (eDNA) present in the external surface of the individuals, a decontamination procedure was included prior to the samples dissection. The importance of this measure lied in ensuring that all taxonomic entities later detected by sequencing come from direct ingestion by the crayfish. Before having their guts removed, each crayfish was first rinsed in distilled water and left immersed in 2.5% bleach for 40 minutes, as suggested by Greenstone et al. (2012).

Dissection was necessary in order to isolate the gut regions containing digested material. The procedure was performed through cuts from telson to rostrum (Figure 3a), allowing for the removal of the stomach and intestine (Figures 3b and 3c), which were kept frozen in 1.5 mL Eppendorf tubes (Figure 3d).

After isolating the gut regions, a homogenization procedure was tested with the goal of breaking the stomach walls and exposing its contents. By grinding the ingested particles into finer fragments, it was hoped that DNA extraction success would increase. This step was performed by bead-beating, using 0.5 mm Zinc oxide beads, for 5 min at 30 Hz, in a Retsch® MM 400 (Retsch, Germany) mixer mill. The beads were removed manually before proceeding to the next step.

2.2.2. DNA extraction kits and amplification markers

Two DNA extraction kits were tested: *E.Z.N.A. Tissue DNA*® (2 € / sample; Omega Bio-tek, Inc., Georgia, USA) and *Quick-DNA Fecal/Soil Microbe Miniprep*® (5.28 € / sample; Zymo Research Corporation, USA) - hereafter referred to as EZN and ZYM, respectively. Both kits were used on all 20 crayfish gut samples.

Since crayfish are generalist consumers, a meaningful assessment of their diet requires a number of barcodes sufficient to guarantee significant taxonomic coverage and resolution. However, as the requirements for coverage and resolution increase, the more time consuming and expensive diet analysis via metabarcoding becomes. Groups of interest were, thus, defined in regards to pre-established expectations about crayfish diet, and included EPTO insects, Plants, Algae, Vertebrates, and Eukaryotes in a broad sense. Five primer pairs were considered for comparison based on taxonomic coverage and references from literature (Table 1).

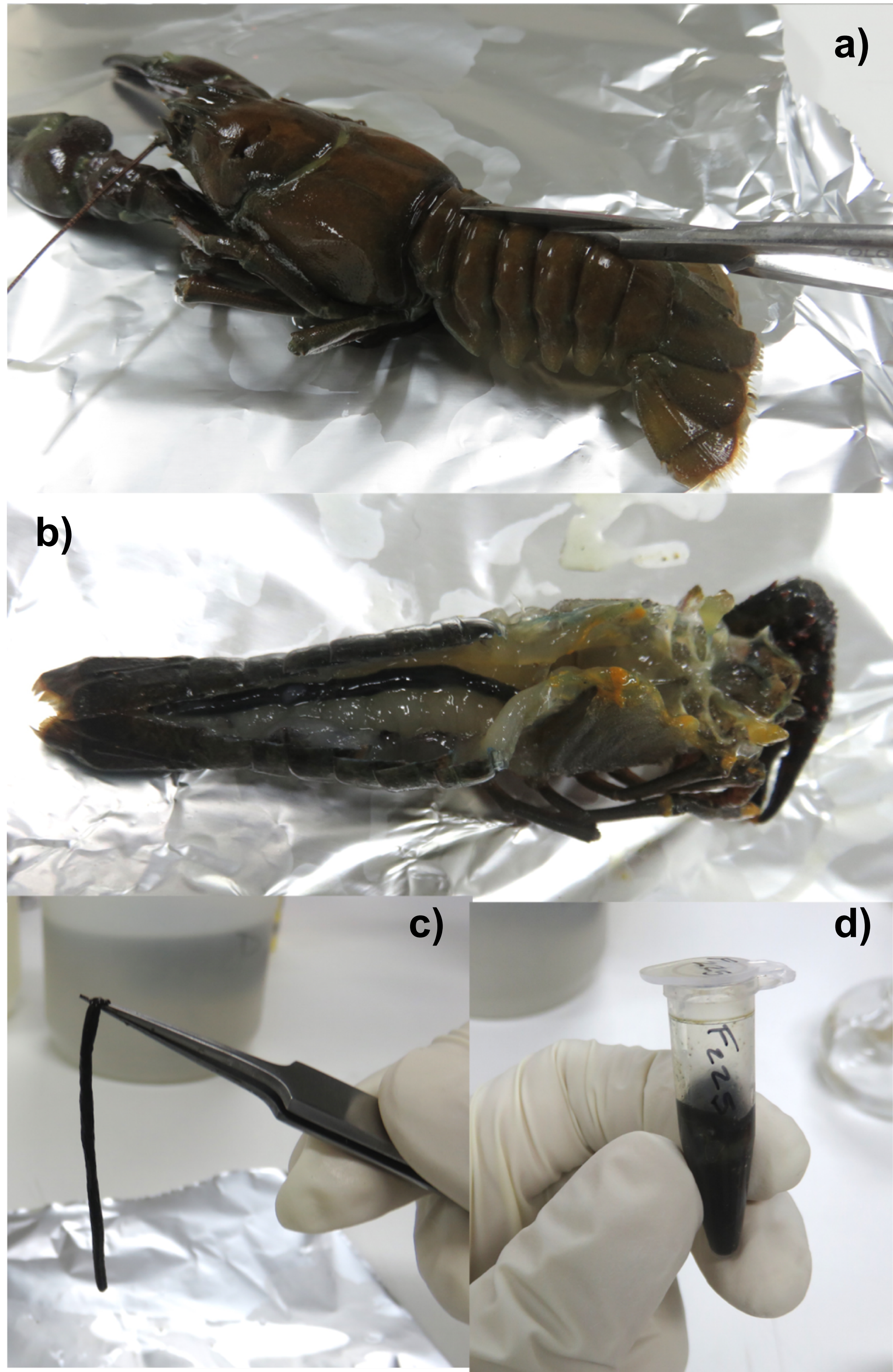

Figure 3 – Steps comprised in crayfish dissection, illustrated by photographs of a signal crayfish (*Pacifastacus leniusculus*) specimen: a) Cutting from telson to rostrum; b) Open crayfish, stomach has already been removed (empty space to the right), intestine remaining; c) isolation of the intestine; d) final sample in an Eppendorf tube.

Table 1 – Sets of primers used in the pilot study.

Primer set	DNA region	Target taxa	**Size** (bp)	Reference
g-h	trnL	Plants	20-80	Taberlet et al., 2007
UniPlant F/R	ITS2	Plants	187-387	Moorhouse-Gann et al., 2018
12SV5.1 F/R	12SV5	Vertebrates	108	Riaz et al., 2011
SSU3' F/R	18S	Eukaryotes / Algae	132	Jarman et al., 2013
Leray-XT	COI	Eukaryotes/EPTO	313	Wangensteen et al., 2018

PCR reactions were performed for DNA amplification with all primers sets, using total volumes of 10 µL comprising 5 µL of QIAGEN® PCR Master Mix (Qiagen,UK), 0.3 µL of each primer, 3.4 µL of ultra-pure water and 1 µL of DNA extract. Primer pair SSU3' F/R represented an exception to this, with the only difference being the use of PHUSION® Hi-Fidelity PCR Master Mix (New England Labs, USA) in place of QIAGEN's.

Primers sets *g-h*, *UniPlant F/R*, *12SV5.1 F/R* and *Leray-XT* shared the same thermal cycling profile (denaturing at 95°C for 10 min; 35 cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 30 s; and a final extension of 60°C for 10 min) while *SSU3' F/R* required its own (an initial denaturing step of 95°C for 15 min; 35 cycles of denaturing at 95°C for 20 s, annealing at 67°C for 20 s, and extension at 72°C for 20 s; and a final extension of 60°C for 10 min).

2.2.3. Library preparation

A second PCR was conducted to add indexes (7 bp dual indexes) and Illumina flow cell adaptors, using 10-µl reactions containing 0.5 µM of each index-primer (indexes were based on Kircher et al., 2012, and Gansauge & Meyer, 2013), 5 µl *2X KAPA HiFi HotStart ReadyMix*® (Kapa Biosystems, USA), and 2 µl of previous PCR product diluted 1:4 with 10 mM Tris. The thermal cycling profile consisted of an initial step of 95°C for 3 min; 10 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 5 min. The resulting PCR product was cleaned using 0.9 X by volume *AMPure XP*® beads (Beckman Coulter, Inc., USA) following the manufacturer's instructions. The concentration of each cleaned PCR product was measured using *Nanodrop 2000* (Thermo Fisher Scientific, USA), and PCR products were normalized to 15 nM using 10 mM Tris pH 8.5. The final pool was created by combining an equal volume from each normalized sample. The concentration of the final pool was assessed by qPCR using *KAPA Illumina Library Quantification*® (Kapa Biosystems, USA) following the manufacturer's instructions. Illumina paired-end sequencing was performed using a 500-cycle *Rapid Run*® kit (Illumina, Inc., USA) on a *Hiseq 2500* sequencer operated by Genewiz (Leipzig, Germany). Demultiplexing was also performed by the sequencing company.

2.2.4. Bioinformatic processing

Sequence data were processed using the software package OBItools (https://git.metabarcoding.org/obitools/obitools). After aligning forward and reverse sequences (command *illuminapairedend*), alignments with an overlapping quality below 40 were discarded. Reads were then assigned to samples and primers were removed (command *ngsfilter*). Finally, reads were collapsed into unique haplotypes and singletons (haplotypes with only one read) were discarded. Haplotypes were mapped against the *nt* database (NCBI Resource Coordinators, 2016), using *megablast* (Morgulis et al., 2008) and 100 results per query were kept. An OTU table was generated and processed in the programming environment R (R Core Team, 2018).. Occurrences with less than 1% of the total read count for the taxa were removed, as well as detections totalling under 20 reads.

2.3. Results & Discussion

2.3.1. DNA extraction kits

The comparison between DNA extraction kits revealed marked differences. A larger count of successful DNA amplifications was obtained from extractions performed with ZYM, deduced by the visualizations in agarose gel (Table S1). Moreover, gel bands resulting from EZN extractions were considerably less clear, suggesting the presence of insufficient PCR product (Figure 4).

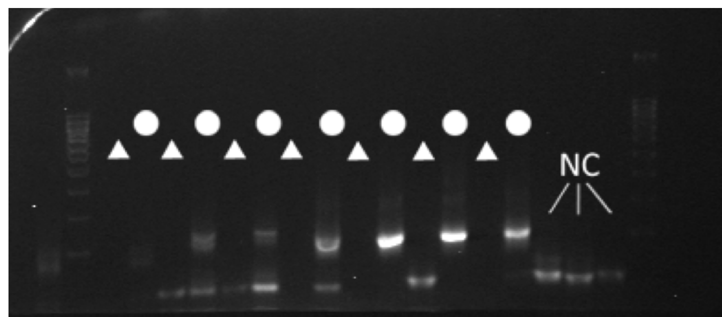

Figure 4– Comparison between EZN and ZYM PCR results, here using the SSU3' F/R primer set (18S). Triangles indicate EZN extractions while circles represent ZYM extractions. NC stands for negative control and refers to the last three rows on the right.

Sequencing results (Table S2) provided confirmation for these observations, by identifying the most taxonomic diverse and resolute samples as those extracted with ZYM. Therefore, ZYM (*Quick-DNA Fecal/Soil Microbe Miniprep®*) was selected as the DNA extractions kit for the final set of samples. Additionally, due to the high levels of lab contamination observed on the pilot samples, all post-dissection tasks to be performed on the final set of samples were relocated to non-invasive laboratory rooms.

The addition of Zinc oxide beads for the homogenization of gut samples proved to be unideal. Although the bead-beating process is effective in releasing the stomach contents, the beads need to be removed manually to avoid interference with DNA extraction reagents. This not only translates into a laborious task, but also implies partial loss of consumed material that adheres to the beads or the removal tools, while increasing chances of contamination. Adding to these observations, a considerable amount of crayfish tissue was present and its detection by sequencing represented a problem. Hence, the post-dissection isolation of the gut contents was redesigned to begin with the perforation of the stomach, allowing only its contents to leak out into an Eppendorf tube, followed by standard DNA extraction using ZYM. Conveniently, that protocol comprises a similar homogenization step, relying on its own beads which need not to be removed and are designed to not interfere with its subsequent steps. However, only 0.25 mL from the leaked stomach contents were ultimately used for DNA extraction, to abide with the protocol's restrictions.

2.3.2. Marker comparison and selection

Based on amplification performances (Table S1), the primer sets *g-h*, *Leray-XT* and *SSU3'F/R* yielded the best results and the respective samples were selected for sequencing. Although agarose gel observations suggested an ineffective amplification of vertebrate DNA by the primer set *12SV5.1*, sequencing was still performed on those samples, due to the interest in vertebrate taxa. Ultimately, the *gh* amplifications would come to be excluded from sequencing during the pilot study, in order to reduce the variance between fragment sizes.

An overview of the sequencing results supported the final decision on which markers were to be used on the final set of samples. *Leray-XT* (COI fragment) (Figure S1) had a strong performance, detecting numerous insect orders, although a notably high frequency of moth species was derived from sample contamination. Detected taxa include insects, fungi, microalgae and one Endangered species of mussel - *Potomida litorallis* (Cuvier, 1798) -, even though crayfish DNA seized up to 60% of reads (not included in Fig. 1 to improve readability). A measure to reduce this high percentage of predator DNA was taken through the development of blocking primers, addressed in the following section.

In turn, *SSU3' F/R* (18S fragment) (Figure S2) provided over 50 OTUs composed by fungi and algae, while also locating mussels and protozoans. The percentage of crayfish DNA detected was low (~10%), tolerating the lack of blocking primers. These results suggest that the combined use of the COI and 18S markers is able to provide coverage for taxa such as insects, algae and lower eukaryotes, potentially important diet components for crayfish.

On the other hand, sequencing with *12SV5.*1 (12S fragment) (Figure S3) proved unreliable for this experiment, due to the marker's susceptibility to laboratory contamination. With the results being dominated by human, wolf, felids and red partridge, virtually all findings were contamination-derived. The only taxonomically plausible detection was that of *Lepomis sp*., although in a very low-read occurrence. A naturally low amount of vertebrate DNA consumed by the crayfish might explain the lack of relevant results, besides leaving room for contamination. Combined with the known limitations of NGS regarding the simultaneous usage of markers of differing length, this primer set was discarded from the final analysis. Nonetheless, detection of vertebrate DNA is still expected through the COI marker.

Finally, although the amplifications of plant DNA were not sequenced during the pilot test, *g-h* was selected for the final experiment, given its robust PCR performance, failed performance of *UniPlant F/R*, and the relevance of the target taxa. Hence, the final set of markers comprised *g-h* (trnL), *Leray-XT* (COI) and *SSU3'F/R* (18S).

2.3.3. Blocking primers

The high percentage of reads (up to 60%) obtained for both crayfish species when using the COI marker represents a problem, since less reads become available for targeted content. As such, one blocking primer for each of the two crayfish species was designed (Table 2) and tested through PCR reactions, maintaining the same thermal cycling profile.

Table 2 – The two COI blocking primers used in this experiment.

COI blocking primer	Target
CCTCCTTTAGCTTCTGCTATTGCTCATGCGGGAGC	*Procambarus clarkii*
CCTCCTCTAGCAGCGGCTATTGCTCATGCAGGGGC	*Pacifastacus leniusculus*

The PCR tests consisted in adding the blocking primers in increasing concentrations (0, 5, 10 and 15 times the concentration of the COI marker) to samples of crayfish DNA (extracted from cuts to the exoskeleton). A positive control was performed using DNA from cow to which crayfish blocking primer was also added. As displayed in Figure 5, removal of virtually all crayfish DNA occurred immediately with the addition of the smallest concentration of blocking primer without negatively affecting the DNA from cow.

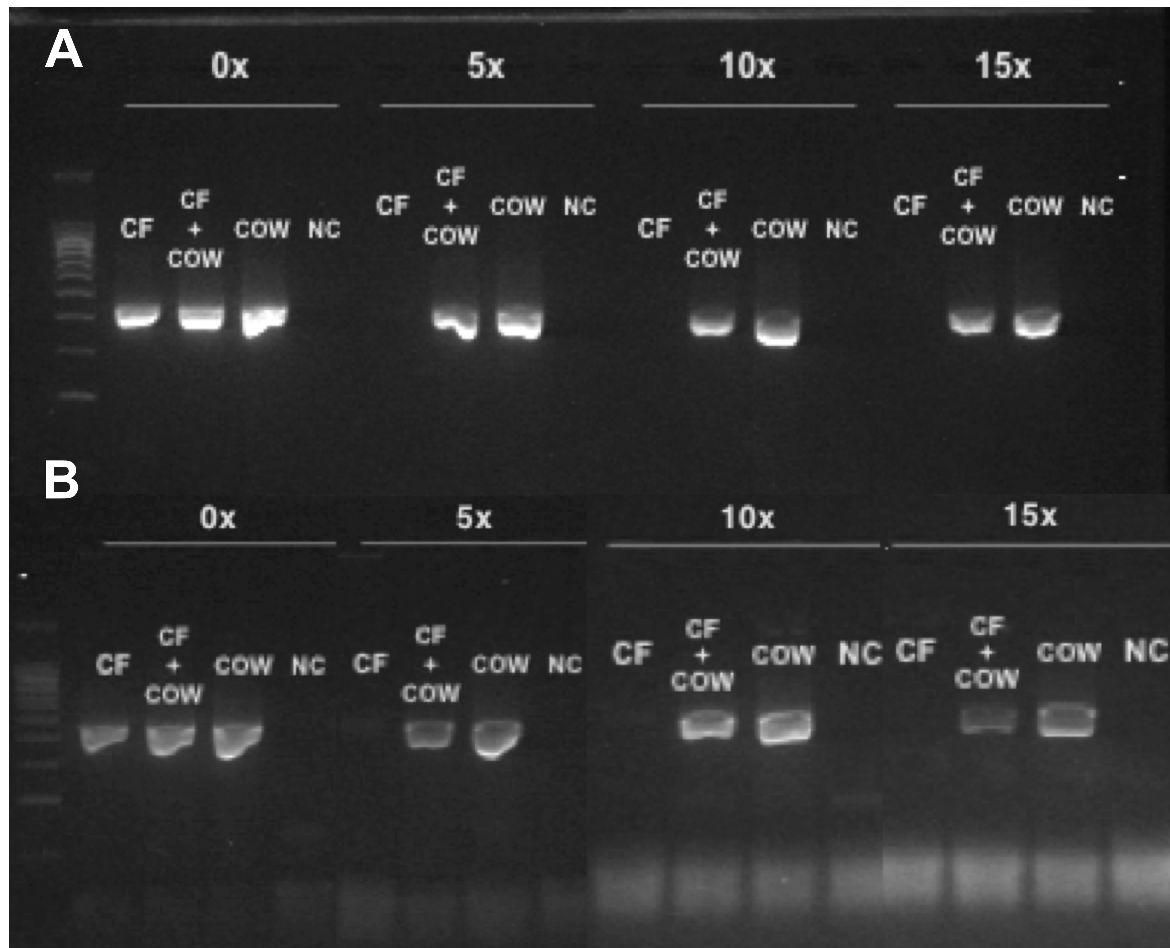

Figure 5 – A: *P. clarkii* blocking test. B: *P. leniusculus* blocking test. Blocking primer concentrations are displayed on top, relative to the COI primer pair (CF = Crayfish DNA, COW = Cow DNA, NC= Negative Control).

Chapter 3: Diet Assessment

3.1. Introduction

The invasion of stream ecosystems by non-native species is a cause of concern worldwide due to its negative environmental and economic impacts (Havel et al., 2015). Freshwater crayfish are among the most problematic invaders, with the red swamp crayfish *Procambarus clarkii* and the signal crayfish *Pacifastacus leniusculus* being two of the most widely distributed species worldwide (Vaeßen & Hollert, 2015; Souty-Grosset et al., 2016). Their predatory nature, along with the potential trophic overlap with native, threatened fauna, calls for immediate risk assessments. Knowledge of the feeding ecology of these two species is, thus, essential for their control and management.

Few instances of coexistence between *P. clarkii* and *P. leniusculus* have been reported in the literature. Coexistence is known to occur in North-eastern Portugal (e.g. Filipe et al., 2017), Spain (Alonso & Martínez, 2006), the U.S.A. (Mueller, 2007), Japan (Nakata et al., 2005), but probably also England and France (cited in Bernardo et al., 2011). Thus, little information is available regarding the interactions between the two invaders, as well as their joint impacts. Although environmental conditions seem to explain why occurrences of coexistence are limited (Bernardo et al., 2011), an eventual trophic niche overlap may also have a meaningful role. Research on such a mechanism could better inform prediction models of range expansions, leading to more efficient management strategies. Furthermore, efforts to protect the native biodiversity can benefit from identifying which taxa are most vulnerable to the two invasive crayfish species.

In the north-east of Portugal, where their ranges are expanding (Bernardo et al., 2011; Filipe et al., 2017), the two crayfish invaders find streams in varying conditions. Human impact can either be absent and systems pristine, or instead highly impacted by the construction of dams and introduction of non-native species. With the two species occurring both in allopatry and sympatry along the watersheds of Tua and Sabor, appropriate conditions are found for the investigation of dietary variations. Such context allows to establish comparisons along gradients of abiotic variables, habitat composition, crayfish coexistence or human-impact.

Thus, in this chapter, the diet composition of the red swamp and signal crayfish is explored using the set of procedures for DNA metabarcoding defined in Chapter 2. Crayfish samples were collected from the Sabor watershed and processed for diet analysis. Descriptions of diet diversity are provided for the two invaders, followed by dietary comparisons between allopatric and sympatric locations and between crayfish species. Diet differences among size classes are investigated to assess the existence of an ontogenetic shift. Finally, the present results are compared with previous studies, pitfalls and future directions are addressed, and management improvements are proposed.

3.2. Material & Methods

3.2.1. Study area and sampling technique

Surveys were performed along the Sabor river basin in North-east Portugal, where the Long-Term Ecological Research (LTER) site of Baixo Sabor is also located. Diverse environmental conditions describe the watershed, such as altitude (100–1500 m above sea level), mean annual temperature (6.9–15.6 °C) and total annual precipitation (443–1163 mm) (Filipe et al., 2017). While the months from October through March present low temperatures and high levels of precipitation, climate from June to September is typically hot and dry, fitting of the Mediterranean setting. Flow regime varies seasonally, with most headwater or smaller streams drying considerably in summer, while the main watercourse and its largest tributaries are maintained (Ferreira et al., 2016).

Collection of crayfish samples took place in 40 locations (Figure 6; Table S3), during July 2018. Locations were based on recent surveys across the watershed by Filipe et al. (2017).Sites were defined as 50-m reaches of streams, where sampling was performed using single anode electrofishing gear (350-750 V, 3–5A, DC), following standard procedures outlined in Ferreira et al. (2016). Electrofishing was done progressively in the upstream direction and always conducted by the same operator, accompanied by a second operator to capture the displaced crayfish. Collected samples were put in individual

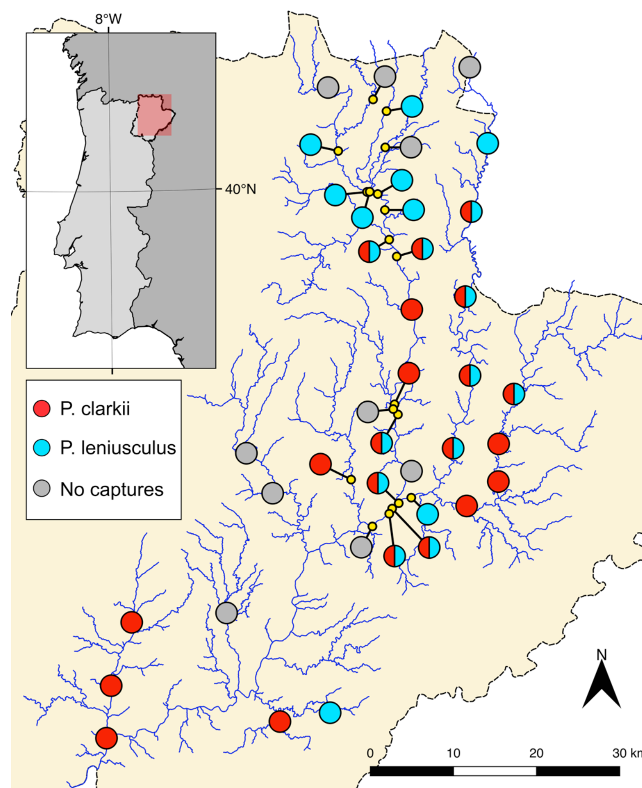

Figure 6 – Map of the Sabor river basin, North-east Portugal. Sampling sites are displayed and coloured according to the detected presence/absence of the two crayfish species.

plastic ziplock bags and stored inside coolers with ice, providing anesthesia and interrupting any physiological processes. To ensure euthanasia and preservation, individuals were moved to a freezer (-20°C) at the end of each sampling day and stored until being processed in the laboratory. Cooling and freezing are the recommended methods of anesthesia and euthanasia for the welfare of freshwater crayfish. (Reilly, 2001; AVMA, 2013; Puri & Faulkes, 2015).

3.2.2. Sample processing

In order to investigate the effect of crayfish size on diet composition, *cephalothorax length* (CL) was measured for all samples with a caliper (Figure 7) and individuals were separated in three size classes (**Small**: CL < 35 mm; **Medium**: 35 mm < CL < 40 mm; and **Large**: CL > 40 mm), defined based on the size-distribution of all captures and previous literature (e.g. Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998; Pérez-Bote, 2004; Usio et al., 2009; Chucholl, 2013).

Figure 7 – Measurement of the cephalothorax length of a signal crayfish (*Pacifastacus leniusculus*) specimen.

Following the procedures described in the pilot study (Chapter 2), samples were first decontaminated by bleaching and dissected. DNA extraction from the gut contents generated 208 DNA samples and 10 extraction controls. Amplification through PCR was performed twice for the three selected markers (trnL, 18S and COI) and generated an additional 48 PCR controls. Thus, a total of 1356 DNA products were sequenced. Library preparation and high-throughput sequencing were performed as defined in the pilot study.

3.2.3. Bioinformatic processing

Sequence data were processed using the MBC pipelines software package (Galhardo et al., in prep.). Within the package, paired-end reads were aligned using flash2 (Magoč & Salzberg, 2011) with the settings: --max-overlap=100 -D -m 10 -t 1. Sequences outside the expected amplicon lengths were removed. Remaining sequences were demultiplexed

using vsearch (Rognes et al., 2016) with fastq_maxee=1 and singletons were removed. The Exact Sequence Variants (ESVs) were mapped against the *nt* database (NCBI Resource Coordinators, 2016), using megablast (Morgulis et al., 2008) and 100 results per query were kept. ESVs were placed in taxonomic bins through the custom R script *bin.blast* (https://github.com/bastianegeter/bin.blast) using the default parameters. The procedure for binning used by this function is to 1) discard hits with less than 70% query cover; 2) discard hits with evalue below 0.001; 3) for each query, keep any hits that had a percentage identity within 1% of the top hit; 4) for each taxonomic level, consider only hits that fall within the specified thresholds (species = 98%, genus = 95%, family = 92%, higher-than-family = 80%); 5) for each query, find the lowest-common-ancestor (LCA) of the remaining hits; 6) if a query results in a species-level LCA, that is the final bin assigned to the ESV; 7) failing a species-level assignation, genus is assigned; 8) failing a genus-level assignation, family is assigned; 9) failing a family-level assignation, order and above are assigned. A table reporting read counts after each of these steps was assembled (Table 3, Steps 1 to 6).

Table 3 - Read count after each bioinformatics processing step after sequencing. Step 1: demultiplexing paired reads; Step 2: paired-end alignment; Step 3: primer trimming; Step 4: filtering by size according to expected amplicon lengths and removal of singletons; Step 5: BLAST; Step 6: removal of sequences with low query cover and evalue; Step 7: applying taxonomic filters.

Fragment	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
18s	18657404	18314784	17257462	16262340	15537935	15535146	13261468
COI	23983196	23225262	20035354	14512943	13932402	13131343	12624501
trnL	10386574	10335180	10129630	8314266	7627290	7627290	6561422

Once taxonomy was assigned, a set of filters was applied in R (R Core Team, 2018) to improve the confidence in the dataset. Firstly, to counter the potential of tag-jumping, when sequences can be incorrectly assigned to the wrong samples due to sequencing error, detections with read counts below 0.15% of the total reads of the relevant taxon were removed from the dataset, as were detections with read counts below 0.15% of the total reads of the relevant sample. Furthermore, absolute read count minimum thresholds were calculated separately for each fragment and were based on reads detected in negative extraction (n=60) and PCR (n=48) controls: 1) detections were kept if the taxon was detected in both PCR replicates and the summed read count was above the specified threshold or 2) detections were kept if present in only one of the PCR replicates, but was above the specified threshold. As such, detections were removed from single replicates if their read count was below 100 for trnL and COI or 500 for 18S, or if the sum of reads obtained between replicates was below 200 for COI and 6000 for trnL and 18S. The number of reads after the application of these filters is reported in Table 3 - Step 7. As a last measure, taxa whose origin was likely derived from lab contamination were excluded from the dataset. This resulted in all 108 controls being free of reads.

3.2.4. Data analysis

All data analyses were performed in R. A matrix was assembled comprising all the detected taxa per crayfish gut sample. Sequence reads were converted to binary presence/absence data and frequencies of occurrence estimated for all taxa. In an attempt to account for ecological context and trophic behaviour, the original matrix was then divided in three functional groups: Animals (preyed macroinvertebrates, fish, amphibians and zooplankton); Plants (aquatic and terrestrial plants, likely consumed as detritus), and Algae (from grazing activity on the periphyton). Subsequent analyses were performed at two taxonomic levels: a) full taxonomic path; and b) Class-level for Algae and zooplankton or Family-level for Plants and the remaining Animals. The usage of class level in *b)* was necessary due to the taxonomic inconsistencies verified below class for algae and zoo plankton. Taxonomic diversity within the three groups was illustrated using sunburst plots (*plotly* package, Sievert, 2018), euler diagrams (*eulerr* package, Larsson, 2019) and venn diagrams (*gplots* package, Warnes et al., 2019).

A principal coordinate analysis (PCoA) was performed to explore dietary variations between crayfish species and size classes, using presence/absence matrixes with the full taxonomic paths detected within each functional group. In order to compare overall diet composition between crayfish species and between size classes, permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) was applied to each functional group, at both taxonomic levels, using the function *adonis* from the *vegan* package (Oksanen et al., 2016). The starting model included site as a random factor and tested the effects of crayfish size class and sex, both nested within crayfish species. The least significant interactions were sequentially removed to improve the models. To complement the PERMANOVAs, analyses of similarities (ANOSIM, Clarke, 1993) were also performed along with similarity percentage analysis (SIMPER; Clarke, 1993) if significant effects were detected, in order to determine the main taxa contributing to such effects. This was achieved using the functions *anosim* and *simper,* respectively, both present in the *vegan* package.

In order to assess differences in species richness between species and size classes, and to investigate potential differences for particular food taxa, generalized linear mixed models (GLMs) were employed through the function *glmer*, from the package *lme4* (Bates et al., 2015).

3.3. Results

3.3.1. Sampling results

Out of the 40 visited sites, 11 had no crayfish captures, while 3 locations provided insufficient samples (<5 allopatric individuals; no minimum number of samples was set as threshold for sympatric occurrences, due to the high interest in these locations). The remaining 26 sites (Table S3) comprised 9 locations where *P. clarkii* was found exclusively, 7 sites containing only *P. leniusculus* and 10 sympatric locations between the two species.

From a total of 596 crayfish specimens collected (250 *P. clarkii* and 346 *P. leniusculus*), 20 served as pilot samples and 208 were used for the final diet assessment. Unused samples included: i) 28 crayfish with CL measuring less than 20 mm, as dissection would prove impractical; and ii) 17 dissected individuals that exhibited empty stomachs. All unspoiled specimens were donated to the Museum of Natural History and Science of the University of Porto.

The final set of 208 samples was designed to include a balanced number of individuals from the three defined size classes (Table 4). As such, each size class was comprised of a selection of 33 samples of each crayfish species, totaling 198 specimens. Finally, 10 large *P. clarkii* from 2 extra allopatric sites were added in an effort to increase spatial representability. The set of 208 specimens was thus completed and comprised 109 samples of *P. clarkii* and 99 samples of *P. leniusculus*, of which 60 and 49, respectively, originated from sympatric sites.

Table 4 – Distribution of CL (cephalothorax length) for the final set of 208 crayfish samples. (*n* refers to the number of samples, while *Min.*, *Max*, *Avg.* and *S. E.* indicate the minimum, maximum, average and standard error for the measurements in each category.

		n	Min. (mm)	Max. (mm)	Avg. (mm)	S. E.
	P. clarkii	109	20.4	54.5	38.98	7.26
Small:	***CL*** < 35 mm	33	20.4	34.9	30.69	3.78
Medium:	35 mm < ***CL*** < 40 mm	33	35.1	39.9	37.75	1.36
Large:	***CL*** > 40 mm	43	41.1	54.5	46.28	3.64
	P. leniusculus	99	22.4	58.4	37.44	7.49
Small:	***CL*** < 35 mm	33	22.4	34.9	29.35	3.54
Medium:	35 mm < ***CL*** < 40 mm	33	35.1	39.7	37.26	1.41
Large:	***CL*** > 40 mm	33	40.2	58.4	45.71	4.38
	TOTAL	208	20.4	58.4	38.25	7.39

3.3.2. Diet assessment results

A total of 368 unique taxa were detected at various taxonomic levels (Table S4), representing the sum of detections using trnL (89), COI (148) and 18S (151), with 20 taxa being detected through more than one marker. Of the 208 crayfish samples, 207 contained at least one valid taxonomic detection. Out of these detections, 91.0% were successfully classified to order level, 69.3% to family level, 52.4% to genus-level and 27.7% to species level (Table 5). Regarding marker performance, trnL was noticeably the most successful in identifying taxa to family level (95.5%). Still, 18S was the fragment revealing the deepest resolution capability, identifying almost a third (32.5%) of its detections to species level.

Table 5 – Number and percentage of taxa detected per marker and taxonomic level.

	Kingdom	Phylum	Class	Order	Family	Genus	Species
All	368	347	340	335	255	193	102
%	(100.0)	(94.3)	(92.4)	(91.0)	(69.3)	(52.4)	(27.7)
trnL	89	89	89	88	85	52	19
%	(100.0)	(100.0)	(100.0)	(98.9)	(95.5)	(58.4)	(21.3)
COI	148	137	136	132	76	56	33
%	(100.0)	(92.6)	(91.9)	(89.2)	(51.4)	(37.8)	(22.3)
18S	151	139	135	131	106	90	49
%	(100.0)	(92.1)	(89.4)	(86.8)	(70.2)	(59.6)	(32.5)

The number of unique taxonomic detections is reduced to 256 when considering only the three defined functional groups: Animals (65 taxa); Plants (103 taxa); and Algae (88 taxa) (Table 6). In regards to frequency of occurrence, Plants and Algae were observed, respectively in 97.1% and 91.3% of samples, while animal taxa occurred in 83.2% of the samples. Most crayfish guts contained taxa from the three functional groups (166 samples or 79.8%) and no evidence was found for exclusive animal consumption (Figure 8). Comparing the two crayfish species, even though a larger number of *P. clarkii* samples contained taxa belonging to the functional groups, *P. leniusculus* displayed a slightly higher diversity in diet (Table 6). Still, more than half (54.7%) of all detected taxa were present in the guts of both species (Figure 9). While female crayfish consistently displayed a more diverse diet than their male counterparts (Table 6), no clear pattern was observed between size classes. Sympatric samples contained a higher diversity of taxa than both allopatric sets combined. (Figure 10). Moreover, the diet diversity of allopatric *P. leniusculus* was twice as high as that of allopatric *P. clarkii*. A total of 19 animal families, 7 zooplanktonic classes, 55 plant families and 18 Algae classes (Figure 10) were found. The most frequent clusters were Caenidae (Ephemeroptera) within Animals, Salicaceae (Malpighiales) among Plants and Chlorophyceae (Chlorophyta) within Algae (Table 7).

Table 6 – To the left, taxonomic diversity as the number of detected taxa (n) per functional group, crayfish species, size class and sex. On the right, frequency of occurrence as the number of samples (N) containing taxa per functional group, crayfish species, size class and sex. Left and right proportions (%) were calculated in relation to the total number of taxa for each row or total samples per category, respectively.

				Taxonomic diversity				**Frequency of occurrence**		
				Animals	**Plants**	**Algae**		**Animals**	**Plants**	**Algae**
All crayfish			n	65	103	88	N	173	202	190
			%	(25.4)	(40.2)	(34.4)	%	(83.2)	(97.1)	(91.3)
Procambarus clarkii	TOTAL		n	49	73	71	N	91	107	98
			%	(25.4)	(37.8)	(36.8)	%	(83.5)	(98.2)	(89.9)
	Size	Large	n	30	43	54	N	35	43	37
			%	(23.6)	(33.9)	(42.5)	%	(81.4)	(100.0)	(86.0)
		Medium	n	29	43	44	N	28	31	33
			%	(25.0)	(37.1)	(37.9)	%	(84.8)	(93.9)	(100.0)
		Small	n	35	37	52	N	28	33	28
			%	(28.2)	(29.8)	(41.9)	%	(84.8)	(100.0)	(84.8)
	Sex	Male	n	35	52	52	N	39	45	37
			%	(25.2)	(37.4)	(37.4)	%	(84.8)	(97.8)	(80.4)
		Female	n	40	57	65	N	52	62	61
			%	(24.7)	(35.2)	(40.1)	%	(82.5)	(98.4)	(96.8)
Pacifastacus leniusculus	TOTAL		n	50	80	73	N	82	95	92
			%	(24.6)	(39.4)	(36.0)	%	(82.8)	(96.0)	(92.9)
	Size	Large	n	28	37	54	N	27	32	31
			%	(23.5)	(31.1)	(45.4)	%	(81.8)	(97.0)	(93.9)
		Medium	n	29	51	56	N	28	31	31
			%	(21.3)	(37.5)	(41.2)	%	(84.8)	(93.9)	(93.9)
		Small	n	31	53	47	N	27	32	30
			%	(23.7)	(40.5)	(35.9)	%	(81.8)	(97.0)	(90.9)
	Sex	Male	n	33	45	55	N	34	38	38
			%	(24.8)	(33.8)	(41.4)	%	(85.0)	(95.0)	(95.0)
		Female	n	39	68	63	N	48	57	54
			%	(22.9)	(40.0)	(37.1)	%	(81.4)	(96.6)	(91.5)

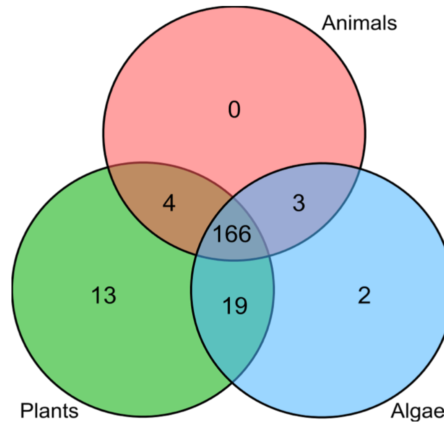

Figure 8 – Venn diagram displaying the number of crayfish samples (n=208) containing taxa from each of the three functional groups.

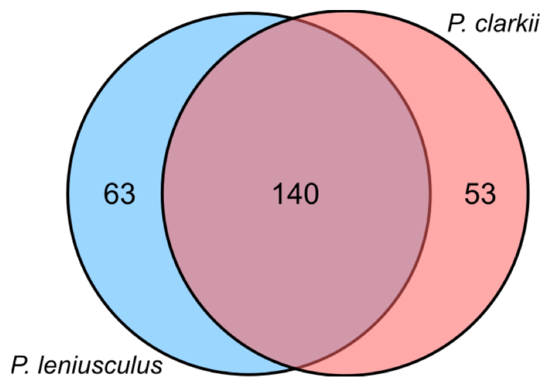

Figure 9 – Euler diagram displaying the number of taxa from the three functional groups detected within samples of both crayfish species.

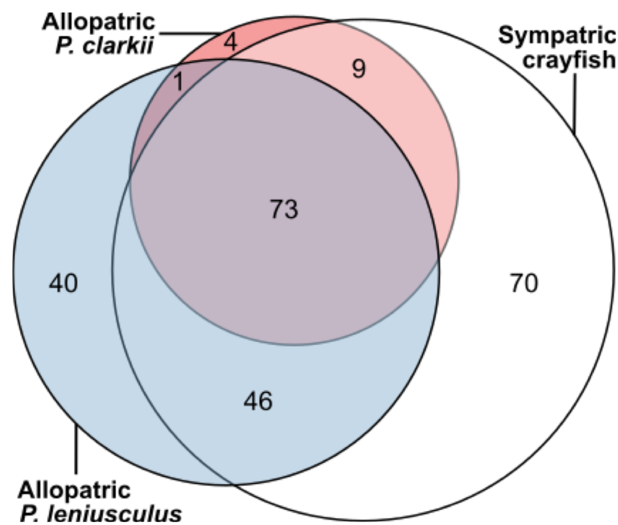

Figure 10 – Euler diagram displaying the number of taxa from the three functional groups detected within crayfish samples collected in sympatry and allopatry.

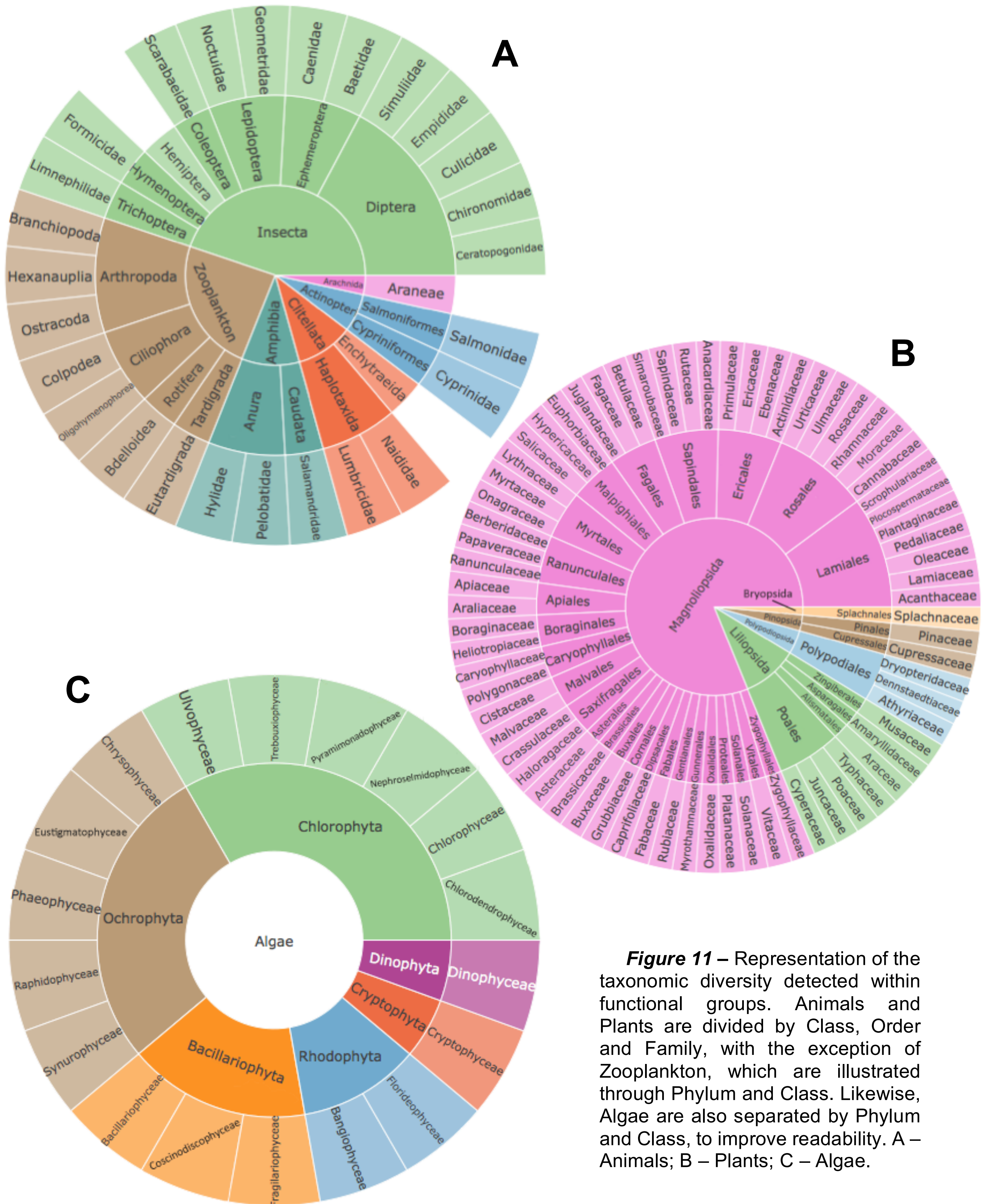

Figure 11 – Representation of the taxonomic diversity detected within functional groups. Animals and Plants are divided by Class, Order and Family, with the exception of Zooplankton, which are illustrated through Phylum and Class. Likewise, Algae are also separated by Phylum and Class, to improve readability. A – Animals; B – Plants; C – Algae.

Table 7 – Top animal families, plant families and algae classes detected. Preceding taxonomic levels were included for context. Each term consists in the class, order and family for Animals and Plants, or phylum and class for Algae. Number of occurring samples (N) and frequency of occurrence (%) on the two columns to the left.

	TAXA	N	%
ANIMALS	Insecta > Ephemeroptera > Caenidae	58	27.88
	Clitellata > Haplotaxida > Lumbricidae	33	15.87
	Insecta > Diptera > Chironomidae	27	12.98
	Insecta > Diptera > Simuliidae	14	6.73
	Branchiopoda > Diplostraca > Daphniidae	13	6.25
	Branchiopoda > Diplostraca > Macrotrichidae	9	4.33
	Amphibia > Anura > Pelobatidae	8	3.85
	Monogononta > Ploima > Brachionidae	7	3.37
	Amphibia > Anura > Hylidae	6	2.88
	Insecta > Trichoptera > Limnephilidae	4	1.92

	TAXA	N	%
PLANTS	Magnoliopsida > Malpighiales > Salicaceae	121	58.17
	Magnoliopsida > Fagales > Betulaceae	95	45.67
	Liliopsida > Poales > Poaceae	45	21.63
	Magnoliopsida > Fagales > Fagaceae	20	9.62
	Magnoliopsida > Apiales > Apiaceae	18	8.65
	Magnoliopsida > Ericales > Primulaceae	17	8.17
	Magnoliopsida > Ranunculales > Ranunculaceae	14	6.73
	Magnoliopsida > Rosales > Rosaceae	14	6.73
	Magnoliopsida > Malvales > Cistaceae	13	6.25
	Magnoliopsida > Fabales > Fabaceae	11	5.29

	TAXA	N	%
ALGAE	Chlorophyta > Chlorophyceae	174	83.65
	Ochrophyta > Phaeophyceae	130	62.50
	Ochrophyta > Eustigmatophyceae	105	50.48
	Chlorophyta > Trebouxiophyceae	87	41.83
	Dinophyta > Dinophyceae	71	34.13
	Ochrophyta > Chrysophyceae	34	16.35
	Rhodophyta > Florideophyceae	33	15.87
	Bacillariophyta > Fragilariophyceae	26	12.50
	Bacillariophyta > Bacillariophyceae	25	12.02
	Ochrophyta > Synurophyceae	15	7.21

Results from the PCoA did not suggest marked differences deriving from the factors crayfish species or size class. All visualisations obtained using the animal (Figure 12), plant (Figure 13) and algae (Figure 14) matrixes displayed axes with low percentages of explained variation (under 30% in all cases). Moreover, samples were generally distributed without a clear clustering pattern in regards to the target factors. Nonetheless, non-tested factors such as habitat traits might be responsible for a potential clustering observed for plants.

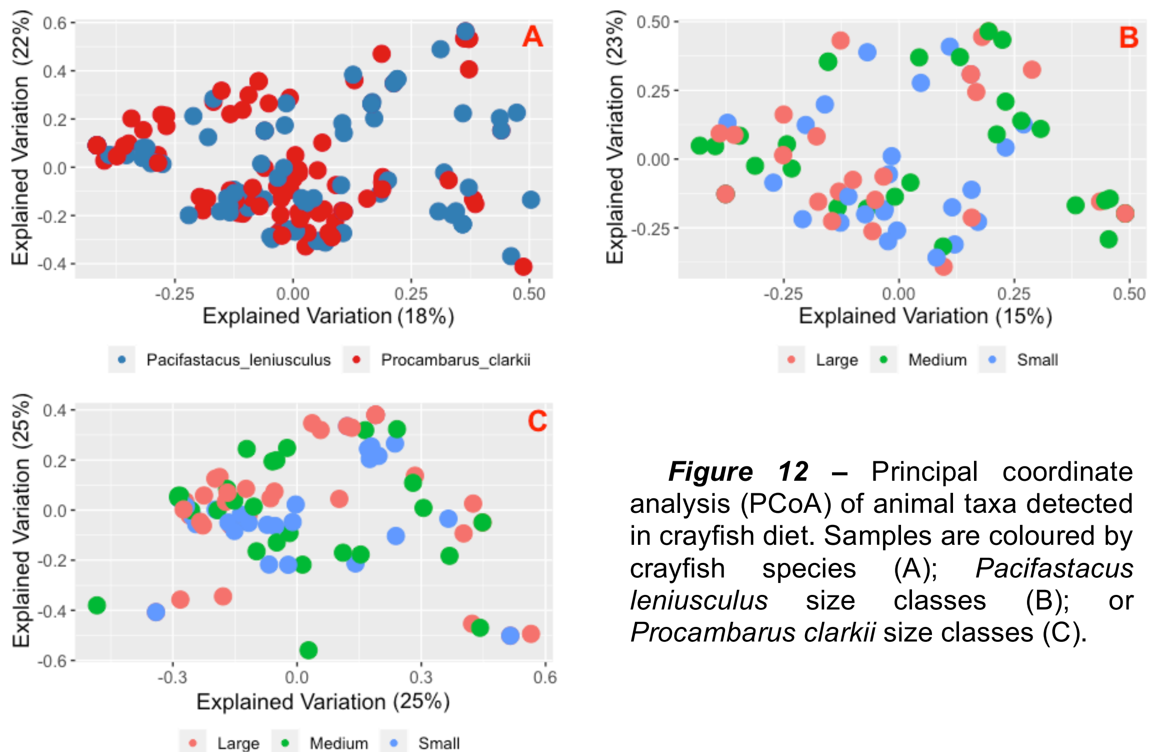

Figure 12 **–** Principal coordinate analysis (PCoA) of animal taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C).

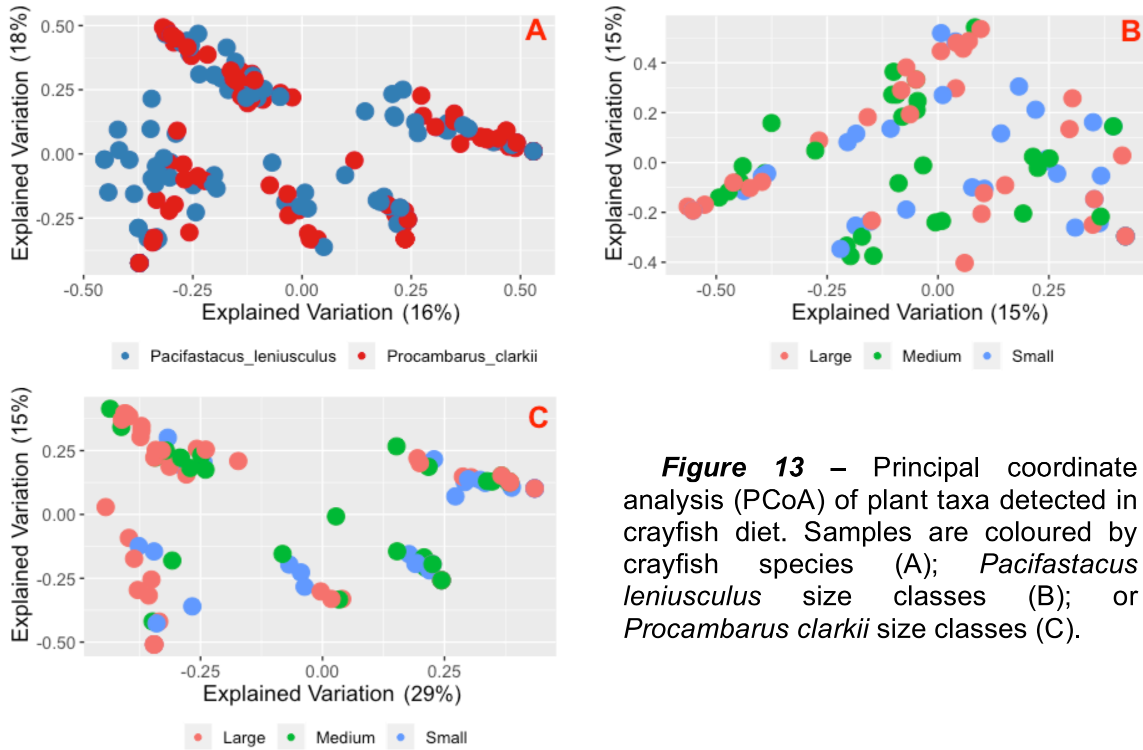

Figure 13 – Principal coordinate analysis (PCoA) of plant taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C).

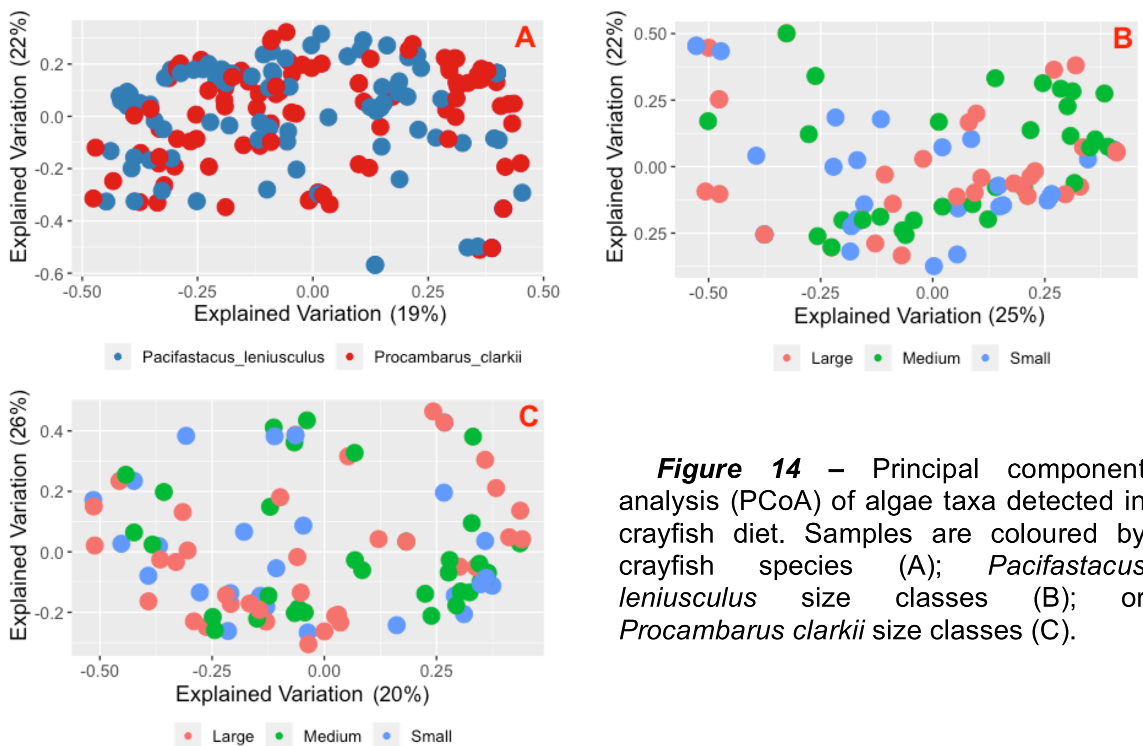

Figure 14 – Principal component analysis (PCoA) of algae taxa detected in crayfish diet. Samples are coloured by crayfish species (A); *Pacifastacus leniusculus* size classes (B); or *Procambarus clarkii* size classes (C).

The results from the statistical comparisons differed among functional groups. No significant effects were detected through the PERMANOVA and GLM tests performed on the Animals or Algae matrixes, at either of the taxonomic resolutions. However, for Plants, a significant effect was noted from the interaction between the factors species and size class. The phenomenon was observed through PERMANOVA, both using the full taxonomic path (p=0.0435) and Family-level (p=0.0090) (Table S5). By applying individual GLMs to all taxa, 18 significant effects were found (Table 8). All three functional groups had taxa among those findings, with 4 animals, 5 plants and 9 algae experiencing significant effects from the tested factors. Important scenarios are painted by these results (Tables 9, 10 and 11), such as the markedly higher consumption of Diptera (278% more) by *P. clarkii*, compared to *P. leniusculus,* or of chironomids (710% more) by male red swamp crayfish, in relation to females. No significant effects were detected through the ANOSIM tests directed at crayfish species and size class, and performed on all functional groups. Nonetheless, through the removal of zooplankton from the animal matrix, a significant difference (p=0.0480) arose between the two crayfish species (Figure 15), when considering the full taxonomic path. As such, a similarity percentage analysis was used to determine the taxa contributing the most to the differentiation (Table 12).

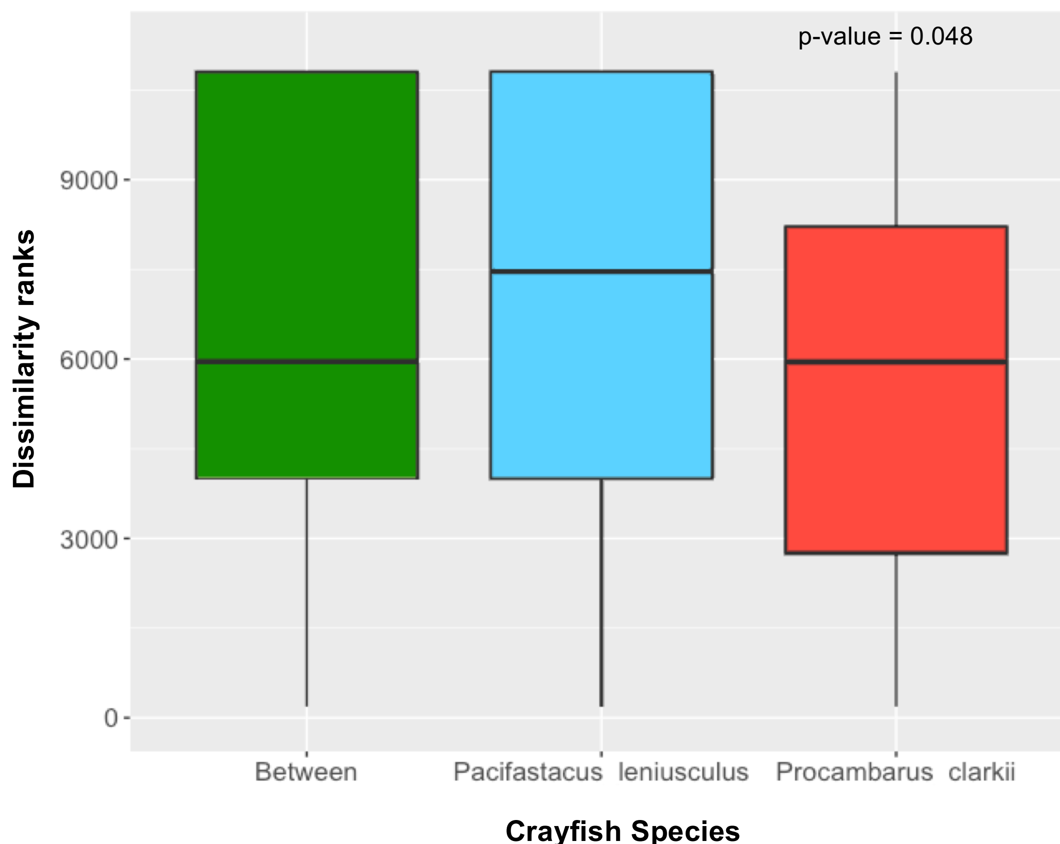

Figure 15 – Boxplot representation of the results from the analysis of similarities investigating the effect of crayfish species and performed using the animal matrix after the removal of zooplanktonic taxa.

Table 8 – Taxa experiencing significant effects from the factors and interactions tested using GLM.

Taxon	Taxonomic level	Functional group	Taxonomic analysis	Significant Factor/Interaction	p-value	Model
Ploima	Order	Animals	Full	Species:Sex	0.0156	Species*Sex
Diptera	Order	Animals	Full	Species	0.0051	Species
Eiseniella tetraedra	Species	Animals	Full	Species	0.0345	Species
Chironomidae	Family	Animals	Family	Species:Sex	0.0447	Species*SizeClass+Species*Sex
Alnus	Genus	Plants	Full	Species:SizeClass Species:Sex	0.0134 0.0266	Species*SizeClass+Species*Sex
Lysimachia	Genus	Plants	Full	Species	0.0182	Species
Alnus serrulata	Species	Plants	Full	Species	0.0419	Species
Betulaceae	Family	Plants	Family	Species:SizeClass	0.0099	Species*SizeClass
Salicaceae	Family	Plants	Family	Species:SizeClass	0.0386	Species*SizeClass
Fasciculochloris boldii	Species	Algae	Full	Species:Sex	0.0203	Species*SizeClass+Species*Sex
Mychonastaceae	Family	Algae	Full	Species:Sex	0.0240	Species*SizeClass+Species*Sex
Tetraedron minimum	Species	Algae	Full	Species:Sex	0.275	Species*Sex
Goniochloridales	Order	Algae	Full	Species:SizeClass	0.0195	Species*SizeClass
Pseudocharaciopsis	Genus	Algae	Full	Species:Sex	0.0158	Species*Sex
Licmophorales	Order	Algae	Full	Species	0.0069	Species
Eustigmatophyceae	Class	Algae	Full	Species	0.0443	Species
Chlorophyceae	Class	Algae	Class	Species:SizeClass	0.0253	Species*SizeClass
Fragilariophyceae	Class	Algae	Class	Species	0.0059	Species

Table 9 – Taxa experiencing significant species-related effects according to the individual GLM tests.

Taxon	Species-related Effect
Alnus serrulata	232% more frequent in *P. clarkii*
Diptera	278% more frequent in *P. clarkii*
Eiseniella tetraedra	266% more frequent in *P. clarkii*
Eustigmatophyceae	28% more frequent in *P. clarkii*
Fragilariophyceae	23% more frequent in *P. clarkii*
Licmophorales	20% more frequent in *P. clarkii*
Lysimachia	16% more frequent in *P. clarkii*

Table 10 – Taxa experiencing significant size-related effects according to the individual GLM tests.

Taxon	Size-related Effect
Alnus	985% more frequent in Small *P. clarkii* than Large
Betulaceae	1094% more frequent in Small *P. clarkii* than Large
Chlorophyceae	15% more frequent in Small *P. clarkii* than Large
Goniochloridales	211% more frequent in Small *P. clarkii* than Large
Salicaceae	19% more frequent in Small *P. clarkii* than Large

Table 11 – Taxa experiencing significant sex-related effects according to the individual GLM tests.

Taxon	Sex-related Effect
Alnus	21% more frequent in male P. clarkii than female
Chironomidae	710% more frequent in male P. clarkii than female
Fasciculochloris boldii	1189% more frequent in male P. clarkii than female
Mychonastaceae	11% more frequent in male P. clarkii than female
Ploima	12% more frequent in male P. clarkii than female
Pseudocharaciopsis	1191% more frequent in male P. clarkii than female
Tetraedron minimum	4% more frequent in male P. clarkii than female

Table 12 – Cumulative contributions of the most influential animal taxa to the differentiation between the two crayfish species.

Highest known resolution	Order/Family	Cumulative contribution
Insecta	-/-	0.1555
Diptera	Diptera/-	0.3102
Caenis luctuosa	Ephemeroptera/Caenidae	0.4537
Eiseniella tetraedra	Haplotaxida/Lumbricidae	0.5581
Hemiptera	Hemiptera/-	0.6190
Hymenoptera	Hymenoptera/-	0.6565
Orthocladius fuscimanus	Diptera/Chironomidae	0.6918
Pelobates cultripes	Anura/Pelobatidae	0.7268

3.4. Discussion

3.4.1. Diet composition

The idea that freshwater crayfish are omnivorous consumers feeding opportunistically on a diverse array of items, has for long been supported by various research techniques. From morphological interpretation of gut contents (e.g. Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998), to the analysis of isotopic signatures (e.g. Stenroth et al., 2006; Alcorlo & Baltanás, 2013) or controlled-environment experiments (e.g. Rebelo & Cruz, 2005; Carreira et al., 2014; Sousa et al., 2019), researchers have generally found the same answer. Although this study represents the first effort to identify the diet composition of crayfish through DNA techniques, the results presented come to reinforce the traditional view. A considerable variety of taxonomic entities was identified, comprising not only fungi, algae and plants, but also animals, ranging from micro- to macroscopical sizes.

Among the three functional groups defined, plants were markedly the most consistently consumed, occurring in 97.1% of the gut samples. Such prevalence is in accordance with results from past studies (Gutiérrez-Yurrita et al., 1998; Alcorlo et al., 2004; Pérez-Bote, 2004; Chucholl, 2013). Within the plants group, the phyla Magnoliophyta dominated the detections, occurring in all 202 plant-containing gut samples in diverse forms. Naturally, abundant riparian families displayed the highest frequencies, such as Salicaceae (58.2%) and Betulaceae (45.7%). Still, a large diversity of plant taxa was observed, comprising a total of 55 families, an unprecedented result in the analysis of crayfish diet. Plants, probably in the form of detritus, are, thus, an important part in the diet of these invaders.

Nonetheless, the high frequencies observed for Animals (83.2%) and Algae (91.3%) help illustrate the generalist nature of the two crayfish species' feeding habits. Again, the verified occurrence values are greater than those of previous studies, where the highest rates reported were closer to 60-70% for animals and 80% for algae (e.g. Gutiérrez-Yurrita et al., 1998; Alcorlo et al., 2004). Besides being vastly occurring, these groups were considerably diverse too. Displaying counts of 65 and 88 unique detections respectively, animals and algae represented individually about a fourth and a third of all taxa identified.

Detections of animal origin were primarily comprised by insects, occurring in 72.6% of all samples, mainly due to a strong Diptera prevalence (63.9%). Although such values comprehend a considerable number of unidentified insects, families with aquatic larvae like Caenidae (Order: Ephemeroptera) and Chironomidae (Order: Diptera) were recognised in 27.9% and 13.0% of all samples, respectively. Previous research had found similar patterns (e.g. Chucholl, 2013), strengthening the notion of an important trophic link between crayfish and sediment-dwelling insects. In addition, zooplanktonic taxa occurred in varying proportions, ranging anywhere from 1-40%, implying different roles as food

items. In that sense, the two most prevalent classes were Hexanauplia (Arthropoda) and Monogonta (Rotifera), respectively occurring in 43.27% and 27.40% of all samples. Along with the observations of larger animal taxa like annelids (16.8%) and amphibians (7.21%), the image of crayfish as powerful, opportunistic feeders is reinforced. Finally, even though the two crayfish species are known predators of local freshwater mussels (Meira et al., 2019; Sousa et al., 2019), no molluscs were detected in this assessment.

Regarding the Algae group, three classes were dominant, each appearing in more than half of all samples: Chlorophyceae (83.6%), Phaeophyceae (62.5%), Eustigmatophyceae (50.5%). Algae have been given little attention in past diet analyses, (e.g. Pérez-Bote, 2004; Barradas et al., 2006), perhaps due to the difficulties related to processing the numerous members of an extremely diverse and site-dependent group. The present study, however, was able to identify 88 unique taxa from 6 Phyla, 18 Classes, 34 Orders and 30 Families. Careful examination of these results is recommended, though: as algae DNA is highly abundant in the water, and most preyed animals are also grazers, consumption analysis is prone to confounding effects such as contamination and secondary predation.

Some of the functional groups occurred at a scale that is divergent from past research. Factors responsible for that deviation might be technical, as, for instance, the ability to identify even the smallest organisms and fragments. Likewise, the greater taxonomic resolution provided by DNA metabarcoding, in comparison to morphological or isotopic assessments, can help explain the larger diversity and higher frequencies reported here. However, detection of cannibalism is not possible through the use of such broad molecular markers, leaving that diet component unaccounted for. Another important aspect to consider is the role played by the particularities of each location or habitat. Past studies were undertaken in a variety of locations, such as Iberian rice fields (Correia, 2002; Alcorlo et al., 2004), temporary marshes and ponds (Gutiérrez-Yurrita et al., 1998; Carreira et al., 2014), lowland rivers (Guan & Wiles, 1998) or central European lakes (Chucholl, 2013; Jackson et al., 2017). As diversity and availability of food items varies, so will diet composition, a phenomenon intensified by seasonality.

Overall, the data retrieved portray *Procambarus clarkii* and *Pacifastacus leniusculus* as omnivorous consumers, able to feed on most organic tissues. From a functional perspective, crayfish are pictured both as primary and secondary consumers, as well as detritivores. Capable of grazing the benthic autotrophs, while also preying on different levels of heterotrophs, connectance within the trophic web is increased and diversity is lost (Chucholl, 2013). Important habitat elements can also be compromised, as is the case for the macrophyte cover that functions as refuge (Carreira et al., 2014), or the accumulated organic matter that represents an important energy reserve during drought season (Geiger et al., 2005).

3.4.2. Species comparison

Few studies in the past established direct connections between *Procambarus clarkii* and *Pacifastacus leniusculus* (e.g. Nakata et al., 2005; Bernardo et al., 2011; Filipe et al., 2017), with the work of Meira et al. (2019) being the only instance where the trophic component was the main focus. The present assessment of both species' diet composition using DNA metabarcoding allowed for a side-by-side analysis of the vast range of items consumed by the two invaders.

While 54.7% of the taxa detected within the functional groups was shared between crayfish species, diversity for *P. leniusculus* (203 taxa) was slightly higher than for *P. clarkii* (193 taxa), even though the latter counted with 10 extra specimens. Plant taxa were the main contributor to this difference, with a total of 7 taxa separating both species. This effect might be related to habitat composition, since the presence of the two crayfish varied along the watershed and half of all samples were collected in allopatry. Glancing at the effects of coexistence, it is relevant to note the rise in diet richness when comparing the combined allopatric-exclusive detections (45) to those obtained only in sympatry (70). Such an observation might indicate that *P. clarkii* and *P. leniusculus* broaden their diet spectrum when in sympatry. Interestingly, the signal crayfish was the main contributor to the total allopatric-exclusive detections (41 taxa), with more than eight times as many items as the red swamp crayfish (5 taxa). The explanation of such disparity might reside in site-related differences, such as habitat characteristics, or in a naturally broader range in the diet of *P. leniusculus*. Still, the proportion of taxa belonging to each of the three functional groups, was generally similar between the two crayfish species. The same proximity was observed in regards to frequency of occurrence, suggesting the two invaders display similar foraging behaviours. Such an assumption is coherent with the idea of freshwater crayfish as opportunistic consumers.

While most statistic tests revealed little effect from crayfish species as a factor differentiating the two diets, some significant cases were found. PERMANOVA results indicated that the set of consumed plants experienced a significant effect from the interaction between the factors species and size class. Although the same PERMANOVA result was achieved using either the full taxonomy or restricting data to the family level, GLM tests did not find significance. This disparity might be related to the different patterns of dietary diversity observed along size classes, when comparing both crayfish species. While *P. clarkii* displayed only a slight increase in the number of plant taxa as size class augmented, *P. leniusculus* experienced a sharp, unpredicted decrease. It is unclear if such observation results from small sample size, habitat differences or a well-defined trophic behaviour.

In general, results from the ANOSIM tests were also unsuccessful in finding differentiation between crayfish species, based on the three main functional groups. However, as zooplanktonic taxa were removed from the animal set, a slightly significant difference arose between crayfish species. The removal of zooplankton was performed based on the idea that, given the small dimensions of those organisms, they might be preyed upon less actively, being ingested in a more passive way. Dwelling and feeding where they find phytoplankton, these minuscule creatures are, perhaps, consumed accidentally, as crayfish graze the periphyton. The remaining animal prey are commonly more mobile, requiring an energy expense from foraging, if preyed alive. Thus, differences in trophic behaviour might become more prominent when isolating such prey. Further exploration of the significant effect detected using SIMPER allowed to look at the taxa contributing more to the differentiation between *P. clarkii* and *P. leniusculus* (Table 11). The top eight taxa, jointly contributing to 70% of the differentiation, comprises 6 insects, 1 annelid and 1 amphibian. However, the three main individual contributors are exclusively composed of members of Class Insecta, with all unassigned insects coming first (contributing 15.47%), followed by the unassigned Diptera (contributing 14.35%), and finally the mayfly *Caenis luctuosa* (Burmeister, 1839) (contributing 10,44%). This result must be taken cautiously, though, as only the first element of that list displayed a considerable difference between crayfish species, in terms of frequency of occurrence. This detection occurred in 39 more samples of *P. clarkii* (74) than of *P. leniusculus* (35), while the other two elements have discrepancies of only a couple of samples. The larger number of insect detections on *P. clarkii* samples might be related to the type of habitat exclusive to this crayfish species. The generally slower water flow and higher temperatures might present better conditions for insects to thrive. Further research, combining habitat characteristics with prey occurrence might be able to provide the necessary answers.

The individual GLM tests per taxa were able to identify effects from crayfish species on 7 instances (Table 9). Although these comprise taxa from all the functional groups, particular interest is found in the effects on Diptera, *Eiseniella tetraedra* (Savigny, 1826), and *Alnus serrulata* (Aiton) Willd., due the great effect they present. All three taxa displayed a frequency in *P. clarkii* that is at least triple that of *P. leniusculus.* Differences in habitat type could help explain some of the differences, although for species like *Alnus sp.*, present in most of the margins along the river extension, some other mechanism might be responsible.

To conclude, although the diets of the two crayfish species appear to be mostly similar in composition, the detection of slight differences in frequency of occurrence asks for a more thorough investigation, accounting for biotic and abiotic variables such as habitat composition, stream size and water flow or temperature.

3.4.3. Ontogenetic shift

The existence of a shift in diet as crayfish grow in size has for long been discussed (Guan & Wiles, 1998; Gutiérrez-Yurrita et al., 1998; Correia, 2002; Alcorlo et al., 2004; Pérez-Bote, 2004; Stenroth et al., 2006; Usio et al., 2009; Alcorlo & Baltanás, 2013; Chucholl, 2013). While certain authors defend that young and smaller crayfish consume animal prey more frequently and diversely (e.g. Guan & Wiles, 1998; Correia, 2002; Chucholl, 2013), others report that adults are not as different (e.g. Stenroth et al., 2006; Usio et al., 2009; Alcorlo & Baltanás, 2013). In general, the results of morphological analyses of gut contents have been favourable of such a shift, contrarily to research using isotopic signatures. Thus, the results obtained through DNA metabarcoding and presented here offer an additional viewpoint for ecologists to consider.

In this assessment, a reduction in diversity of animal taxa was, indeed, observed as crayfish size increased for both crayfish species, although only accompanied by a decrease in frequency of occurrence for *P. clarkii*. Diversity within the other two functional groups, however, did not exhibit a clear trend as crayfish size varied. Moreover, frequency of occurrence oscillated erratically for plants and algae, often differing only for medium-sized crayfish. Between crayfish species, both occurrence and diversity values were similar among the same size classes, supporting the observations from the previous section. A single exception was the interspecific comparison between small individuals in regards to diversity of plant taxa. small-sized *P. leniusculus* consumed a total of 53 unique taxa, compared to the 37 taxa ingested by *P. clarkii*. No broader tendencies were deduced from these numbers, implying that no clear differences in taxa occurrence and diversity exist between size classes.

In addition, all statistical tests directed at the three main functional groups failed to find significant differentiation among size classes. Only by applying individual GLM tests to all taxa did significant effects arise (Table 10). These, however, comprised only taxa from the Plants and Algae groups, contrarily to the expected effects on Animals based on the literature. Interestingly, the taxa with the most marked size-related effects were the Family Betulaceae and Genus *Alnus*, being much more frequent in small-sized red swamp specimens. Once again, these represent extremely common trees in the study area, meaning such a strong size-related effect can derive from a mechanism intrinsic to crayfish and not just habitat variation. If that was to be the case, though, expectations based on the general concept of ontogenetic shift would lead us to believe that such an increase in consumption would be inversely related to size. On the other hand, the fact that some locations only provided samples belonging to a particular size class might translate into a sampling bias that would explain the detected effect, if those locations were to have a markedly stronger Betulaceae presence.

3.4.4. Sex comparison

The concept of a sex-driven diet differentiation in *P. clarkii* and *P. leniusculus* has been seldom addressed in the literature. The few instances it is mentioned depict discordance, with Gutiérrez-Yurrita et al. (1998) reporting no differences and Pérez-Bote (2004) detecting variation in a year-round experiment. The latter claims that, during breeding season in spring, females see their mobility reduced in comparison to males, which, in turn, also benefit from having larger pincers used to forage larger agile prey. As such, gut samples from males are expected to display more animal content, while females should contain comparably more plants. Interestingly, the present observations fit with the hypothesis described, as males exhibit a slightly larger frequency of occurrence for animals, and the inverse being true for females and plants. Still, occurrence values are close, which is not the case in terms of taxonomic diversity. Females exhibited more diversity in regards to all groups, displaying up to 23 additional taxa when compared to male crayfish. With most of those taxa belonging to Algae and Plants, the idea of sex-driven diet differentiation is supported. Nonetheless, it is important to remember that the present study took place after the typical breeding period, so differences between sexes might not be related to that life-history event.

Although testing the effects of sex was not the main focus of this study, some significant cases were detected through the individual GLM tests (Table 11). Large effects were detected for the Genus *Alnus*, Family Chironomidae and the Algae *Fasciculochloris boldii* and *Pseudocharacipsis*. Among these, the chironomids are the main case to relate with the hypothesis of sex-driven diet differentiation, as an instance of animal prey occurring more frequently in male crayfish.

3.4.5. Sampling effort

Perhaps due to the high levels of rainfall verified during the first semester of 2018, the number of crayfish samples collected was much lower than the expectations based on previous years. The larger volume of water running off and strong flow rate caused the waterlines across the Sabor basin to spend the summer inundated by sediments in suspension. In some locations, electrofishing was, thus, compromised by the lack of visibility. This resulted in certain sites not reaching the expected minimum number of specimens, although others provided an excess in crayfish. While that proved a constraint during the selection of the final samples, an attempt was made to maximize spatial representability by including samples from the largest number of sites without creating imbalances in the setup.

3.4.6. Shortcomings of the approach

While the benefits from assessing the diet of freshwater crayfish using DNA metabarcoding techniques have been previously discussed, their integration in the present approach did not come without drawbacks.

First, this DNA-based method is not able to distinguish between cannibalistic consumption and the predator's own tissue. As such, an apparently important component in the diet of crayfish (e.g. Guan & Wiles, 1998; Pérez-Bote, 2004) was ignored. This not only makes the numerous detections of crayfish DNA uninformative, but also prejudicial, as read counts for other taxa are reduced. Interspecific predation between crayfish species was, too, excluded from the analysis, as most detections were likely from lab contamination. Additionally, the high read counts for crayfish DNA indicate that the blocking primers were not as successful as suggested by the pilot tests. A second DNA-related limitation can be found in the lack of vertebrate-specific markers, which might have caused an underestimation of the occurrence of groups such as fish and amphibians. Still, it must be noted that marker bias is an intrinsic characteristic of DNA metabarcoding assessments that should always be accounted for while interpreting results. Similarly, the quality of DNA databases is deeply connected to the accuracy of taxonomic assignments. As such, and in face of the high numbers of unassigned reads, efforts must be made to develop custom databases more capable of taxonomic identification.

Furthermore, there are also shortcomings related to the experimental design. The role of detritus as a diet component might have not been entirely addressed due to the exclusion of taxa such as fungi and bacteria. Since these groups are extremely diverse, not fully present in the taxonomic databases and frequent as contaminants, their exclusion meant to reduce the weight of low-confidence assignments on the analyses. To add to this, it was not possible to separate the status at which groups such as amphibians and fish were consumed. Thus, the detrital component could be much more prevalent, combining organisms from a vast range of taxonomic and functional groups.

Finally, the reduced temporal window in which sampling took place could mean the present results are not representative of the entire dietary diversity. As seasons change, so do important environmental variables like precipitation and temperature, factors likely affecting the availability of food items. In the same note, the life-cycle of crayfish was not fully sampled, although the focused season is the one during which their activity is highest.

Some of these limitations could be compensated by a previous step of morphological analysis of gut contents. Quantitative information, as well as taxonomic confirmation could be gained from performing and comparing the two approaches. However, given the required time and expertise to undertake such a laborious task, the procedure was not included.

3.4.7. Final remarks

The present study provided a first glimpse into the diverse taxonomy within the diet of the red swamp *Procambarus clarkii* and signal crayfish *Pacifastacus leniusculus*. Although no considerable differences were found in the items consumed by the two invaders, the vast range of taxa identified indicate the predators play a significant role in the ecosystem. As expected from freshwater crayfish, *P. clarkii* and *P. leniusculus* were shown to interact with many levels of the trophic network, which implies a serious disruption of the invaded systems. Besides the great taxonomic diversity affected, the detection of sensible taxa such as Ephemeroptera and Trichoptera (bioindicators of good water quality), as well as of the Endangered mussel *Potomida litorallis* (Cuvier, 1798) are worrying. Adding to the high occurrence of organisms with important ecosystem functions (e.g. Chironomidae, here largely consumed by male *P. clarkii*), the need for monitoring and management of the two crayfish species is stressed, to avoid further deterioration of native systems.

The present results suggest both invaders should be managed with equal priority, and at all life-stages. This is a critical consideration, since the signal crayfish was until recently being introduced as a potential barrier to the spread of the red swamp crayfish (cited in Alonso & Martínez, 2006), but is here depicted as being at least as influential as its counterpart. Efforts should also be applied to avoid the invasion of pristine ecosystems, where a delicate trophic balance may exist, since both *P. clarkii and P. leniusculus* display traits and habits prone to cause irreversible change.

Chapter 4: References

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Chapter 5: Supplementary Material

5.1. Supplemetary Tables

Table S1 – Summary of agarose gel results after PCR, for the marker comparison and selection, in the context of the Pilot Study.

Target group	PLANTS				VERTEBRATES		EUKARYOTES/EPTO		EUK/MACROALGAE		EPTO	
Primer pair	gh F/R (trnL)		UniPlant (ITS2)		12SV5.1 (12S)		Leray-XT (COI)		Round1-18Sf/r-msq (18S)		FwhF2/R2n (COI)	
Sample	Band	Quality	Band	Quality	Band	Quality	Band	Quality	Band	Quality	Band	Quality
A-EZNA	Present	Good	Absent	Bad	Present	Bad	Absent	Bad	Absent	Bad	Absent	Bad
A-ZYMO	Present	Good	Present	Bad	Present	Good	Present	Good	Present	Bad	Absent	Bad
B-EZNA	Present	Good	Absent	Bad	Present	Bad	Absent	Bad	Absent	Bad	Absent	Bad
B-ZYMO	Present	Good	Present	Bad	Present	Good	Present	Good	Present	Bad	Absent	Bad
C-EZNA	Present	Bad	Absent	Bad	Present	Bad	Present	Bad	Absent	Bad	Absent	Bad
C-ZYMO	Present	Bad	Absent	Bad	Present	Good	Absent	Bad	Present	Good	Absent	Bad
D-EZNA	Present	Good	Absent	Bad	Present	Good	Present	Good	Absent	Bad	Absent	Bad
D-ZYMO	Present	Good	Absent	Bad	Present	Bad	Present	Good	Present	Good	Absent	Bad
E-EZNA	Present	Good	Absent	Bad	Present	Bad	Absent	Bad	Absent	Bad	Absent	Bad
E-ZYMO	Present	Good	Present	Bad	Present	Bad	Present	Good	Present	Good	Absent	Bad
F-EZNA	Present	Good	Present	Bad	Present	Bad	Present	Bad	Absent	Bad	Absent	Bad
F-ZYMO	Present	Good	Present	Bad	Present	Bad	Present	Good	Present	Good	Absent	Bad
G-EZNA	Present	Good	Absent	Bad	Absent	Bad	Absent	Bad	Absent	Bad	Absent	Bad
G-ZYMO	Present	Good	Absent	Bad	Present	Bad	Present	Good	Present	Good	Present	Good
H-EZNA	Present	Good	Present	Bad	Present	Bad	Present	Good	Absent	Bad	Present	Good
H-ZYMO	Present	Good	Absent	Bad	Present	Bad	Present	Good	Present	Good	Present	Good
Extraction kit	Bands	Good	Bands	Good	Bands	Good	Bands	Good	Bands	Good	Bands	Good
EZNA	8	7	2	0	7	1	4	2	0	0	1	1
ZYMO	8	7	4	0	8	3	7	7	8	6	2	2

Table S2 – Taxonomic resolution displayed by the primer sets tested during the Pilot Study, using both extraction kits.

	12SV5.1 F/R		SSU3' F/R		LERAY-XT	
Extraction Kit	**EZN**	**ZYM**	**EZN**	**ZYM**	**EZN**	**ZYM**
Order	7	6	0	64	10	32
Family	7	7	0	56	7	46
Genus	9	7	0	63	8	66
Species	5	5	0	42	8	61

Table S3 – List of sites visited for the collection of crayfish samples.

Site	Latitude	Longitude	*P. clarkii*	*P. leniusculus*
A-SABOR-01	41.794358	-6.703803	0	37
A-SABOR-02	41.791792	-6.688394	0	46
A-SABOR-03	41.774242	-6.678625	0	43
A-SABOR-04	41.741961	-6.67355	1	40
A-SABOR-05	41.563311	-6.672347	7	0
A-SABOR-06	41.552197	-6.667769	10	18
A-SABOR-07	41.456011	-6.670094	17	6
A-SABOR-08	41.450428	-6.679769	9	2
A-SABOR-09	41.444881	-6.684186	8	6
ANGUEIRA_02	41.570842	-6.4994170	41	4
ANGUEIRA_04	41.450694	-6.5723812	32	0
ANGUEIRA_05	41.476381	-6.5255436	7	0
ANGUEIRA_06	41.517059	-6.5236584	6	0
AZIBO_03	41.470151	-6.8508093	0	0
AZIBO_06	41.514129	-6.8871848	0	0
BACAL_03	41.894447	-6.6914494	0	0
IGREJAS_03	41.881346	-6.6724223	0	6
INFERNO_01	41.222338	-6.8485439	9	0
MACAS_01	41.592027	-6.5619675	2	5
MACAS_03	41.513703	-6.5894988	10	6
MACAS_07	41.677867	-6.5654319	14	10
MACAS_09	41.769596	-6.5536158	3	29
MACAS_11	41.843267	-6.5267581	0	4
MACAS_12	41.926328	-6.5497060	0	0
MACAS_13	41.461667	-6.651856	0	1
ONOR_01	41.842100	-6.6756525	0	0
PONTE_01	41.23074	-6.776373	0	41
SABOR_01	41.558197	-6.6743835	0	0
SABOR_12	41.431609	-6.7089003	0	0
SABOR_15	41.488745	-6.6590081	0	0
SABOR_19	41.665447	-6.6435618	13	0
SABOR_20	41.723545	-6.6633425	2	1
SABOR_22	41.794469	-6.6999407	0	20
SABOR_23	41.839562	-6.7439223	0	21
SABOR_25	41.908577	-6.7559123	0	0
VALE-MOINHOS_01	41.482986	-6.7377040	2	0
VILARICA_03	41.208896	-7.0979753	21	0
VILARICA_06	41.265591	-7.0895379	30	0
VILARICA_08	41.333701	-7.0582233	6	0
ZACARIAS_03	41.341584	-6.9212177	0	0
		TOTAL	250	346

Table S4 – List of all taxa detected in gut samples. On the right, number of samples in which the taxa occurred (N) and frequency of occurrence (%).

Phylum	Class	Order	Family	Genus	Species	N	%
Amoebozoa						69	33,17
	Corycida					1	0,48
	Unknown Class						
		Himatismenida				6	2,88
			Cochliopodiidae			6	2,88
				Cochliopodium		6	2,88
		Longamoebia				14	6,73
			Acanthamoebidae			12	5,77
				Acanthamoeba		2	0,96
					Acanthamoeba_hatchetti	1	0,48
				Protacanthamoeba		9	4,33
					Protacanthamoeba_bohemica	8	3,85
		Physariida				41	19,71
			Didymiaceae			10	4,81
		Plasmodiophorida	Plasmodiophoridae	Polymyxa		11	5,29
					Polymyxa_graminis	11	5,29
		Unknown Order	Alphamonaceae	Alphamonas	Alphamonas_edax	2	0,96
	Variosea	Unknown Order	Unknown Family			17	8,17
				Filamoeba	Filamoeba_nolandi	2	0,96
				Ischnamoeba	Ischnamoeba_montana	17	8,17
Annelida	Clitellata					35	16,83
		Enchytraeida				1	0,48
		Haplotaxida				34	16,35
			Lumbricidae			33	15,87
				Aporrectodea		1	0,48
				Eiseniella	Eiseniella_tetraedra	32	15,38
			Naididae	Nais		1	0,48
Aphelidiomycota	Aphelidiomycetes					1	0,48
Apicomplexa	Conoidasida	Eucoccidiorida	Adeleidae	Adelina		1	0,48
Arthropoda						167	80,29
	Arachnida					7	3,37
		Araneae				4	1,92
	Branchiopoda	Diplostraca				45	21,63
			Daphniidae	Daphnia		13	6,25
			Macrotrichidae			9	4,33
				Macrothrix		4	1,92
	Hexanauplia					89	42,79
		Cyclopoida				83	39,90
			Cyclopidae	Acanthocyclops	Acanthocyclops_americanus	1	0,48
	Insecta					151	72,60
		Coleoptera				6	2,88
			Scarabaeidae			1	0,48
		Diptera				133	63,94
			Ceratopogonidae	Culicoides	Culicoides_imicola	1	0,48
			Chironomidae			21	10,10
				Corynoneura	Corynoneura_scutellata	3	1,44
				Orthocladius	Orthocladius_fuscimanus	12	5,77
				Polypedilum		1	0,48
				Rheotanytarsus	Rheotanytarsus_pentapoda	13	6,25
				Tanytarsus		5	2,40
			Culicidae			1	0,48
			Empididae	Hemerodromia	Hemerodromia_baetica	1	0,48
			Simuliidae			14	6,73
				Simulium		12	5,77
					Simulium_intermedium	3	1,44
					Simulium_velutinum	6	2,88
		Ephemeroptera				60	28,85
			Baetidae			1	0,48
			Caenidae	Caenis	Caenis_luctuosa	58	27,88
		Hemiptera				22	10,58
		Hymenoptera				11	5,29
			Formicidae	Lasius		1	0,48
		Lepidoptera				10	4,81
			Geometridae	Neognopharmia	Neognopharmia_stevenaria	2	0,96
			Noctuidae	Agrotis	Agrotis_catalaunensis	1	0,48
		Trichoptera				8	3,85
			Limnephilidae	Micropterna	Micropterna_fissa	4	1,92
	Ostracoda					4	1,92
		Podocopida	Cyprididae	Cypridopsis		3	1,44
Ascomycota						101	48,56
	Dothideomycetes					10	4,81
		Capnodiales				6	2,88
		Pleosporales				3	1,44
			Leptosphaeriaceae			1	0,48
			Phaeosphaeriaceae			1	0,48
			Pleosporaceae			1	0,48
	Eurotiomycetes					49	23,56
		Chaetothyriales				15	7,21
			Herpotrichiellaceae			1	0,48
		Eurotiales	Aspergillaceae			36	17,31
				Aspergillus		2	0,96
				Penicillium		27	12,98
					Penicillium_glabrum	23	11,06
			Trichocomaceae	Talaromyces		3	1,44
	Lecanoromycetes	Ostropales				3	1,44
	Leotiomycetes					21	10,10
		Helotiales				2	0,96
			Sclerotiniaceae			1	0,48
	Pezizomycetes	Pezizales	Sarcosomataceae			1	0,48
	Saccharomycetes	Saccharomycetales				14	6,73
			Debaryomycetaceae	Kurtzmaniella	Candida_zeylanoides	1	0,48
			Dipodascaceae	Geotrichum	Geotrichum_candidum	12	5,77
			Phaffomycetaceae			1	0,48
	Sordariomycetes					30	14,42
		Glomerellales				2	0,96
		Hypocreales				12	5,77
			Nectriaceae			2	0,96
				Calonectria	Calonectria_colhounii	1	0,48
				Fusarium		1	0,48
				Emericellopsis	Emericellopsis_minima	3	1,44
		Sordariales	Chaetomiaceae			15	7,21
Bacillariophyta						52	25,00
	Bacillariophyceae					2	0,96
		Bacillariales				3	1,44
			Bacillariaceae			1	0,48
		Cymbellales				21	10,10
			Gomphonemataceae	Gomphonema	Gomphonema_parvulum	7	3,37
		Naviculales				3	1,44
			Sellaphoraceae	Sellaphora		1	0,48
	Coscinodiscophyceae					4	1,92
		Melosirales	Melosiraceae	Melosira	Melosira_varians	2	0,96
		Thalassiosirales	Thalassiosiraceae	Thalassiosira	Thalassiosira_tenera	2	0,96
	Fragilariophyceae					26	12,50

		Fragilariales				2	0,96
		Licmophorales				24	11,54
Basidiomycota						36	17,31
	Agaricomycetes					7	3,37
		Agaricales	Psathyrellaceae			1	0,48
		Thelephorales				5	2,40
			Thelephoraceae			2	0,96
	Cystobasidiomycetes	Unknown Order	Unknown Family	Buckleyzyma	Buckleyzyma_aurantiaca	2	0,96
	Malasseziomycetes	Malasseziales	Malasseziaceae	Malassezia	Malassezia_globosa	1	0,48
	Microbotryomycetes					19	9,13
		Sporidiobolales				16	7,69
			Sporidiobolaceae			2	0,96
				Rhodotorula	Rhodotorula_mucilaginosa	1	0,48
	Pucciniomycetes	Pucciniales	Melampsoraceae	Melampsora		1	0,48
	Tremellomycetes					12	5,77
		Cystofilobasidiales	Mrakiaceae			2	0,96
		Tremellales				5	2,40
Blastocladiomycota						4	1,92
	Blastocladiomycetes	Blastocladiales				3	1,44
			Blastocladiaceae	Blastocladiella		1	0,48
			Catenariaceae			1	0,48
	Physodermatomycetes	Physodermatales	Physodermataceae	Paraphysoderma		1	0,48
Bryophyta	Bryopsida					2	0,96
Cercozoa	Unknown Class					84	40,38
		Cercomonadida				21	10,10
			Cercomonadidae			20	9,62
				Cercomonas		14	6,73
					Cercomonas_celer	5	2,40
				Eocercomonas	Eocercomonas_exploratorii	3	1,44
				Neocercomonas	Neocercomonas_tuberculata	1	0,48
		Thaumatomonadida	Thaumatomastigidae			11	5,29
		Unknown Order	Unknown Family	Kraken	Kraken_carinae	2	0,96
Chlorophyta						178	85,58
	Chlorodendrophyceae	Chlorodendrales				1	0,48
	Chlorophyceae					174	83,65
		Chaetophorales	Chaetophoraceae			5	2,40
		Chlamydomonadales				121	58,17
			Chlamydomonadaceae			7	3,37
				Chlamydomonas		6	2,88
			Chlorococcaceae	Fasciculochloris	Fasciculochloris_boldii	15	7,21
			Dunaliellaceae	Hafniomonas		7	3,37
			Volvocaceae	Lobomonas	Lobomonas_monstruosa	5	2,40
		Oedogoniales	Unknown Family	Oedogonium		12	5,77
					Oedogonium_angustistomum	6	2,88
					Oedogonium_cardiacum	2	0,96
		Protosiphonales	Protosiphonaceae			8	3,85
				Protosiphon		8	3,85
		Sphaeropleales				156	75,00
			Bracteacoccaceae	Bracteacoccus		1	0,48
			Hydrodictyaceae			8	3,85
			Mychonastaceae			68	32,69
				Mychonastes		48	23,08
					Mychonastes_homosphaera	2	0,96
			Neochloridaceae			80	38,46
				Botryosphaerella	Botryosphaerella_sudetica	9	4,33
				Echinosphaeridium	Echinosphaeridium_nordstedtii	15	7,21
				Neochloris		72	34,62
			Pseudomuriellaceae	Pseudomuriella		1	0,48
			Scenedesmaceae			89	42,79
				Desmodesmus		74	35,58
				Scenedesmus		33	15,87
					Scenedesmus_acutus	1	0,48
				Tetradesmus	Tetradesmus_obliquus	1	0,48
			Sphaeropleaceae	Tetraedron	Tetraedron_minimum	17	8,17
		Tetrasporales		Paulschulzia		5	2,40
	Nephroselmidophyceae					4	1,92
	Pyramimonadophyceae	Pyramimonadales				3	1,44
	Trebouxiophyceae					87	41,83
		Chlorellales				85	40,87
			Chlorellaceae			78	37,50
				Pseudochlorella	Pseudochlorella_subsphaerica	1	0,48
			Oocystaceae	Tetrastrum	Tetrastrum_staurogeniiforme	2	0,96
		Trebouxiales	Trebouxiaceae	Trebouxia		5	2,40
					Trebouxia_arboricola	3	1,44
	Ulvophyceae	Scotinosphaerales	Scotinosphaeraceae	Scotinosphaera		2	0,96
Chordata						40	19,23
	Actinopteri					3	1,44
		Cypriniformes	Cyprinidae	Rutilus	Rutilus_rutilus	1	0,48
		Salmoniformes	Salmonidae	Salmo		1	0,48
	Amphibia					15	7,21
		Anura				14	6,73
			Hylidae	Hyla	Hyla_meridionalis	6	2,88
			Pelobatidae	Pelobates	Pelobates_cultripes	8	3,85
		Caudata	Salamandridae	Lissotriton	Lissotriton_boscai	1	0,48
Ciliophora						5	2,40
	Colpodea					3	1,44
		Colpodida	Colpodidae	Colpoda	Colpoda_inflata	1	0,48
		Grossglockneriida	Grossglockneriidae			3	1,44
				Mykophagophrys	Mykophagophrys_terricola	2	0,96
				Pseudoplatyophrya	Pseudoplatyophrya_nana	1	0,48
	Litostomatea	Haptorida	Didiniidae			1	0,48
	Nassophorea	Nassulida	Nassulidae	Obertrumia	Obertrumia_georgiana	1	0,48
	Spirotrichea	Sporadotrichida	Gonostomatidae	Gonostomum		1	0,48
Cnidaria	Myxozoa	Bivalvulida	Myxobolidae	Myxobolus	Myxobolus_pavlovskii	2	0,96
Cryptophyta	Cryptophyceae					7	3,37
		Cryptomonadales				4	1,92
		Pyrenomonadales				3	1,44
Dinophyta	Dinophyceae					71	34,13
		Peridiniales				15	7,21
			Amphidiniopsidaceae			8	3,85
			Kryptoperidiniaceae	Unruhdinium		7	3,37
		Suessiales	Suessiaceae			15	7,21
				Asulcocephalium	Asulcocephalium_miricentonis	11	5,29
		Thoracosphaerales	Thoracosphaeraceae	Thoracosphaera	Thoracosphaera_heimii	22	10,58
Gastrotricha	Unknown Class	Chaetonotida				13	6,25
			Chaetonotidae			5	2,40
Magnoliophyta						202	97,12
	Liliopsida					68	32,69
		Asparagales	Amaryllidaceae	Allium	Allium_sativum	5	2,40
		Poales				54	25,96
			Cyperaceae	Carex		2	0,96
			Juncaceae	Juncus		3	1,44
			Poaceae			45	21,63
				Bromus	Bromus_ramosus	1	0,48
					Bromus_riparius	1	0,48
				Holcus		1	0,48
				Oldeania		1	0,48
				Paspalum		32	15,38
					Paspalum_dilatatum	5	2,40
			Typhaceae			8	3,85
				Sparganium		7	3,37
					Sparganium_americanum	2	0,96

		Zingiberales	Musaceae			1	0,48
	Magnoliopsida					195	93,75
		Apiales				26	12,50
			Apiaceae			18	8,65
				Lomatium	Lomatium_triternatum	3	1,44
			Araliaceae			9	4,33
				Hydrocotyle		2	0,96
		Asterales	Asteraceae			7	3,37
		Boraginales	Heliotropiaceae			1	0,48
		Brassicales	Brassicaceae			7	3,37
		Buxales	Buxaceae	Buxus		4	1,92
		Cornales	Grubbiaceae	Grubbia	Grubbia_rosmarinifolia	1	0,48
		Dipsacales	Caprifoliaceae	Lonicera		3	1,44
					Lonicera_chrysantha	1	0,48
		Ericales	Ebenaceae			21	10,10
			Ericaceae			3	1,44
			Primulaceae	Lysimachia		17	8,17
		Fabales	Fabaceae			11	5,29
				Lens		1	0,48
		Fagales				109	52,40
			Betulaceae	Alnus		95	45,67
					Alnus_serrulata	87	41,83
				Betula		95	45,67
			Fagaceae			20	9,62
				Quercus		6	2,88
			Juglandaceae			5	2,40
		Gentianales	Rubiaceae	Galium		5	2,40
		Lamiales				13	6,25
			Lamiaceae			1	0,48
			Pedaliaceae			2	0,96
			Plantaginaceae			6	2,88
				Callitriche		3	1,44
				Plantago		1	0,48
				Veronica		3	1,44
			Scrophulariaceae			4	1,92
				Verbascum		1	0,48
		Malpighiales				122	58,65
			Euphorbiaceae	Chrozophora		1	0,48
			Hypericaceae			1	0,48
			Salicaceae			121	58,17
				Populus		42	20,19
					Populus_alba	1	0,48
		Malvales	Cistaceae			13	6,25
				Cistus		4	1,92
				Fumana	Fumana_baetica	9	4,33
		Myrtales				9	4,33
			Lythraceae			7	3,37
				Lythrum	Lythrum_salicaria	2	0,96
				Pemphis	Pemphis_acidula	1	0,48
				Punica	Punica_granatum	1	0,48
			Myrtaceae			1	0,48
			Onagraceae			1	0,48
		Oxalidales	Oxalidaceae	Oxalis		1	0,48
		Ranunculales				16	7,69
			Berberidaceae	Epimedium	Epimedium_koreanum	1	0,48
			Papaveraceae	Papaver	Papaver_somniferum	2	0,96
			Ranunculaceae			14	6,73
		Rosales	Cannabaceae			39	18,75
			Moraceae			5	2,40
			Rhamnaceae			2	0,96
				Frangula		1	0,48
				Ziziphus	Ziziphus_jujuba	1	0,48
			Rosaceae			14	6,73
				Fragaria	Fragaria_viridis	1	0,48
				Prunus		4	1,92
				Rubus	Rubus_idaeus	1	0,48
			Ulmaceae			9	4,33
			Urticaceae	Parietaria	Parietaria_judaica	1	0,48
		Sapindales				7	3,37
			Anacardiaceae			5	2,40
			Sapindaceae	Acer		1	0,48
			Simaroubaceae	Ailanthus	Ailanthus_altissima	1	0,48
		Saxifragales	Crassulaceae	Sedum	Sedum_rupestre	1	0,48
			Haloragaceae	Myriophyllum	Myriophyllum_alterniflorum	1	0,48
		Solanales	Solanaceae			7	3,37
				Capsicum		2	0,96
		Vitales	Vitaceae			11	5,29
				Ampelocissus	Ampelocissus_tomentosa	1	0,48
				Cissus		2	0,96
Mollusca	Bivalvia	Pholadomyoida				2	0,96
Mucoromycota	Mucoromycetes	Mucorales				1	0,48
Nematoda	Chromadorea					18	8,65
		Rhabditida				17	8,17
		Strongylida				1	0,48
Ochrophyta						156	75,00
	Chrysophyceae					34	16,35
		Chromulinales				30	14,42
	Eustigmatophyceae					105	50,48
		Eustigmatales				39	18,75
			Monodopsidaceae	Nannochloropsis	Nannochloropsis_limnetica	4	1,92
			Pseudocharaciopsidaceae	Pseudocharaciopsis		32	15,38
			Unknown Family	Pseudellipsoidion		2	0,96
		Goniochloridales				59	28,37
			Unknown Family	Vacuoliviride	Vacuoliviride_crystalliferum	5	2,40
	Phaeophyceae					130	62,50
		Ectocarpales				117	56,25
		Fucales				12	5,77
		Laminariales				1	0,48
	Raphidophyceae	Chattonellales				4	1,92
	Synurophyceae	Synurales				15	7,21
Pinophyta	Pinopsida					12	5,77
		Cupressales	Cupressaceae	Platycladus	Platycladus_orientalis	4	1,92
		Pinales	Pinaceae	Pinus		9	4,33
Platyhelminthes						6	2,88
	Cestoda	Bothriocephalidea	Bothriocephalidae			1	0,48
	Monogenea	Dactylogyridea				4	1,92
			Ancyrocephalidae			1	0,48
			Dactylogyridae	Dactylogyrus	Dactylogyrus_falsiphallus	3	1,44
	Trematoda	Strigeidida				1	0,48
Pteridophyta	Polypodiopsida	Polypodiales	Athyriaceae	Athyrium		8	3,85
			Dennstaedtiaceae			1	0,48
			Dryopteridaceae			1	0,48
Rhodophyta						34	16,35
	Bangiophyceae	Bangiales				1	0,48
	Florideophyceae					33	15,87
		Batrachospermales				29	13,94
			Batrachospermaceae			14	6,73
				Batrachospermum	Batrachospermum_helminthosum	13	6,25
			Lemaneaceae	Lemanea		1	0,48
		Gigartinales				4	1,92
Rotifera						69	33,17
	Bdelloidea					20	9,62

		Philodinida				2	0,96
	Monogononta					57	27,40
		Flosculariaceae	Flosculariidae			2	0,96
				Beauchampia	Beauchampia_crucigera	1	0,48
				Floscularia	Floscularia_bifida	1	0,48
		Ploima				55	26,44
			Brachionidae			7	3,37
				Brachionus	Brachionus_calyciflorus	1	0,48
				Euchlanis	Euchlanis_dilatata	6	2,88
			Epiphanidae	Epiphanes	Epiphanes_senta	1	0,48
			Gastropidae	Ascomorpha	Ascomorpha_ovalis	1	0,48
			Lepadellidae	Lepadella	Lepadella_patella	1	0,48
Tardigrada	Eutardigrada	Parachela				5	2,40
			Hypsibiidae	Hypsibius		2	0,96
					Hypsibius_convergens	1	0,48
			Macrobiotidae	Dactylobiotus		3	1,44
					Dactylobiotus_parthenogeneticus	1	0,48
Unknown Phylum	Ichthyosporea					16	7,69
		Dermocystida	Unknown Family	Amphibiocystidium		1	0,48
		Ichthyophonida				15	7,21
			Unknown Family	Anurofeca		13	6,25
					Anurofeca_richardsi	4	1,92
			Unknown Family	Psorospermium		2	0,96
Unknown Phylum	Oomycetes					143	68,75
		Lagenidiales	Lagenidiaceae	Lagenidium		1	0,48
		Olpidiopsidales				1	0,48
		Peronosporales				68	32,69
			Peronosporaceae			67	32,21
				Phytophthora		2	0,96
					Phytophthora_amnicola	1	0,48
				Plasmopara		59	28,37
		Pythiales				80	38,46
			Pythiaceae			28	13,46
				Pythium		23	11,06
		Saprolegniales				15	7,21
			Saprolegniaceae	Aphanomyces		6	2,88
					Aphanomyces_astaci	5	2,40
					Aphanomyces_euteiches	1	0,48
Unknown Phylum	Unknown Class	Eccrinales				18	8,65
			Eccrinaceae	Arundinula		17	8,17
		Fonticulida				1	0,48
Zoopagomycota	Basidiobolomycetes	Basidiobolales				1	0,48

Table S5 – PERMANOVA table, assessing the effects of the interaction between species and size class, within the Plants functional group.

		Df	Sumsofsqs	Meansqs	F.model	R2	PR(>F)
FULL TAXONOMY	**Species**	1	1.865	1.86528	5.4785	0.02623	0.18741
	SizeClass	2	1.126	0.56324	1.6543	0.01584	0.36332
	Species:SizeClass	2	1.389	0.69457	2.0400	0.01953	0.04998 *
	Residuals	196	66.733	0.34048		0.93840	
	Total	201	71.114			1.00000	
		Df	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
FAMILY LEVEL	**Species**	1	1.775	1.77477	5.9667	0.02849	0.186407
	SizeClass	2	0.914	0.45692	1.5362	0.01467	0.440780
	Species:SizeClass	2	1.594	0.79725	2.6803	0.02560	0.008996 **
	Residuals	195	58.002	0.29744		0.93123	
	Total	200	62.285			1.00000	

5.2. Supplementary Figures

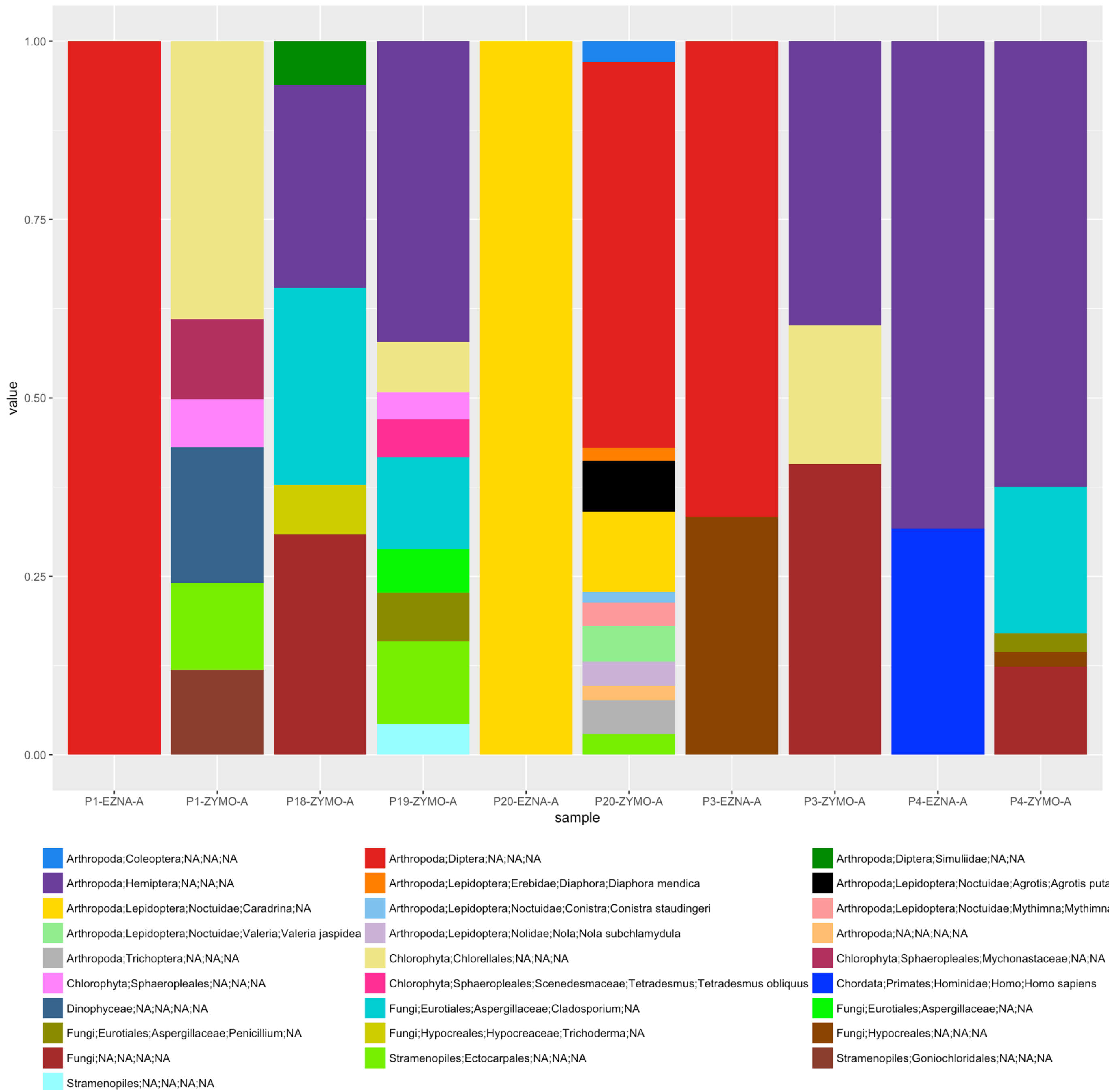

Figure S1 – Sequencing results from amplification of crayfish gut contents using the primer set Leray-XT (COI), during the Pilot Study.

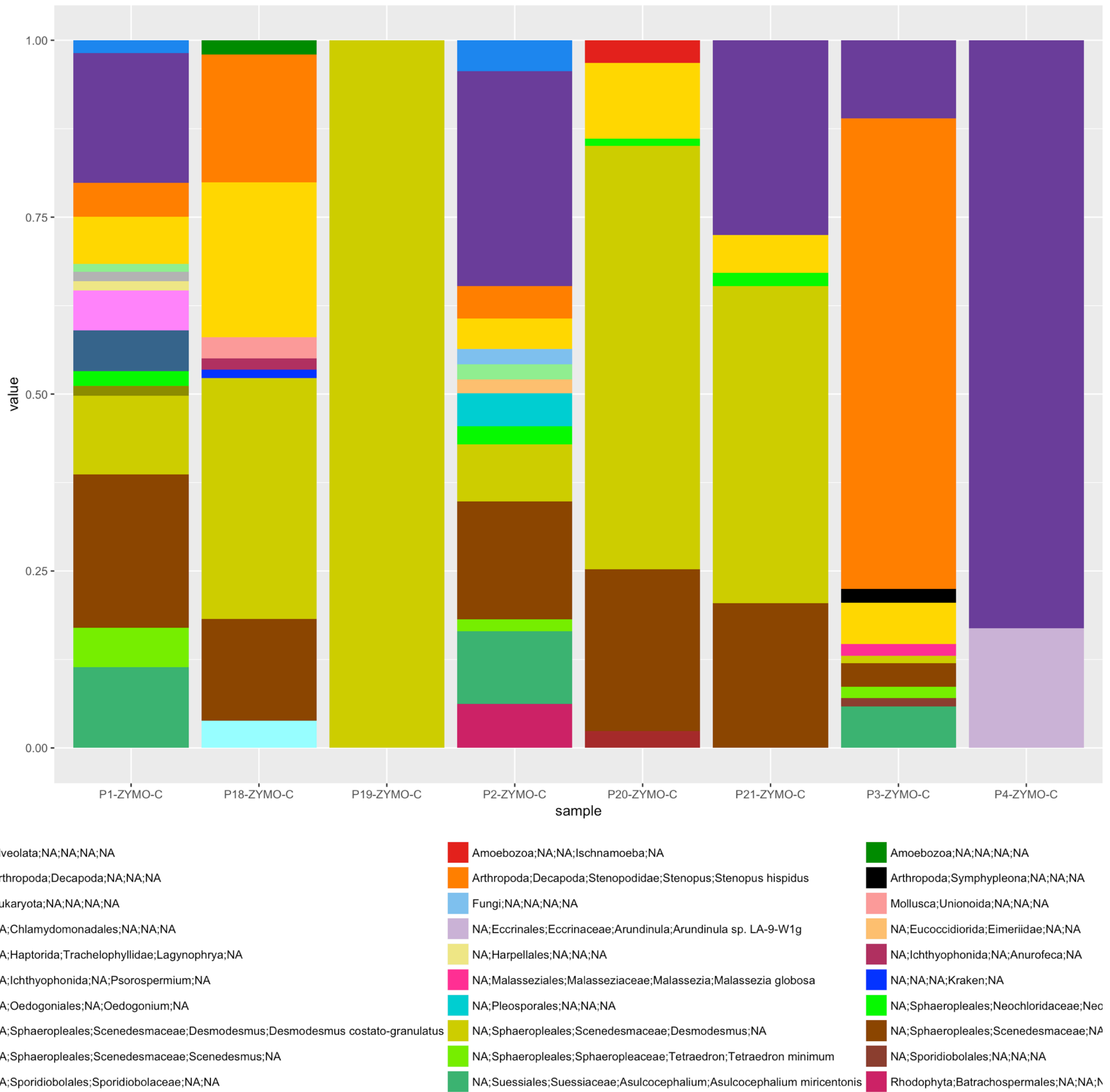

Figure S2 – Sequencing results from amplification of crayfish gut contents using the primer set SSU3' F/R (18S) , during the Pilot Study.

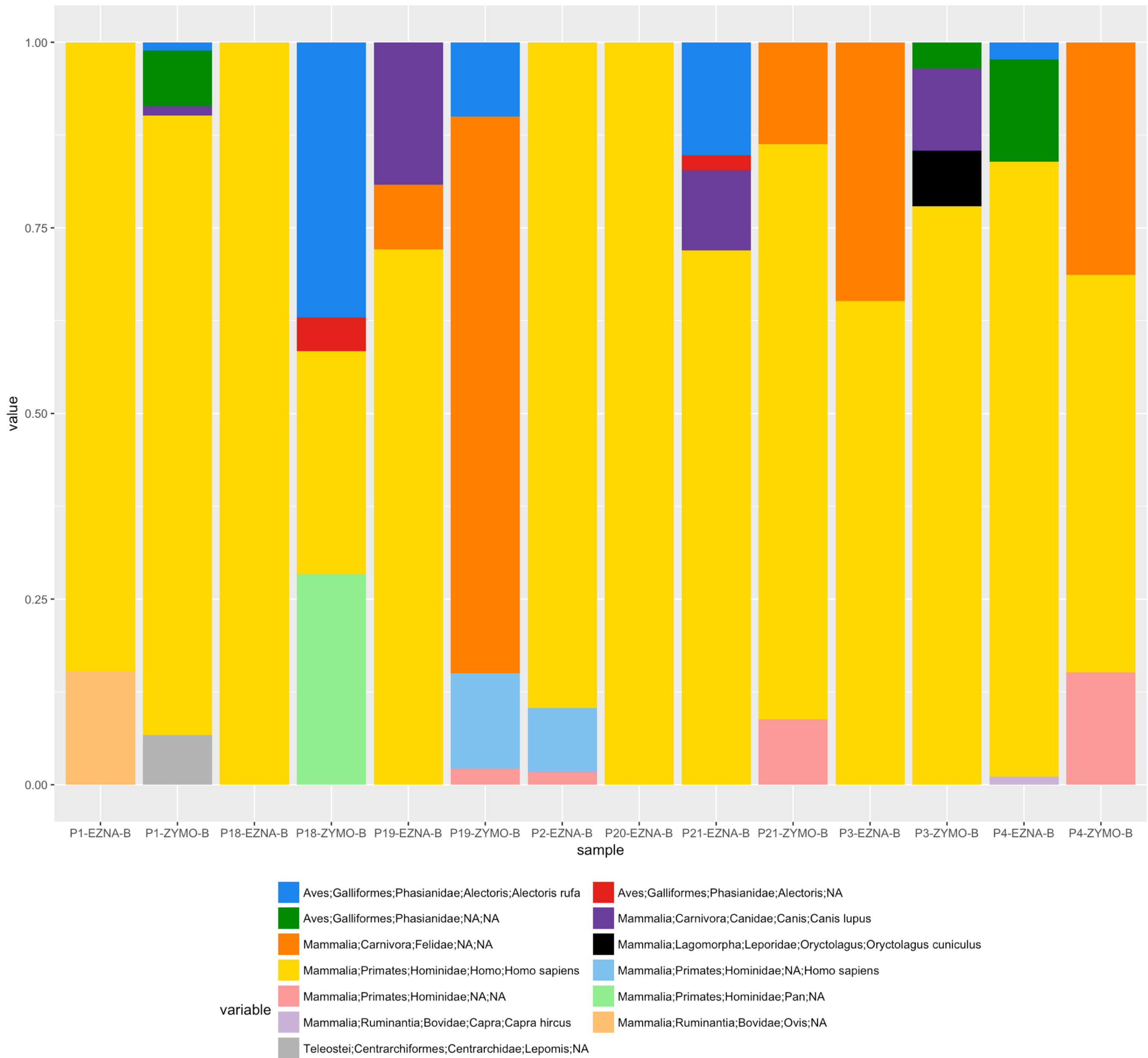

Figure S3 – Sequencing results from amplification of crayfish gut contents using the primer set 12SV5.1 (12S), during the Pilot Study.