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Fourier Transform Infra-red spectroscopy of Crustaceans

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Master's degree in Aquaculture & Fisheries

(Field of Aquaculture)

Under the supervision of:

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Florbela Soares (IPMA)

2018/2019

Macroinvertebrate fauna assemblage on macroalgae within an IMTA earthen pond system

Use of three Crustacea species for aquaculture using Fourier Transform Infra-red spectroscopy

Academic Thesis: Declaration of Authorship

I declare being the author of this work, which is original and unique. Authors and works consulted are properly cited in text and included in the list of references

(Rafael José Tomé Vieira)

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Resumo

Atualmente reconhece-se a importância dos ecossistemas costeiros, quer a nível ecológico, quer para atividades humanas, tais como lazer ou para produção em aquacultura. A Ria Formosa é considerada uma das lagoas costeiras mais importantes da Península Ibérica onde existem sistemas de aquacultura em tanques de terra. Estes sistemas de produção são caracterizados pelos seus efluentes, ricos em matéria orgânica. Novos sistemas de produção têm sido desenvolvidos como forma de minimizar os impactos provocados por produções aquícolas, sendo que um deles é a produção em sistemas de aquacultura multi-trófica integrada que é conseguido utilizando culturas, em simultâneo, de organismos consumidores (por ex. peixes) que libertam elevadas quantidades de matéria orgânica e espécies capazes de assimilar matéria orgânica (por ex. bivalves) e inorgânica (macroalgas). As macroalgas são reconhecidas pela sua grande capacidade como depuradores biológicos. A sua presença em sistemas de produção multi-tróficos é, portanto, muito importante. As comunidades de macroinvertebrados constituem um elo intermédio nas teias tróficas e são importantes como integradoras das condições ambientais de um determinado habitat e como acumuladores de matéria orgânica pronta a ser transferida para níveis superiores. Certos grupos de invertebrados são utilizados na alimentação de espécies criadas em aquacultura, mas alternativas com elevados valores nutricionais devem continuar a ser estudadas com o objetivo de melhor a performance de espécies cultivadas. Com isto em mente, este estudo sobre organismos macroinvertebrados associados a macroalgas foi elaborado para complementar o conhecimento relativamente a sistemas de produção multi-trófica, bem como o emprego da técnica de espectroscopia de infravermelhos (FTIR) para estudar o perfil metabólico através da identificação de compostos e grupos funcionais.

A amostragem de invertebrados ocorreu no tanque de decantação da estação piloto de piscicultura de Olhão (IPMA), onde as macroalgas são abundantes. Três ocasiões de amostragem foram realizadas ao longo das estações do ano (Outono, Inverno e Primavera) com o propósito de observar se estas comunidades apresentam variações sazonais. As algas amostradas passaram por um processo de secagem, permitindo a análise da densidade de organismos por peso seco de alga. Algumas espécies de anfípodes e um isópode foram processados para análise espectrográfica, sendo primeiramente liofilizados e moídos. Esta moagem com adição de brometo de potássio (KBr) permitiu a criação de "pastilhas", utilizadas na obtenção de espectros de absorvância.

Os resultados obtidos mostram a dominância de *Ulva* spp., sendo a comunidade de invertebrados dominada por anfípodes, gastrópodes e isópodes. Foi possível observar variação sazonal da biomassa de algas por metro cúbico de água, da biomassa total de organismos, das densidades e dos índices de diversidade. Apesar da diminuição em biomassa de alga amostrada por volume entre o Outono e a Primavera (de 1820g m^{-3} para 852 g m^{-3}), todos os outros parâmetros acima referidos apresentam a tendência inversa. As amostras recolhidas durante a Primavera parecem ser consideravelmente distintas das recolhidas no Outono, não só em termos de densidades de invertebrados, mas também na composição de espécies. A análise de ordenação MDS permitiu a observação de grupos por estação do ano, mas o mesmo não foi possível na diferenciação de zonas do tanque. Os resultados espectrográficos apresentam diferenças entre grupos taxonómicos processados (anfípodes e isópode), mas não entre fêmeas e machos da mesma espécie.

Os resultados observados indicam que as macroalgas servem como fonte de habitat e alimento (direta ou indiretamente) para uma comunidade de invertebrados abundante e variada. Apesar disso os valores de diversidade e riqueza especifica são mais baixos comparativamente aos encontrados em estudos realizados em macroalgas e ervas marinhas em ambiente natural, podendo-se concluir que a comunidade presente no tanque de decantação é composta por organismos especializados. As espécies encontradas são na sua maioria detritívoras ou de pequenos herbívoros, estes organismos em associação com macroalgas podem contribuir para o tratamento de efluentes de aquacultura e promover o crescimento de alga no tanque. Tudo isto aliado à variação observada na comunidade de invertebrados ao longo do ano poderá ser explorada em termos de produção acessória, como alimento a fornecer na produção de espécies de interesse comercial.

Abstract

Earthen pond aquaculture farms may cause environmental impacts. Integrated multi-trophic aquaculture (IMTA) systems are a possible solution in the reduction of these impacts. These production systems are generally accompanied by the presence of macroalgae, making them ideal sites for studies of associated macroinvertebrate assemblages. Knowing the macroinvertebrate community may also potentiate an accessory production, currently overlooked by most farmers. The present work aims to evaluate the macroinvertebrate assemblage in earthen pond systems. Sampling took place on the settling pond of IPMA's aquaculture research station, in Olhão between October 2018 and June 2019. Invertebrate assemblages were investigated in relation with the quantity of algae sampled and species richness. Seasonal variation of species composition and densities were also studied. Two amphipod and one isopod species were analysed by Fourier transform infrared (FT-IR) allocating peak bands to functional groups and classes of biomolecules.

Environmental factors measured (temperature, salinity, pH, dissolved oxygen) did not vary greatly, except for water temperature. Seasonal variation was observable in terms of density, biomass and diversity, which all attained higher values for spring. Density of invertebrates varied between 29977 ind m^{-3} in autumn and 90250 ind m^{-3} in Spring, biomass between 105g m^{-3} in autumn and 464g m^{-3}. Results from the MDS analysis suggest differentiation between seasons but not between sites within the settling pond. Two non-indigenous species of invertebrates were found. Amphipods were the most abundant group and their high nutritional value can be exploited. The fact that these species are naturally present on the facility can be utilized, both in the improvement of effluent water quality and as suitable ingredients for the nutrition of fish and cephalopod species.

Keywords: Integrated multi-trophic aquaculture; macroalgae; macroinvertebrate assemblage; amphipods; Fourier transform infrared spectroscopy; non-indigenous species.

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1 Introduction

1.1 Characterization of the environment

Coastal lagoons are important ecosystems with high productivity. Even though only covering *ca* 5% of the European coast, are considered high primary and secondary production areas, incrementing value for anthropogenic activities such as aquaculture (Kjerfve 1994). The Ria Formosa is considered as one of the most important coastal lagoons in Southwest Iberia and is a relevant source for local populations, both ecologically and socio-economically (Rosa et al. 2019). Although the anthropogenic pressure in this system is high, a complex community has been observed and pointing towards an elevated ecological status with primary production being dominated by macrophytes and macroalgae (Gamito 2008).

Earthen ponds for aquaculture present specific characteristics and conditions making them comparable with confined lagoon systems (Gamito 2008; Machado et al. 2014). In systems with high daily water renewal, the seasonal environmental variability is low (Gamito 2008). However, some aquacultures may have a low water renewal, with high daily and seasonal environmental variability, leading to the presence of organisms which developed adaptations to that variability, as in estuaries and lagoons with reduced communication with the adjacent sea (Barnes 1979). The outcome is a less diverse community in comparison to other aquatic systems (Elliott and Whitfield 2011).

Bottom-up trophic base succession is observable in salt marshes where fauna responds to developing food sources availability due to increasingly algae, plant and detritus. showcasing that primary producers are linked with increase of consumer organisms (Nordström et al. 2014).

1.2 Integrated Multi-Trophic Aquaculture

Integrated multi-trophic aquaculture (IMTA) systems come together as a way of cultivating species using techniques that are both ecologically efficient and environmentally friendly, whilst remaining profitable (Chopin 2013). This is achieved by cultivating organisms of different trophic levels, making it possible to utilize both organic and inorganic nutrients, that are usually lost from the fed species, by converting it into a product (Chopin 2006; Barrington et al. 2009). This is attained by combining the cultivation, in optimal proportions, of fed species with extractive species (Figure 1), which can be reared at different locations as long as they remain connected through the water in terms of nutrient and energy transfer (Barrington et al. 2009; Chopin 2013).

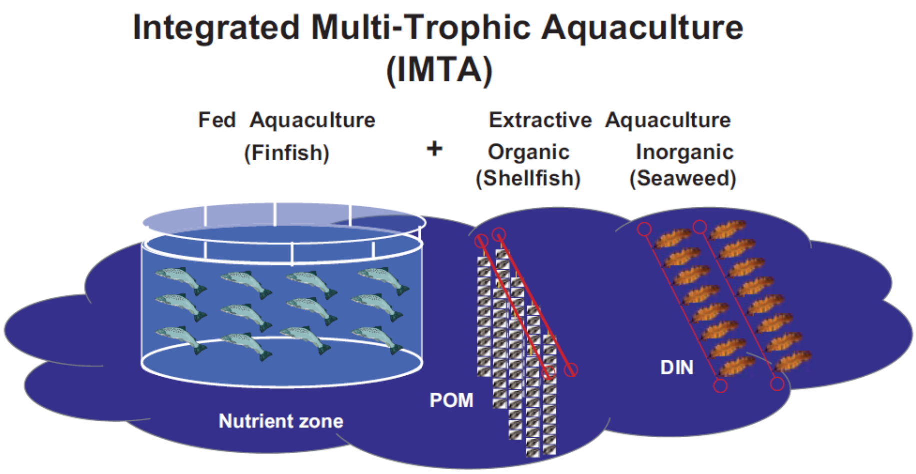

Figure 1. Diagram of integrated multi-trophic aquaculture (IMTA) with a combination of fed fish with particulate organic matter (POM) extractive organisms (shellfish) and dissolved inorganic nutrients (DIN) extractive culture (seaweeds). Source: (Chopin 2006).

The production of fed species, such as finfish and shrimps, induces an increase on the concentration of dissolved nutrients, bacteria and other suspended matter in effluent water (Ziemann et al. 1992). Since traditional water treatment methods have been proven to be inefficient and not cost effective (Hopkins et al. 1995), alternatives such as the treatment of effluent water using macroalgae were tested, showing better results on the assimilation of both organic and inorganic nutrients (Ryther et al. 1981; Pedersen 1994).

Macroalgae potential goes beyond acting as a biological filter since some species, such as *Ulva lactuca*, showed positive results, by restricting the growth of several species of phytoplankton that generates harmful algal blooms, which can inhibit the commercialization of reared species (Tang and Gobler 2011). Besides, seaweeds have shown an important role as a food product and source of non-food products for pharmaceuticals.

1.3 Macroalgae for invertebrates

Algae has proved to be an important food source to many invertebrates (Dudley et al. 1986) both directly (to herbivores) or indirectly by providing a substrate for attachment of other organisms and the creation of a periphytic material (Brönmark 1989). Previous studies have shown a relation between the presence of vegetation and an abundance and diversity increase of invertebrates (Downing 1986) although some show different results (Rookie 1984).

It is also known that the physical structure of the habitat influences the community composition. This is due to the fact that higher densities are attained in complex habitats where the surface area is bigger (Downes et al. 2000),in which case, variations in surface area covered by macroalgae could affect the animal composition associated with it.

Besides their importance as a food source, algae can aid in the treatment of aquaculture effluents by bioremediation of nitrogenous and phosphorous compounds generally present in those waters whilst enriching them by releasing O_2 (Guttman et al. 2018). Therefore, the integration of macroalgae within aquaculture systems are both beneficial for the algae and the organisms present. The effect of bioremediation is particularly efficient in static system such as those present in decantation ponds (Skriptsova and Miroshnikova 2011).

1.4 Macroinvertebrate fauna and importance for aquaculture species

Studies of macroinvertebrate fauna are very important to understand the relationship between organisms and habitat. Macroinvertebrate fauna is important regarding estuarine productivity showing that the macrobenthic organisms, when present, create high secondary production in estuaries and coastal lagoons, which can be used, for example, by reared fish (Wilson 2002)

A previous study, performed in the EPPO, has shown that the macrobenthic community in fish earthen ponds is diverse and mainly composed of polychaeta, amphipods and insects (Carvalho et al. 2006).

Invertebrates have commonly been used as feed for reared species. This is particularly important in early stages of development and the most commonly used ones have been rotifers, *Artemia* and copepods (Dhont et al. 2013). But these can prove to be either of low nutritional value or hard to produce, hence creating the need to procure different alternatives (van der Meeren et al. 2014; Hamre 2016). However, other macroinvertebrates have been used to study their potential as aquaculture, both as live and formulated feeds (Moren et al. 2006; Baeza-Rojano et al. 2010; Vargas-Abúndez et al. 2018). Techniques such as lipid extraction can be used to study the nutritional values of organisms (Maazouzi et al. 2007; Legeżyńska et al. 2012), but they may be expensive and time consuming.

1.5 Fourier transform infrared spectroscopy

With that in mind, Fourier transform infrared (FT-IR) spectroscopy can be used for metabolic profiling by identifying compounds, that enables a rapid and non-destructive analysis of a large array of organic and inorganic samples (Çakmak et al. 2003). The analysis of a spectrum, representing the “fingerprint” of samples, is possible since different molecules produce different spectral fingerprints (Theophanides 1984; Aharoni et al. 2002) allowing for composition analysis of organisms (Prabu 2012; Simon et al. 2016). In the case of biological samples this technique is often less expensive and time consuming as compared to studies of metabolic profiling, where a quantitative estimate of all metabolites present on a sample is the focus (Dunn and Ellis 2005).

2 Objectives

The main objective of this work was to acquire knowledge regarding the invertebrate assemblage in macroalgae, within a biomitigation pond of an IMTA system of earthen ponds. Consequently, the community was studied in terms of seasonal variation with the intention of better understanding the role of macroalgae for small invertebrates and the perspective of using the latter as an accessory production. Thus, Fourier transform infrared spectroscopy was employed to make a screening of biomolecules functional groups, in some invertebrates found during the study.

3 Materials and methods

3.1 Sampling periods

Sampling was undertaken in three different periods, occurring during the months of October-November 2018, February-March 2019 and June 2019. For each sampling period, four replicates were performed, labelled as Aut1-4 (22,29,30 Oct. and 6 Nov.), Win1-4 (25,26,27 Feb and 1 March) and Spr1-4 (3-6 June). Invertebrates for Fourier-Transform infrared spectroscopy analysis were picked in august 2019.

3.2 Sampling Area

This work was conducted at IPMA's aquaculture research station (EPPO), located on an old salt pan, at Parque natural da Ria Formosa, Olhão. It is composed of a water reservoir connected to the Ria, a pumping system to provide water for the 17 production ponds and a settling pond where water outflows back to the natural environment. The study was performed on the settling pond with an approximate area of 3700 m^2, rich in macroalgae due to its richness of nutrients and organic matter (Figure 2).

Figure 2. The research station production area, composed of water reservoir (WR) with connection to the Ria Formosa, 17 rearing ponds and lastly, where samples were performed, settling pond divided in three areas (A to the right of the rearing tanks outflowing water channel; B area proximal to the channel; C area to the left of the channel). Solid arrow represents inflowing water and dotted arrows outflowing water. Source: Google earth V 9.3.94.1.

3.3 Sampling and sorting of macrofauna

Before each sampling occasion, using a Hanna multiparameter waterproof meter, the following parameters were measured: water temperature, salinity, dissolved oxygen (%) and pH.

Macroalgae and associated macroinvertebrates quantitative sampling was carried out using an O-shaped sweep net with 20cm opening mouth and from a depth of about 35cm to the surface. Total water volume per replicate was $0.011m^{-3}$. The samples were subsequently filtered with the help of a mesh sieve (500 µm) to retain invertebrates and macroalgae. The biological material was placed on plastic trays with water to sort out macroalgae and invertebrates. All invertebrates were handpicked and preserved in 70% ethanol for later taxonomic analysis. Furthermore, macroalgae collected was wet weighed (g) followed by drying at approximately 70ºC for 24 hours in order to obtain its dry weight (g). All measurements were carried out using a precision balance with two decimals.

The identification of species was made to the lowest taxonomic level possible. Sexual dimorphism, as well as the percentage of ovigerous females, were recorded whenever possible. This work was performed using: Leica S8 APO stereomicroscope (Germany), Leica M80 stereomicroscope (Germany), Wild M3Z Heerbrug stereomicroscope (Switzerland) and Leitz Laborlux K microscope (Germany) and with the application of the following keys: (Bellan-Santini et al. 1982; Hayward and Ryland 1995; Gil 2011).

Invertebrates were wet weighed using a precision balance, after removal of excess water with absorbent paper and total biomass of each taxon was estimated by multiplying the average individual weight by the total number of individuals.

3.4 Sampling for Fourier Transform infrared (FT-IR) spectroscopy

Three invertebrate species were selected based on their abundance, time of the year and importance for the study. A targeted sampling was performed in August 2019. From these species, one was separated in triplicates of males (C1-C3) and females (F1-F3), the second in triplicates of alpha males (P1-P3) and others (includes females, beta and gamma males. Pf1-Pf3). The third species was sampled in quadruplicates, without sex separation (M1-M4). Organisms were collected and placed in 1.5 mL colourless Eppendorf Tubes® (Eppendorf Quality™).

3.5 Fourier transform infrared FT-IR spectroscopy

After selection of organisms and tube labelling the samples were frozen at -80ºC, using liquid nitrogen, followed by freeze-drying in a vacuum freeze dryer (Savant RVT 400-120), for three days. After lyophilization samples were ground and reduced to a fine dry powder. Each of this samples were then mixed with KBr (≈100mg KBr/1g sample) utilizing an agate pestle and mortar until a homogeneous fine powder was attained. Small portions of this mix were placed in in a die (13mm diameter) and subjected to a pressure of *circa* 7.5 X 10^6 Pa for 3 minutes, in order to obtain clear 1mm pellets for further IR spectroscopy analysis.

Four spectra, at different points of each pellet, were acquired using a "TENSOR" FT-IR equipment (Bruker) coupled with OPUS control/analysis software (Bruker). Each reflectance spectrum was obtained by running 25 scans covering the 400-4000 cm^{-1} range at a resolution of 4 cm^{-1}.

3.6 Data treatment

After sample collection and taxonomic verification all data was organized using Microsoft Office Excel. This data was then transferred to pivot tables to acquire the matrixes needed for further analysis. Total number of invertebrate species, density (individuals per volume and per algae dry weight), biomass (g m^{-3}), species richness and Margalef index (based on abundance data) were estimated. Seasonal variation of dried algae biomass ($g_{algaeDW}$) and density was analysed.

Primer-e software (V 6.1.13) was used to study diversity within samples and seasons, assessed by Margalef's diversity index. In order to evaluate possible seasonal or site differentiation of invertebrate communities associated with macroalgae, a multidimensional scaling (MDS) was carried out using Bray Curtis dissimilarity index after square root transformation, utilising the same software.

FT-IR spectroscopy data point tables were exported from the OPUS software into excel. The Rstudio software (V 1.2.1335) was then used for further treatment of this data. Firstly, the four replicates obtained per pellet were averaged, yielding the average spectrum for each sample. Two matrices were created, one with the averaged spectra of reflectance (R) and a second with the absorbance spectra (A) obtained by calculating log10(1/R). In order to standardize the spectra a standard normal variation (SNV) transformation was applied to the absorbance spectra. Lastly, principal component analysis (PCA) was performed to the SNV results using the function prcomp from Rstudio.

4 Results

4.1 General analysis

Dissolved oxygen was always high, above 84%. Temperature and salinity had higher mean values during Spring, following seasonal variations (Table 1).

Table 1. Mean, maximal and minimal values of water temperature (Temp, ºC), salinity (Sal), dissolved oxygen (DO%) and pH per season of sampling

		Autumn	**Winter**	**Spring**
	min	17.20	15.84	23.09
Temp	mean	17.39	16.36	24.27
	max	17.61	17.00	25.14
	min	35.45	35.50	36.90
Sal	mean	35.82	35.65	37.09
	max	36.20	35.80	37.20
	min	83.50	89.90	92.00
DO	mean	108.88	100.18	100.98
	max	130.60	114.20	109.90
	min	8.01	8.01	8.28
pH	mean	8.10	8.02	8.31
	max	8.21	8.04	8.35

Two types of algae were collected, *Ulva* spp. with 11 samples, and 1 sample of *Chaetomorpha* sp., sampled on the fourth winter replicate (Win4).

Arthropoda was the most abundant phylum with a mean density of 41848 individuals per volume of water (ind m^{-3}, ≈72%), followed by Mollusca with 13826 ind m^{-3} (≈24%) and Cnidaria with 2348 ind m^{-3} (≈4%). The other groups, Annelida, Chordata and Echinodermata, contributed with less than 0.3% (Figure 3).

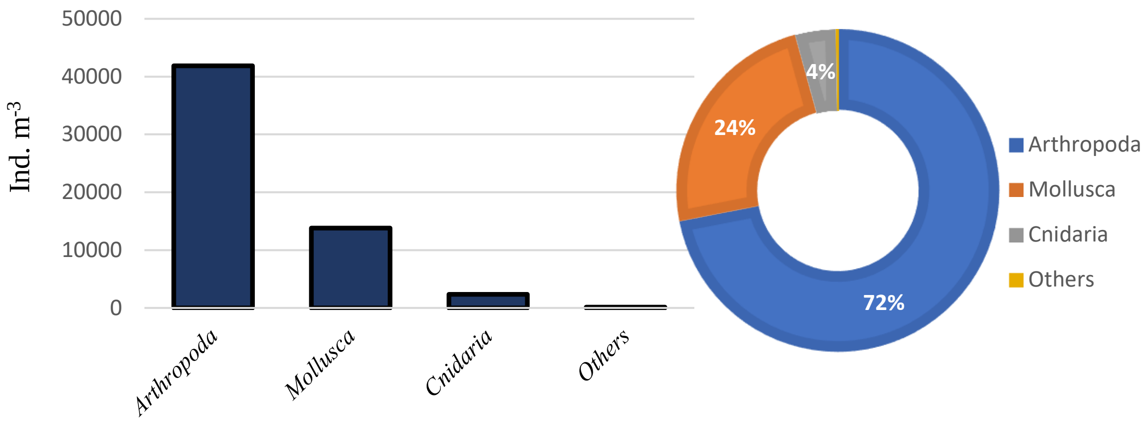

Figure 3. Mean density of macroinvertebrates by volume of water (ind m^{-3}) and percentage of phyla sampled. Others include the phyla: Annelida, Chordata and Echinodermata.

Table 2 shows the seasonal observations of abundance, biomass and density. In a total of 7678 invertebrates, six phyla, 20 families and 30 different species were identified. From these, 23 were identified to the specific name, 2 to the genus and the rest to higher taxa. The most abundant species was *Monocorophium insidiosum* (24%) followed by *Hydrobia glyca* (19.5%), *Paracerceis sculpta* (18%) and *Grandidierella japonica* (11.6%). Total estimated biomass of organisms was 781 g m^{-3}, with the highest contributor being *Paracerceis sculpta* (197.133g m^{-3}), *Tritia corniculum* (156.466g m^{-3}), *Cymadusa filosa* (113.325g m^{-3}), *Hydrobia glyca* (110.368g m^{-3}) and *Grandidierella japonica* (86.840g m^{-3}). These five species make up 85% of the total biomass sampled.

Seasonal variation was observed in terms of organisms sampled. Spring showed the highest values of density with 90250 ind m^{-3} (52%) followed by winter with 54273 ind m^{-3}(31%) and lastly autumn with 29977 ind m^{-3} (17%).

Table 2. Total abundance (number of individuals), total biomass (g) and seasonal variations of density (ind m^{-3}) and biomass (g m^{-3}), sampled for each taxon. Empty values indicate no presence.

Phylum/Subphylum	Group	Species name	N total (nr individuals)	Organisms total biomass (g)	Abundance (g m^{-3})			Organisms biomass (g m^{-3})		
					Autumn	Winter	Spring	Autumn	Winter	Spring
Crustacea	Amphipod	Monocorophium insidiosum	1839	0.7647	3750	10159	27886	1.559	4.224	11.595
		Grandidierella japonica	890	3.8209	14386	5136	705	61.763	22.051	3.025
		Cymadusa filosa	520	4.9863	318	477	11023	3.051	4.577	105.697
		Microdeutopus gryllotalpa	488	0.4357	1273	2886	6932	1.136	2.577	6.189
		Elasmopus rapax	110	0.4143	45	2045	409	0.171	7.704	1.541
		Monocorophium acherusicum	109	0.0453	182	1932	364	0.076	0.803	0.151
		Gammarella fucicola	74	0.2896	68		1614	0.267		6.314
		Melita palmata	30	0.0462	114	23	545	0.175	0.035	0.839
		Gammarus insensibilis	15	0.1500			341			3.409
		Monocorophium sextonae	7	0.0029		159			0.066	0.000
	Decapoda	Palaemon varians	3	0.3400			68			7.727
	Isopoda	Paracerceis sculpta	1383	8.6739	386	20909	10136	2.423	131.137	63.573
	Mysida	Heteromysis formosa	35	0.2100		795			4.773	
		Gastrosaccus spinifer	1	0.0100		23			0.227	
	Nebaliidae	Nebalia bipes	12	0.0240	23	0	250	0.045		0.500
	Tanaidacea	Tanais dulongii	1	0.0001			23			0.002
Mollusca	Gastropoda	Hydrobia glyca	1501	4.8562	8477	1795	23841	27.426	5.809	77.132
		Tritia corniculum	147	6.8845	68	182	3091	3.193	8.515	144.758
		Peringia ulvae	142	0.5680	682	591	1955	2.727	2.364	7.818
		Haminoea sp.	31	0.7750	23		682	0.568		17.045
		Bittium reticulatum	4	0.1600			91			3.636
Annelida	Polychaeta	Neanthes acuminata	7	0.0700			159			1.591
		Spio decorata	2	0.0002			45			0.005
Arthropoda	Insecta	Insecta larvae	4	0.0160	91			0.364		
		Insecta sp1	1	0.0040	23			0.091		

Phylum/Subphylum	Group	Species name	N total (nr individuals)	Organisms total biomass (g)	Abundance (g m^{-3})			Organisms biomass (g m^{-3})		
					Autumn	Winter	Spring	Autumn	Winter	Spring
Arthropoda	Insecta	Insecta sp2	1	0.0040	23			0.091		
		Insecta sp3	1	0.0040	23			0.091		
Cnidaria		Cnidaria sp.	310	0.7585	23	7023		0.056	17.183	
Echinodermata	Ophiuroidea	Amphipholis squamata	3	0.0600			68			1.364
Tunicata	Ascidiacea	Molgula sp.	7	0.0070		136	23		0.136	0.023
Grand Total			7678	34.3812	29977	54273	90250	105.274	212.182	463.935

4.2 Season and Spatial evaluation

Density of macroinvertebrates was studied in terms of number of individuals by volume of water (ind m^{-3}) and by algae dry weight (ind $g^{-1}_{algaeDW}$). Both the density of individuals by volume of water and by algae dry weight (DW) attains higher values for spring replicates (Figure 4).

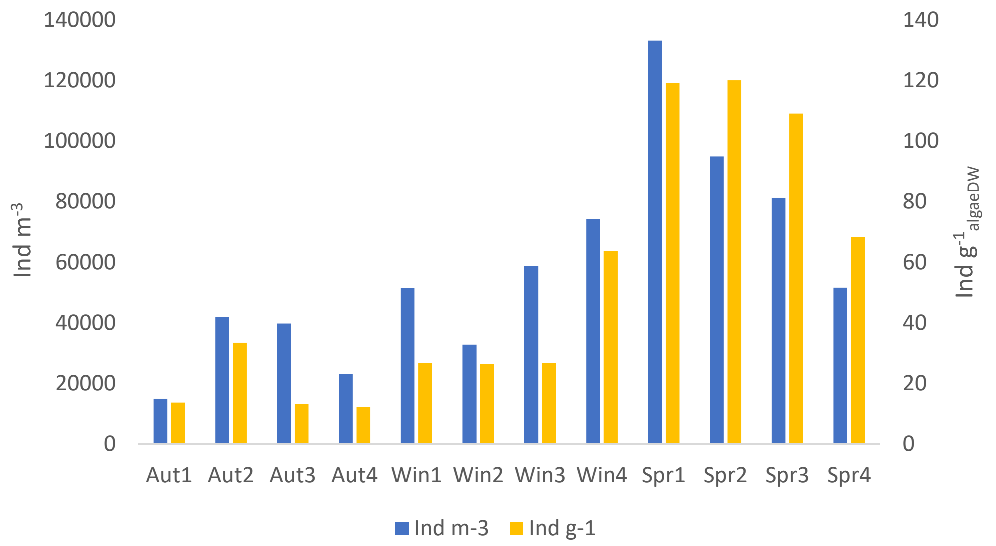

Figure 4. Density of macroinvertebrates by water volume (ind m^{-3}) and by dry weight of algae (Ind $g^{-1}_{algaeDW}$) per sampling occasion. Four samples for each season: autumn (Aut1-4), winter (Win1-4) and spring (Spr1-4).

The analysis of dry algae biomass shows a decrease throughout the sampling periods, from autumn to spring. Conversely, biomass of invertebrates, density of invertebrates per dry weight of algae and by volume of water increased. Variation of density by algae DW seems to be more accentuated between winter and spring whereas for density by volume of water is smoother (Figure 5).

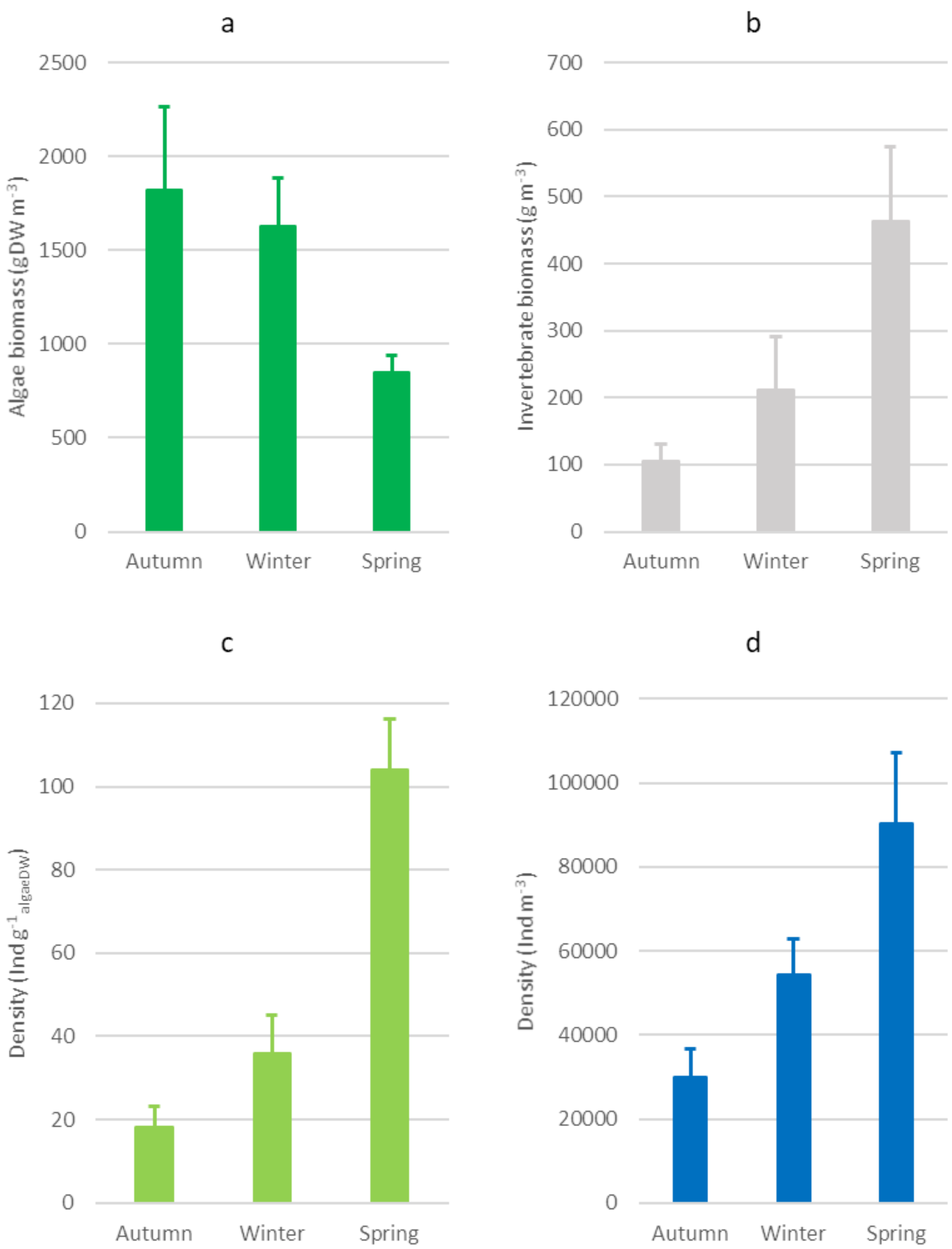

Figure 5. Seasonal variation plots with standard error for: a - dried algae biomass by cubic metre of water; b - invertebrates' biomass by cubic metre of water; c - density by gram of algae dry weight; d - density by cubic metre of water.

The scatter distribution of algae dry and wet weight can be found in annex A 1. Seasonal scatter distribution, represented in Figure 6, of number of individuals per algae DW was used to study the relationship between these two variables. Although a positive relationship between algae DW and nr of individuals is observable for autumn and spring, no relationship is suggested for winter samplings. The goodness of fit is low for autumn and winter, 20.27% and <0.01% respectively, and high for Spring, 76.72%.

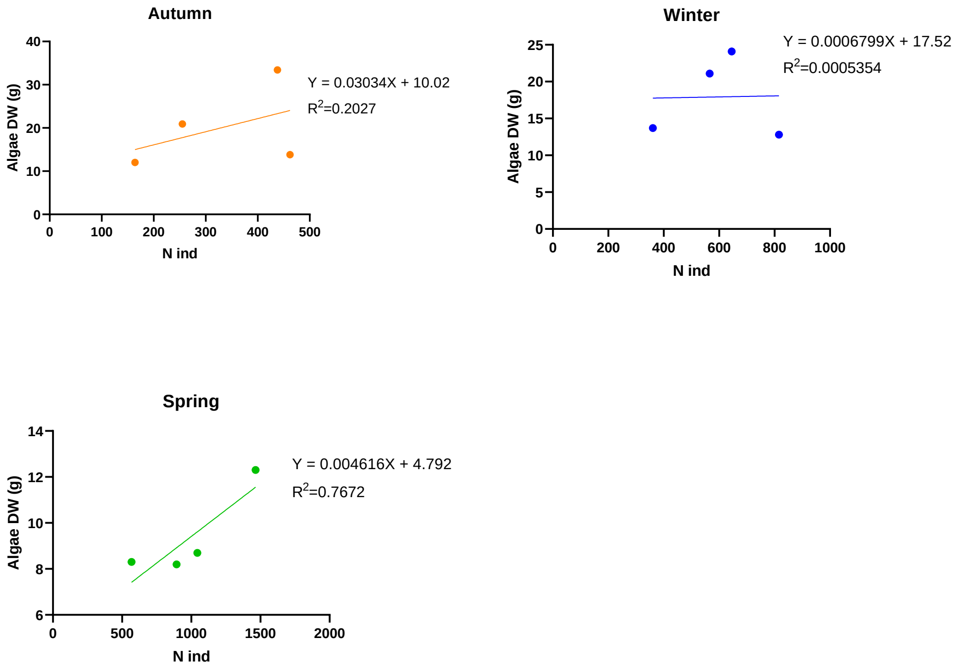

Figure 6. Scatter plots of number of individuals relatively to algae dry weight, per season.

Values of density, for the six most abundant species, were averaged to evaluate variation between seasons. *M. insidiosum*, *C. filosa* and *M. gryllotalpa* were increasingly abundant from autumn to spring whereas *G. japonica* showed the opposite trend. *H. glyca* was almost absent in winter, reappearing in Spring and the isopod *P. sculpta* peaked in winter samples. Both units of density show similar results although the error observed is smaller for density per alga DW (Figure 7).

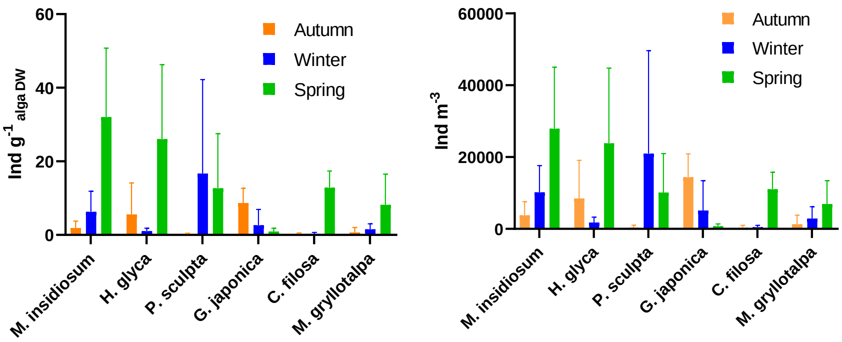

Figure 7. Density per gram of dry alga (left) and per cubic metre (right). Variation, per season, for the six most abundant species.

The analysis of sex dimorphism, possible for amphipod species, showed that most species have female dominated populations. It was also possible to observe that ovigerous females were observed throughout all seasons although spring was the one that presented the highest number of species with high percentages of ovigerous females (Table A 1).

4.3 Multivariate analysis

Analysis through multidimensional scaling (MDS) was unable to show groups based on the region of the tank sampled (Figure 8, left). As for the seasonal analysis, it was possible to observe grouping of samples from the same season, as a result of higher similarity between each other (Figure 8, right). Sample Win4 was very distant from the others, probably as a reflex of it being the only sample of *Chaetomorpha* sp. present in the study.

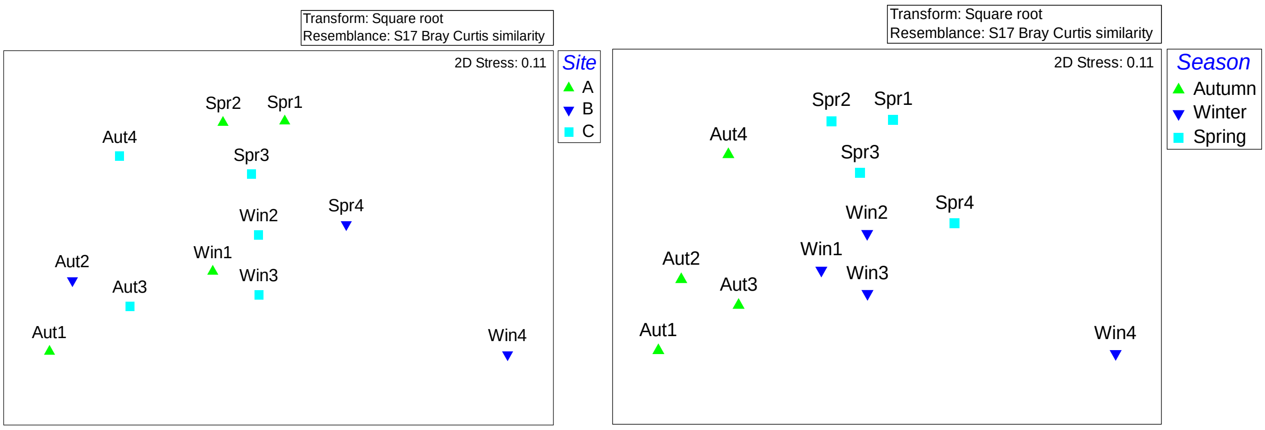

Figure 8. 2D multidimensional scaling (MDS) carried out with abundance data set, to observe the differences between sites (left) and Season (right). Bray Curtis similarity index and root-transformation, 12 sampling occasions. Stress level 0.11. Four samples for each season: autumn (Aut1-4), winter (Win1-4) and spring (Spr1-4).

4.4 Diversity analysis

Species richness (S) shows its highest value during spring with 22 species sampled. The average values of species richness ($\bar{S}$) and Margalef index ($\bar{d}$) show an increasing trend across the year (Figure 9).

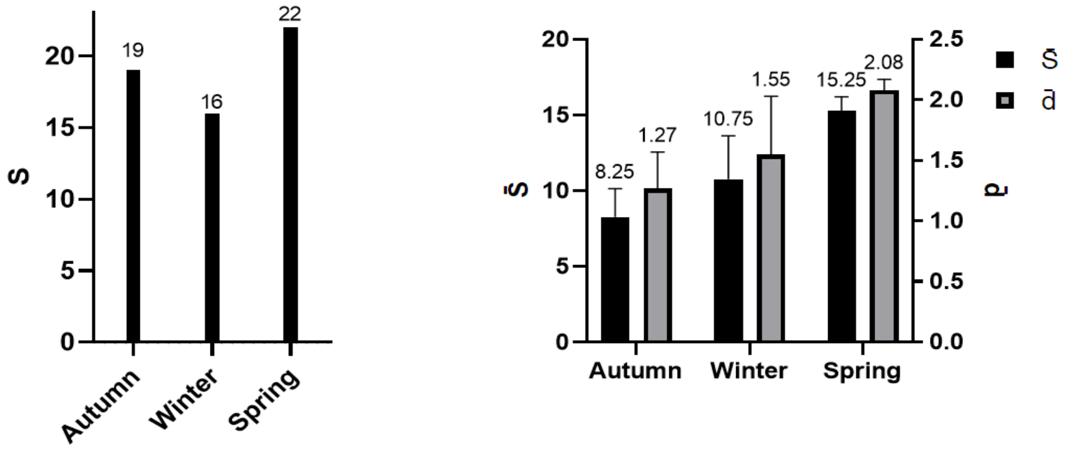

Figure 9. Left: seasonal species richness (S). Right: variation of average species richness (S̄) and average Margalef's index (d̄), applied to the total number of individuals.

4.5 Selection of species for FT-IR spectroscopy

Invertebrates for FT-IR spectroscopy analysis were chosen based on their abundance within the decantation pond, taking into consideration the targeted sampling occurred in summer as well as their importance for the study. From the five most abundant species (Table 2) three crustacean species were selected, the amphipods *Monocorophium insidiosum* and *Cymadusa filosa* and the isopod *Paracerceis sculpta*. The amphipod *Grandidierella japonica* was not abundant at the time of targeted sampling for FT-IR spectroscopy analysis.

The sexual differentiation observed for the isopoda *Parcerceis sculpta*, was only possible for alpha males, whilst females were grouped with beta and gamma males since we were not able to differentiate them in this study. These results are summarized in Table 3.

Table 3. Total number of individuals sampled, by sex, for the species where sex differentiation was studied, using FT-IR spectroscopy. F: females of *Cymadusa filosa*; M: males of *C. filosa*, F+: females plus beta and gamma males of *Paracerceis sculpta*, Mα: alpha males of *P. sculpta*.

Sp. Name	*Sex*	*N Total*
Cymadusa filosa	F	388
	M	132
Paracerceis sculpta	F+	1221
	Mα	162

4.6 FT-IR spectroscopy

The average absorbance spectra for each sample, obtained using FT-IR spectroscopy, in the 3600-800 cm^{-1} spectral region can be observed in Figure 10. The assignments of major bands observed in the figure are related to classes of biomolecules, present in Table 4, which was created by compilated information from different sources (Prabu 2012; Ceylan et al. 2014; Silva et al. 2014; Vongsvivut et al. 2014; Hardoim et al. 2016). Figure 10 and Table 4 must be observed in parallel in order to establish a relationship between them. This allows the observation of the presence of certain functional groups belonging to biomolecules existing in the samples which may aid, for instance, in the search for certain essential fatty acids such as the oleic and linoleic acids (Peak/Band 3).

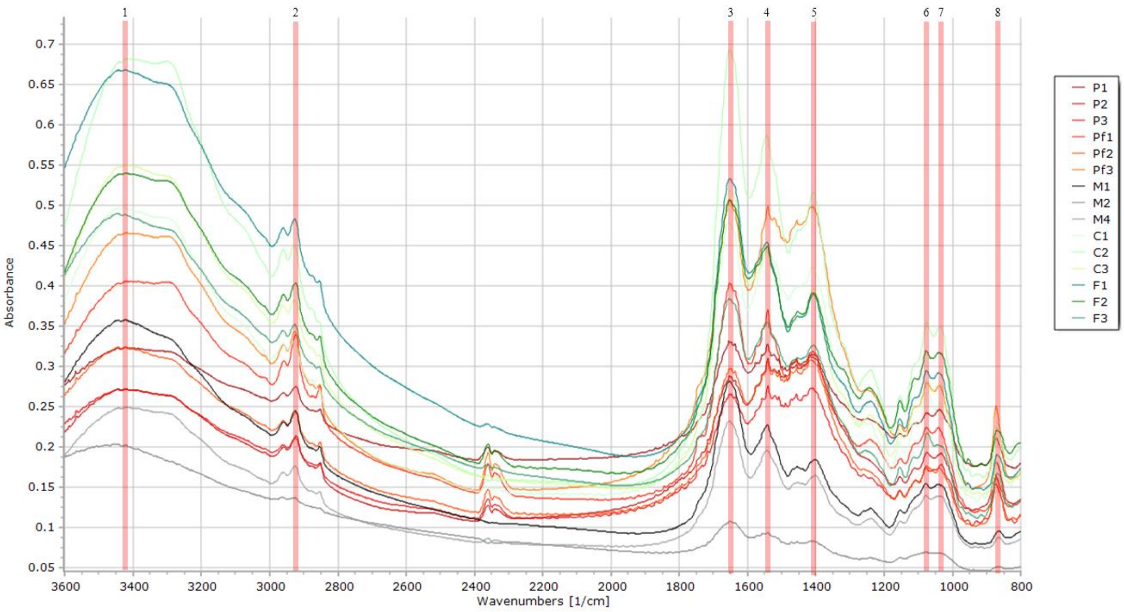

Figure 10. Absorbance spectra for each sample with the 8 major absorption bands. P#: triplicates of *P. sculpta* α males; Pf#: triplicates of *P. sculpta* “Others”; M#: triplicates for *M. insidiosum*; C#: triplicates of *C. filosa* males; Cf#: triplicates of *c. filosa* females.

Table 4. Main spectral band assignments for functional groups detected in the 3600-800 cm^{-1} range.

Peak #	Wavenumber (cm^{-1})	Associated functional group vibration modes	Main components	Other components
1	3418 - 3421	O–H stretching; N–H stretching; C–H stretching	Carbohydrates and glycoconjugates	Alfa-chitin
2	2923 - 2929	CH_2, CH_3 asymmetrical stretching	Lipids	
3	1651 - 1655	C=C stretching; C=O stretching; NH_2 scissoring	Oleic and linoleic acids	Unconjugated olefins
4	1541 - 1544	C–N stretching and N–H bending of amides (amide II peak)	Proteins	Aromatics
5	1402 - 1417	COO^- symmetric stretching	Fatty acids, amino acids. olefins	Carboxylates
6	1073 - 1079	C-O stretching of glycogen; PO_2^- symmetric stretching	Carbohydrates, nucleic acids	Phospholipids, aromatics
7	1033 -1039	inorganic phosphate; C–C skeletal vibrations	AA aromatic side chains	Polyphenols, phosphate
8	864 - 871	carbonate; C-C skeletal vibrations; C-H out-of-plane bend	lipids	Aromatics, Carbonate

The PCA analysis on the SNV transformed data only provides a separation between the isopod *P. sculpta* and the amphipods. Differentiation between alpha males and “Others” was not possible for the isopod, nor for males and ovigerous females of *C. filosa* (Figure 11)

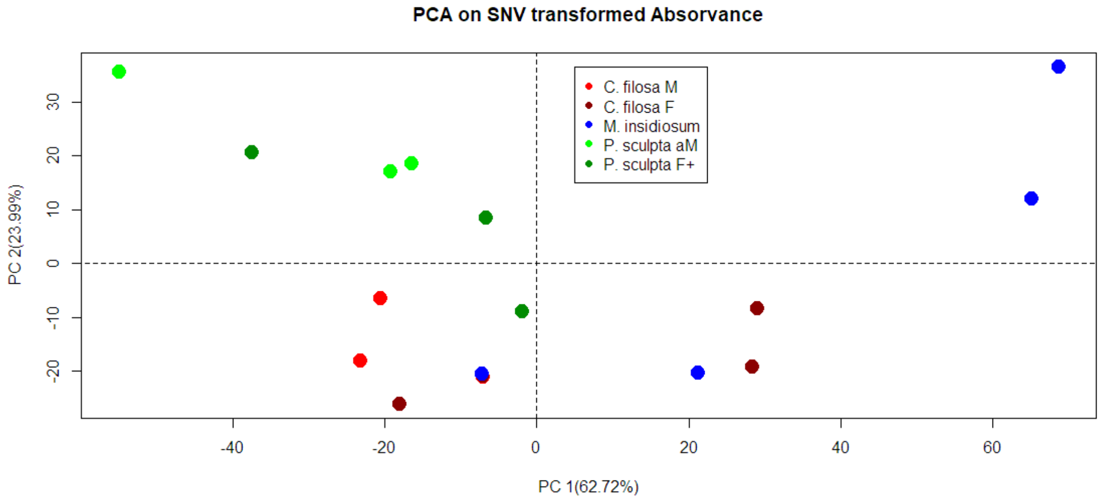

Figure 11. Principal component analysis (PCA) applied to the standard normal variate (SNV) of absorbance values for each sample. *C. filosa* "M" & "F": samples of males and females of *Cymadusa filosa*, respectively; *M. insidiosum*: samples of *Monocorophium* insidiosum*;* *P. sculpta* "aM" & "F+": samples of alpha males and "others" of *Paracerceis sculpta*, respectively.

5 Discussion

5.1 Sample characterization and species composition

The measured parameters did not vary greatly, in fact, water temperature was the only value that showed some differences with values ranging from an average of 16.36ºC in winter and 24.27ºC in the spring. The dissolved oxygen saturation was high, probably caused by the quantity of algae in the pond and time of sampling (between 10 and 12 a.m.).

This pilot study of macroalgae as a substrate for invertebrates, present within IMTA's earthen pond, where 7678 individuals were sampled, with an average density of 58167 individual per cubic metre of water sampled (Ind m^{-3}), shows a community composed mainly by the phyla Arthropoda and Mollusca, which is in accordance to a study performed on different macroalgae as substrate for invertebrates (Saarinen et al. 2018). From these two groups, it is important to point out most arthropods found belong to the order Amphipoda and Isopoda, whereas for molluscs the majority of individuals belong to the order Littorinimorpha in accordance to a previous study by Branoff et al (2009) for invertebrate assemblages on seaweed. Previous studies, performed on the benthic community, in earthen ponds, show slightly different results, which is expected due to different environment and substrate. Then again, some of the groups in those studies are also present in this one showing that certain organisms are present both in the bottom and in association with algae (Carvalho et al. 2006; Gamito 2006; Machado et al. 2014). The sex ratio observed for amphipods had different results depending on species, although larger samples point out to female-dominated populations as observed by Dick and Elwood (1996).

It is important to refer that two non-indigenous species were found: the amphipod *Grandidierella japonica* Stephensen, 1938 and the isopod *Paracerceis sculpta*, Holmes 1904. The latter has already been reported in the Iberian Peninsula and in the Ria Formosa, near Faro by Martínez-Laiz et al (2018). The species is known as the most widespread species within the genus and generally shipping and recreational boating are referred as the main vectors (Martínez-Laiz et al. 2018). In contrast and to the best of our knowledge this is the first record of *G. japonica* in the Ria Formosa. The species was reported to be spreading in the West coast of France (Jourde et al. 2013; Droual et al. 2017) and present in the Adriatic Sea (Munari et al. 2016). The genus *Grandidierella* can be confused with the genus *Unciolella* and *Microdeutopus* leading to misinterpretations, but is characterized by the presence of stridulating organs in male gnathopod 1, two additional teeth on the carpus besides the antero-distal and uniramous uropod 3 (see appendix:A *2*). The main vector of introduction is suggested as the commercial transfer and production of *Crassostrea gigas*. Since this oyster species is present in the research station and widespread in the Ria Formosa, we can assume this may be the vector of transmission.

The settling pond is clearly dominated by amphipods, with 4082 organisms (30924 ind m^{-3}) and a total of 10 species identified. The corophiid *Monocorophium insidiosum* was the most abundant of them. This species, described by Crawford (1937), is characterized by building tubes of mud on macroalgae and being a selective deposit feeder of suspended particles. A previous study by Sheader (1978) on the reproductive biology of *M. insidiosum* suggested two generation per annum, reflected by peaks of recruitment during June and February with most individuals in autumn being in a "resting" immature state. The same can be proposed for the population in this study since the peaks found for this species occurred in winter samplings (Feb-March) and especially in spring (June).

Seasonal influence within this macroinvertebrate assemblage can be observed. Autumn had the lowest values of biomass, density and diversity, whereas spring was clearly characterized by the highest values of biomass, density and diversity of species. Looking at the variation of density for the seven most abundant species it is also possible to infer that some species are clearly influenced by season, reflected in dominant species differentiation per time of the year.

In line with what was previously said, ordination analysis through MDS allowed for the identification of groups when season is used as a factor, making it possible to infer that the community does in fact, vary throughout the year. The most isolated point is visible for the sample "Win4", showing that faunal assemblage in *Chaetomorpha* sp. had no correlation with the one observed in *Ulva* spp. On the other hand, it was not possible to define groups based on site of collection, which can possibly be explained by the fact that no stratification was found within the settling pond, since it was evenly covered by algae.

This was the first pilot study performed on faunal assemblage within IMTA earthen ponds, with macroalgae as a substrate and showed that macroalgae is a valuable substrate for epifauna with a diverse community, which suffers variations throughout the year. From the two species of macroalgae collected *Ulva* spp. was the most abundant over the course of all samples taken on the pond. In fact, 11 of the 12 samples taken were of *Ulva* spp. and one of *Chaetomorpha* sp. Some studies on faunal assemblages on different species of algae, as well as microbenthic communities in earthen ponds point towards high presence of amphipods, isopods and molluscs (Pedersen 1994; Carvalho et al. 2006).

The relationship between algae dry weight and abundance, per season, shows a positive relationship, although with low values of goodness of fit (R^2) for autumn and spring, increasing sample size could accentuate this relationship. A study of macroalgae complexity in epifaunal assemblage by Veiga et al (2014), shows that the relationship between algae dry weight and number of individuals varies depending on the species of algae and, contrary to our results, a positive relationship between dry weight and epifaunal taxa richness.

Algae was observed as a direct source of shelter for species such as *C. filosa* which, at time of sampling, was usually seen wrapped around the edges of *Ulva* spp. collected, accordingly to the species tube building observation in *Ulva lactuca* by Appadoo and Myers (2003). However, algae may not only be useful as a structure for the settlement and growth of these organisms, but also as a source of food, both directly and indirectly. The present study shows amphipod species known for occupying a large spectrum of feeding habits in trophic pathways, although most of them are detritivores (Legeżyńska et al. 2012; Guerra-García et al. 2014). Indeed, most of the invertebrates found are detritivores and grazers. Although not measured, rigidity changes on *Ulva* spp. were observed, structure was seemingly more rigid for algae sampled in spring as compared to the other seasons. This can be a result of seasonal variation, or the effect of invertebrates feeding on the epiphytes present on the algae as showed in a study by Kamermans et al (2002). These effects on seaweed growth, resulting in food availability shifts for mesograzers (herbivores), by altering the presence/absence of their preferred type of epiphytic algae, leads to shifts in macroinvertebrate communities (Vázquez-Luis et al. 2013; Machado et al. 2017).

5.2 Invertebrates as feed for aquaculture

Species present in this study such as *P. ulvae, M. palmata, Corophium* spp. and other gammarideans have already been reported by Cabral (2000) in the diet of *Solea senegalensis* from their nursery area in the Tagus estuary, proving that some of these organisms can be a nutritional source of live feed for relatively small individuals.

Several studies show the importance of small invertebrates in the diet of many wild fish and cephalopods with interest for aquaculture. Some examples are sole and seabass, two species well established in the industry, as well as some ornamentals such as seahorses and even in emerging species such as cuttlefish and octopus (Pinczon Du Sel et al. 2000; Jimenez-Prada et al. 2015; Vargas-Abúndez et al. 2018). These studies also refer that different species of invertebrates are predated at different development stages of the predator. Keeping in mind the possible accessory production of invertebrates, it is essential to know the community and its variations. This may also aid in the planning of uses for these invertebrates, taking into consideration the needs of the targeted fish/cephalopod species being reared, as well as the harvest of such invertebrates, according to different development stages of the reared species.

The fact that amphipods are found in the natural diet of many fish and cephalopods accentuates the interest in studying the biochemical composition of these organisms. So far, a study performed on caprellid and gammarid amphipods of the strait of Gibraltar, revealed that amphipods from this region have high levels of protein and lower levels of lipids. In contrast, deep-sea or Arctic and Antarctic species of amphipods show higher lipid content, as a result of unpredictable food availability in the environment (Baeza-Rojano et al. 2014). Further studies should take into consideration the different food availability throughout the year, as well as the impact of those yearly fluctuations on the biochemical composition of invertebrates, allowing for an informed selection of the best possible species for their potential use in aquaculture, either as live prey, or added in formulated feeds.

Amphipods have been referred to as a group of organisms occupying several levels of the trophic web, but detritus remains the principal food item in the majority of species, especially detritus from animal matter and epiphytes, strengthening the advantages of these organisms being present with macroalgae (Guerra-García et al. 2014). Hence, amphipods are not only relevant as a nutritious source included in fish's diet, but also in the uptake of organic waste as biofilters, emphasizing their potential as an accessory production in integrated multi-trophic aquaculture where they can be used to enhance water quality and later converted into a source of protein (Fernandez-Gonzalez et al. 2018).

Analysis of the PCA applied to the FT-IR spectroscopy was performed to search for differences between some taxonomic groups, between sexes and between ovigerous females and males. Differences between taxonomic groups was possible, the isopod *P. sculpta* wass clustered and separated from the amphipod species, denotating different spectral composition. On the other hand, a differentiation between the 2 species of amphipods was not possible. Lastly, the possible differentiation between males and females was also not evident, even when using ovigerous females of *C. filosa*.

6 Conclusions and final remarks

The averaged diversity indexes were not as high as what has been found in some studies in the natural environment, pointing out to a particular community in the settling pond. The dominant group of organisms are low biomass amphipod species, characterized by direct development that are mainly female dominant; therefore, we can assume these are opportunistic species, with low individual biomass and high growth rates, characteristic of r-strategists, allowing them to cope with the high anthropogenic organic loads typical of aquaculture systems.

Carvalho et al (2009) refers the importance of settling ponds in reducing organic matter and nutrients from aquaculture effluent waters, resulting in less environmental impacts on adjacent lagoon systems. In this regard, macroinvertebrate composition on this pond strengthens the idea of a multitrophic approach where organisms previously undervalued could be important both in environmental impact reduction and later potentiated into a protein source. Seasonal variation of organisms was observable both in terms of species composition, and particularly in terms of densities. For example, spring was characterized by a fivefold increase in density (Ind $^{g-1}$ $_{DWalgae}$) in comparison to autumn. If exploited, these populations could lead to more than one production per annum, with different species harvested depending on time of the year. Meaning that, considering the amphipod *C. filosa*, a species found mainly in spring, could be harvested at a ratio of ~44g per kilogram of fresh algae. Another advantage is the fact that different species of invertebrates could be used in different life cycle stages of reared species.

The great advantage of FT-IR spectroscopy is the rapid screening and profiling of functional groups that can be used prior to any targeted metabolite analysis. Consequently, the results obtained in this study should be further analysed with other metabolic profiling techniques such as chromatography and lipid extraction. The results of this study do not point to differences between ovigerous females and males, contrary to what was conjectured. In the future, and for a stronger analysis, it is recommended to increase the number of samples and study variations per season, which was not possible in this study due to time and material constraints.

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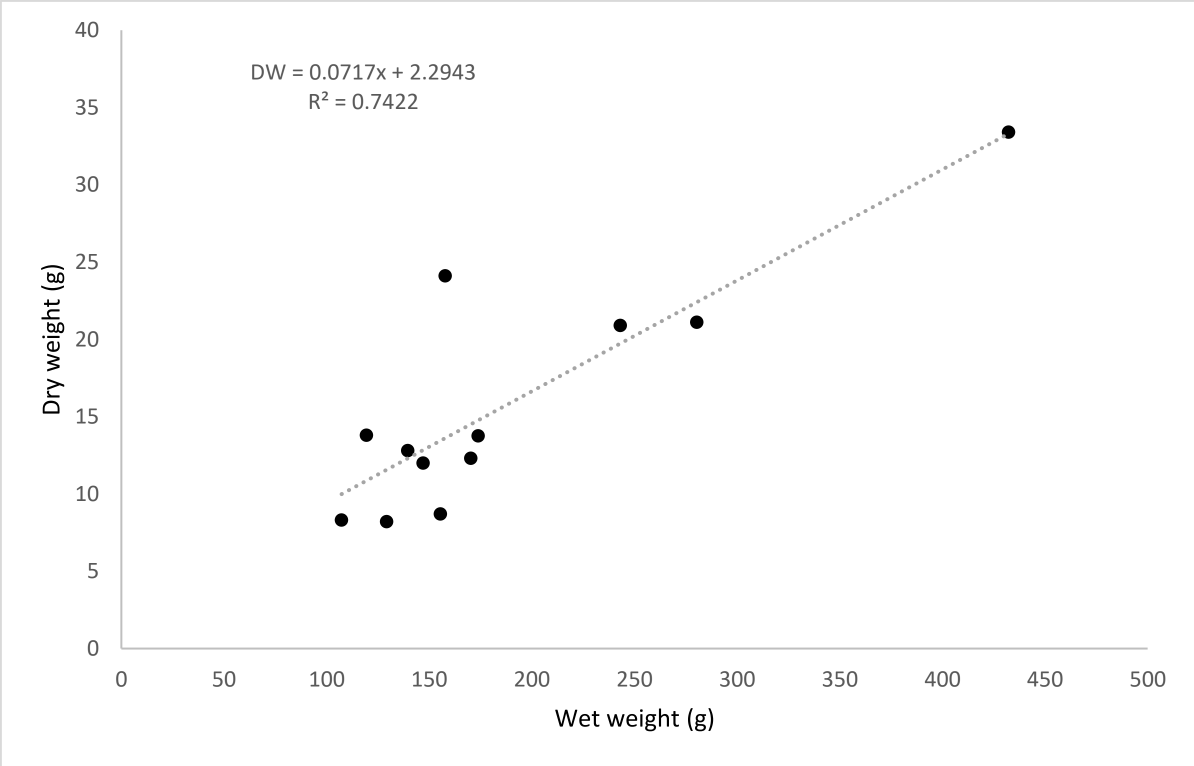
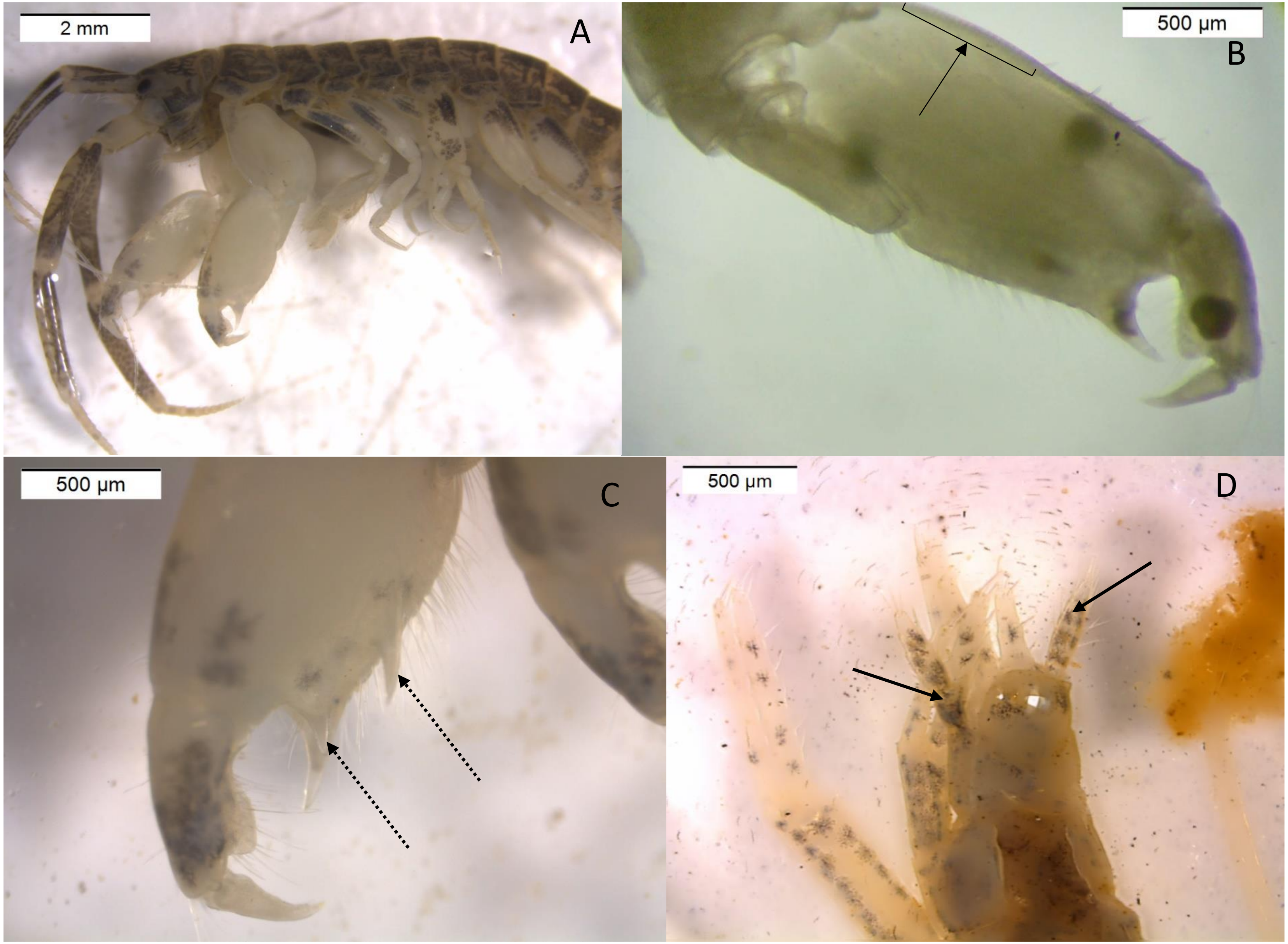
8 Annex



A 1. Correlation between wet and dry algae weight (g), goodness of fit 74%.

Table A 1. Female and males sampled for amphipod species, per season. Ovigerous females per season, in percentage and female male ratios, ratio>1 identify female dominance and vice-versa.

Species Name	Autumn			Winter			Spring			F/M ratio
	F	M	Ovigerous	F	M	Ovigerous	F	M	Ovigerous	
Cymadusa filosa	7	7		16	5	37.5%	365	120	10.7%	2.9
Elasmopus rapax	1	1	100.0%	14	8	14.3%	11	7	63.6%	1.6
Gammarella fucicola	2	1					40	31	35.0%	1.3
Gammarus insensibilis							7	8	42.9%	0.9
Grandidierella japonica	504	125	18.5%	160	66	20.6%	19	12	15.8%	3.4
Melita palmata	4	1	75.0%	1		100.0%	15	9	6.7%	2.0
Microdeutopus gryllotalpa	37	19	51.4%	94	33	55.3%	203	101	62.6%	2.2
Monocorophium acherusicum	3	5	33.3%	36	49	38.9%	12	4	33.3%	0.9
Monocorophium insidiosum	119	46	34.5%	295	152	55.3%	700	527	61.1%	1.5
Monocorophium sextonae				4	3	50.0%				1.3
Grand Total	677	209		738	397		1373	896		



A 2. *Grandidierella japonica* Stephensen, 1938: male (A). Male gnathopod with arrow and bracket showing the stridulating organs (B) and male gnathopods with dotted arrows showing the teeth on the carpus (C) and posterior body end with arrows showing uniramous uropod 3 (D).