

Valorisation of agricultural and mussel waste through the development of experimental diets and analysis of their effect on the growth of the polychaete *Hediste diversicolor* (O.F. Müller, 1776)

Diogo Miguel Carreiras Teles

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Abstract

The agriculture and aquaculture industries are essential for food production, but they also produce waste that can harm nearby ecosystems. Therefore, new strategies must be created and adopted to valorise the different types of waste produced by various industries, in line with the principles of the circular economy.

The polychaete *Hediste diversicolor* is a commercially valuable organism used as fishing bait and supplementary aquaculture feed. Furthermore, this species has been utilised by several researchers as a means of recycling waste from various species within the context of aquaculture production, as well as waste from biogas production. This study aimed to use *H. diversicolor* to transform two types of waste — olive oil production waste (wet olive pomace) and mussel production waste — into valuable biomass. To this end, two trials were conducted.

In the first trial, four diets with two different lipid compositions (6% and 12%) and sources (wet olive pomace and rapeseed oil) were tested directly on polychaetes. The second trial involved testing IMTA cultivation of this polychaete species alongside the mussel *Mytilus edulis* through three treatments involving different quantities of cells (1×10^7 , 5×10^7 or 20×10^7 cells per individual) of *Tetraselmis chuii* and *Chaetoceros gracillis* (in a 50:50 ratio). At the end of both trials, the polychaetes from each treatment were captured and weighed to calculate their survival and growth parameters (SR, SGR, AGR, final biomass and mean wet weight).

In the first trial, the survival rate was similar for diets 2 and 4 (respectively 6% and 12% lipids, without wet olive pomace), but significantly lower for diet 3 (12% lipids and with wet olive pomace) compared to the other treatments (p-value < 0.05). The highest growth was observed in diet 2, and the diet with the worst results in relation to these parameters was treatment 3.

In the second trial, there were no high mortality rates in any treatment, and polychaete growth was higher in treatments (2 and 3) with greater amounts of food supplied to the bivalves, with most parameters being statistically higher than in treatment 1 (p-value < 0.05).

Thus, the first trial of this study concluded that wet olive pomace is not an ideal ingredient for a *H. diversicolor* diet. However, further testing of smaller amounts of this waste in polychaete diets

would be necessary to better assess whether this is an effective method of treating this type of waste. The second trial concluded that the polychaete *H. diversicolor* could successfully feed on waste produced by *M. edulis* mussels, and that this type of IMTA has potential for mussel waste treatment, since treatment 3 (20×10^7 cells/ind.) produced good growth parameters.

Keywords: Growth parameters; *Mytilus edulis*; Wet olive pomace; IMTA.

Resumo

As indústrias da agricultura e da aquicultura são essenciais para a produção de alimentos, mas também produzem resíduos que podem prejudicar os ecossistemas próximos. Portanto, novas estratégias devem ser criadas e adotadas para valorizar os diferentes tipos de resíduos produzidos pelas várias indústrias, em conformidade com os princípios da economia circular.

O poliqueta *Hediste diversicolor* é um organismo comercialmente valioso, utilizado como isca de pesca e alimento suplementar para a aquicultura. Além disso, esta espécie tem sido utilizada por vários investigadores como meio de reciclagem de resíduos de várias espécies no contexto da produção aquícola, bem como de resíduos da produção de biogás. Este estudo teve como objetivo utilizar a *H. diversicolor* para transformar dois tipos de resíduos — resíduos da produção de azeite (bagaço de azeitona húmido) e resíduos da produção de mexilhões — em biomassa valiosa. Para tal, foram realizados dois ensaios.

No primeiro ensaio, foram testadas quatro dietas com duas composições lipídicas diferentes (6% e 12%) e duas fontes (bagaço de azeitona húmido e óleo de colza) diretamente em poliquetas. No segundo ensaio, foi testado o cultivo IMTA desta espécie de poliqueta em conjunto com o mexilhão *Mytilus edulis*, através de três tratamentos com diferentes quantidades de células (1×10^7 , 5×10^7 or 20×10^7 células por indivíduo) de *Tetraselmis chuii* e *Chaetoceros gracilllis* (numa proporção de 50:50). No final de ambos os ensaios, as poliquetas de cada tratamento foram capturadas e pesadas, de modo a calcular os respetivos parâmetros de sobrevivência e crescimento (SR, SGR, AGR, biomassa final e peso médio húmido).

No primeiro ensaio, a taxa de sobrevivência foi semelhante para os poliquetas alimentados com as dietas 2 e 4 (6% e 12% de lípidos, respetivamente, sem bagaço de azeitona húmido), mas significativamente inferior para a dieta 3 (12% de lípidos e com bagaço de azeitona húmido) em comparação com os outros tratamentos (p -value < 0,05). O maior crescimento foi observado nos poliquetas alimentados com a dieta 2 e os piores resultados em relação a estes parâmetros foram verificados nos poliquetas alimentados com a dieta 3.

No segundo ensaio, não se registaram taxas de mortalidade elevadas em nenhum tratamento e o crescimento dos poliquetas foi mais elevado nos tratamentos 2 e 3, que consistiram na administração de maiores quantidades de alimento aos bivalves, com a maioria dos parâmetros a apresentar valores estatisticamente superiores aos do tratamento 1 (p-value < 0,05).

Assim, o primeiro ensaio deste estudo concluiu que o bagaço de azeitona húmido não é um ingrediente ideal para a dieta do *H. diversicolor*. No entanto, são necessários mais testes com quantidades menores deste resíduo nas dietas dos poliquetas para avaliar melhor a eficácia deste método de tratamento. O segundo ensaio concluiu que o poliqueta *H. diversicolor* consegue alimentar-se com sucesso dos resíduos produzidos pelos mexilhões *M. edulis* e que este tipo de IMTA tem potencial para o tratamento de resíduos de mexilhões, visto o tratamento 3 (20×10^7 células/ind.) ter produzido bons parâmetros de crescimento.

Palavras-chave: Parâmetros de crescimento; *Mytilus edulis*; Bagaço de azeitona; IMTA.

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List of Acronyms:

e.g.- for example

IMTA- Integrated Multi-Trophic Aquaculture

RAS- Recirculating Aquaculture System

SGR- Specific growth rate

AGR- Absolute growth rate

SR- survival rate

EPA- Eicosapentaenoic acid

DHA- Docosahexaenoic acid

MARE-IPLeiria- Marine and Environmental Sciences Centre at the Polytechnic University of Leiria.

pH- Potential of Hydrogen

DM- Dried matter

1. Introduction

1.1. Some of the major challenges in today's society

Today's society faces many challenges, but two of the most pressing and globalized issues are hunger and waste disposal from various industries. Despite progress in recent years, around 821 million people were affected by food shortages in 2024, jeopardizing the United Nations' goal of ending hunger by 2030 (Wirajing *et al.*, 2024). It is estimated that around 95% of the food consumed worldwide is produced through agricultural activity, including crops and animal products (Fuglie *et al.*, 2024). However, this activity is also responsible for a quarter of greenhouse gas emissions, as well as the contamination of surface and groundwater with fertilizers and pesticides, and the production of other harmful waste products (Fuglie *et al.*, 2024).

Furthermore, the global population is growing and is expected to reach 9.7 billion by 2050 (United Nations, 2019), meaning that demand for food will also increase. Aquaculture is another form of food production that falls under the umbrella of the agricultural sector, and that is currently expanding, which means it can help to meet this food demand. In 2016, this sector surpassed fisheries in terms of catches for human consumption (FAO, 2020a) and by 2022, it accounted for 51 per cent of the total weight of aquatic animals consumed by humans (FAO, 2024).

In the European Union, productivity per hectare of agricultural production has increased considerably due to irrigation systems, monoculture farming, and the increased use of pesticides and fertilizers. This has generated greater amounts of agricultural waste and environmental costs (Bonciu *et al.*, 2021). Aquaculture is in some cases also responsible for multiple negative environmental impacts, usually caused by the emission of effluents into surrounding ecosystems. These impacts include the pollution of water intended for human consumption, the eutrophication and nitrification of surrounding water ecosystems, and even the release of drugs through inadequate medication practices (Avnimelech, 2009; Martinez-Porchas & Martinez-Cordova, 2012).

Therefore, given the current outlook, it is crucial to not only increase food production but also improve the efficiency of the various processes involved and create waste reduction strategies.

1.2. Subjects covered in this work

1.2.1. Olive oil industry

Olive oil is one of the world's most widely recognised high-value agricultural products, and Europe is its largest producer, with around 4 million hectares of olive groves accounting for 67% of global production (Rapa & Ciano, 2022).

Olive oil production has several stages such as cultivation and harvesting, extraction of the oil from the olives, packaging and waste treatment (Cappelletti *et al.*, 2017). The type of olive mill waste produced depends on the extraction method used. Three-phase extraction generates olive oil, olive pomace (comprising olive stones, skins and pulp) and olive mill wastewater, whereas in two-phase extraction, only olive oil and wet olive pomace (a mixture of olive pomace and olive mill wastewater) are produced (Brito, 2016).

In Portugal, the most widely used extraction method is the two-phase method, which accounts for around 87% of national production, and although this method results in less wastewater, the wet olive pomace produced by it is more difficult to process, recycle and add value to (Muniz, 2021). This virgin pomace generally poses a challenge when it comes to its disposal, as it is challenging to handle due to the elevated levels of mill wastewater present in the composition. It also dries out very slowly, becoming a source of environmental pollution and having a substantial impact on land and water environments (Caputo *et al.*, 2003; Alfano *et al.*, 2011).

Several studies have already proven the existence of these negative effects on microbial soil populations (Paredes *et al.*, 1987; Sidari *et al.*, 2010), aquatic ecosystems (DellaGreca *et al.*, 2001), and the air (Rana *et al.*, 2003). The direct application of liquid and solid olive mill waste to soil has a detrimental effect on its structural stability, microbial activity, and plant germination and growth (Tejada *et al.*, 1997). This is mainly due to the waste's phytotoxic and antimicrobial phenolic compounds, and its fatty acid content and organic composition (González *et al.*, 1990; Riffaldi *et al.*, 1993; Ciafardini & Zullo, 2003; Albuquerque *et al.*, 2004; Roig *et al.*, 2006).

For all the previously mentioned reasons, the olive oil industry has long struggled with waste disposal (Nalbantoglu & Tawfiq, 2006). However, many of these waste products have an interesting nutritional profile or other useful properties (antioxidants, antibacterials, fatty acids, etc.) and can be reused as by-products. Nowadays there are other ways to reuse and add value to this residue, such as generating thermal and/or electrical energy, extracting olive cake, reusing the waste as a soil improver, and reusing its water and nutrients (e.g. as livestock feed), or even other possibilities like extracting or generating by-products with high added value, such as antioxidants, enzymes and activated carbon (Sánchez-Sánchez, 2020).

Although there are already possibilities for reusing olive mill waste, most of these do not happen on an industrial scale and are still in the development stage. In the coming years, olive oil production is expected to increase in southern Europe, and since existing solutions for dealing with waste resulting from olive oil extraction have only alleviated the problem so far, a huge environmental issue is anticipated regarding the management of this type of waste (Sánchez-Sánchez, 2020).

1.2.2. Mussel and other bivalve aquaculture waste

In terms of volume, the bivalve and aquatic organisms with the highest production in Europe are mussels (*Mytilus edulis* Linnaeus 1758) and Mediterranean mussels (*Mytilus galloprovincialis* Lamarck 1819), with 533 000 tonnes produced in 2018 (Hough, 2022). Unlike finfish farming, which requires nutrient-rich feed, shellfish farming, such as mussel and oyster aquaculture, relies more on phytoplankton and the organic matter found in the environment (Burkholder & Shumway, 2011). Consequently, the adverse environmental effects of bivalve aquaculture are generally considered to be less severe than those of salmon farming (Mirto *et al.*, 2000; La Rosa *et al.*, 2002; Yokoyama, 2002; Crawford *et al.*, 2003).

The impact of shellfish farming on nutrient cycling and food web dynamics is a topic of mixed reports. Some authors even argue that farms with high bivalve densities have a positive effect on controlling eutrophication, despite the addition of bio-deposits rich in organic matter (such as bivalve faeces and pseudofaeces) to the bottom sediments, and that mussel aquaculture represents a self-regulating production system (Folke & Kautsky, 1989).

Others have suggested that shellfish cultures may increase eutrophication due to the high regeneration of nutrients by shellfish (Baudinet *et al.*, 1990), by decreasing nutrient limitation and stimulating algal growth rates (Prins *et al.*, 1998). While this effect (eutrophication) is usually aggravated in the area by the decreased water movement and current velocity caused by the structural characteristics of shellfish farms (Burkholder & Shumway, 2011).

Both hypotheses are valid. In moderation, enriching the aquatic environment with nutrients promotes beneficial increases in the production of phytoplankton and benthic algae, which in turn increases the production of zooplankton, macroinvertebrates, finfish and bivalves, as these species feed on the primary producers (Reitan *et al.*, 1999; Zeldis *et al.*, 2008; Burkholder *et al.*, 2008). However, if nutrients are added in excess, this can cause algal overgrowth, and at night, the resulting oxygen depletion in the water due to algae respiration can have a negative effect on the other organisms living in the ecosystem. Furthermore, the bio-deposits of organic matter produced by bivalves can be subject to decomposition by fungi and aerobic bacteria, which in turn can also result in oxygen depletion (Burkholder & Shumway, 2011).

If this over-enrichment becomes chronic, it can lead to significant changes in ecosystem functioning. This often results in high biomass of a few tolerant species and a loss of overall biodiversity (Burkholder & Shumway, 2011). In other words, shellfish farms with high stocking densities located in poorly flushed coastal environments can alter the energy flows of the ecosystem by depleting phytoplankton, zooplankton and Seston from the water column through filter feeding. They can also alter the energy flows by causing high sedimentation through the biodeposition of their faeces and pseudofaeces (Callier *et al.*, 2008).

One increasingly popular method of helping to solve this problem is integrated multi-trophic aquaculture (IMTA). In this type of system, uneaten feed and nutrient losses can be recaptured by other organisms and converted into valuable nutrients for harvestable seafood and crops (Chopin, 2013).

1.2.2.1. Blue Mussel *Mytilus edulis* biology and ecology

1.2.2.1.1. Distribution and brief description

The blue mussel (*M. edulis*), also known as the common mussel (Figure 1.1), is a bivalve mollusc belonging to the Mytilidae family and the Mollusca phylum. Its shell is equivalve and of an elongated nature, displaying a brownish or bluish hue and with a pointed and curved umbo, and adults can have a size between 30 and 140 mm (de Oliveira, 2012). This organism has a lifespan ranging from 18 to 24 years (FAO, 2009), and it is one of the longest harvested marine organisms, since there is evidence of blue mussel shells being present in kitchen middens with dates going back to 6000 BC.



Figure 1.1- Blue mussel *Mytilus edulis*.

This organism is found in nature on a global scale. It is native to the North Atlantic Ocean, specifically the area stretching from Newfoundland and Iceland to the White Sea, Cape Hatteras in North Carolina, and the Bay of Biscay on the French–Spanish border (Hilbush *et al.*, 2000). However, due to its production in aquaculture and its accidental transport in ballast water, this species is now found in various areas of the world in both the southern and northern hemispheres, and hybridisation with other species of the genus *Mytilus* can also occur (Hilbush *et al.*, 2000; Westfall *et al.*, 2010).

In Portugal, it occurs naturally, particularly along the northern and central Atlantic coast. It is less common in the Algarve (de Oliveira, 2012). However, although less abundant, it is still found in southern Portugal, where hybridisation with *Mytilus galloprovincialis* has been observed (Hilbush

et al., 2000). As mentioned above, this organism is cultivated in aquaculture facilities in Europe and other parts of the world, including Chile, which makes it economically important globally (Westfall *et al.*, 2010).

1.2.2.1.2. Habitat and ecological role

The blue mussel dwells in the area extending from the high intertidal to subtidal zones and can even be encountered at depths of up to 40 metres (de Oliveira, 2012; FAO, 2009). They have a benthic lifestyle, living attached to rocks or other solid surfaces through byssal filaments produced by their feet (de Oliveira, 2012). These organisms are filter feeders that consume phytoplankton and other organic matter suspended in the water (Ramalho, 2022).

They are also highly tolerant of changes in salt levels (being euryhaline), dryness, heat (being eurythermal) and oxygen, meaning it can easily live in a broad range of microhabitats from estuaries (brackish water) to the open sea (salt water), in areas where the temperature is between 5-20 °C (FAO, 2009). Since *M. edulis* is so resistant to climatic variations, it is thought that its distribution is mainly controlled by biological factors such as predation and competition for food. In the absence of predators, subtidal aggregations of these organisms can be up to 1.2 metres thick (FAO, 2009).

In this way, mussels are important organisms in terms of the interaction between the water column and the benthic environment since they live in high densities, are highly adaptable and feed through filtration, increasing the sedimentation of particulate matter (Prins & Smaal, 1994). Throughout their life cycle, they also serve as an important food source for many organisms, including various species of jellyfish, starfish, conchs, crabs and seabirds, which prey on them (Kitching *et al.*, 1959).

1.2.2.1.3. Reproduction, life cycle and aquaculture

This species is gonochoric and reproduces externally, releasing its gametes into the water column. In nature, the reproductive cycle occurs from March to April and from September to October, with two peaks in gamete release: one in spring and one in autumn (Silva & Batista, 2008).

Approximately 24 hours after fertilization, the embryo develops into a lecithotrophic trochophore larva, e.g. a larva that still obtains energy from the yolk, and after a few more days, this trochophore larva becomes a veliger (or D-larva) (Gazeau *et al.*, 2010). The veliger larvae feed's on phytoplankton for several weeks to gain weight before reaching the pediveliger stage, and once at this stage, they search for a suitable location to settle (Gazeau *et al.*, 2010). When the conditions for settlement are favourable, metamorphosis occurs and the post-larvae attach themselves to the substrate through secreting byssus filaments, it is also at this point that juveniles start to secrete their adult shells (Gazeau *et al.*, 2010). This is one of the most critical stages in the life cycle of many bivalves, and in the case of aquaculture, it marks the beginning of the production cycle.

In 2017 bivalve production in Portugal contributed 48.5 million euros in a total of 83.2 million euros, which is 58 % of the total value of aquaculture production in the country (FAO, 2018; Brito *et al.*, 2023). In terms of volume, the bivalve and aquatic organisms with the highest production in Europe are mussels (*M. edulis*) and Mediterranean mussels (*M. galloprovincialis*), with 533 000 tonnes produced in 2018 (Hough, 2022).

In most aquaculture production systems around the world, the *M. edulis* spat is obtained through nature since the supply is generally abundant (FAO, 2009), however nowadays more hatcheries are producing juveniles (so called seed) of this species, particularly in countries such as Portugal where bivalve aquaculture is a major activity. Producing spat in this way has multiple advantages, such as a faster and more efficient production cycle, reduced exposure to potential contaminants and pathogens, and precise delivery of feed to organisms, maximising growth and minimising waste.

In these hatcheries, the breeding mussels undergo conditioning and, once mature, are subjected to thermal shock/stripping to release their gametes (FAO, 2009). The larvae are fed *ad libitum* until they settle on ropes, thus growing much faster than in nature. After settling, the mussels are transferred to a nursery where they remain until they reach 6-10 mm (seed) and are then transferred outdoors into grow-out systems (FAO, 2009).

These grow-out systems can be of several different types, each with its own principles and techniques, some examples are on-bottom cultures, Bouchot cultures, longlines and rafts (FAO,

2009). Most commercial facilities take approximately two years for growing the mussels until they reach commercial size (FAO , 2009).

1.3. IMTA aquaculture

IMTA systems, or integrated multi-trophic aquaculture systems, have become increasingly popular over the last decade due to consumers' growing concern for sustainability/Circular Economy, which is nothing more than minimising waste and keeping resources in use for as long as possible. In a typical fish farm feed wastage can reach up to 38%, making it one of the most significant sources of pollution in the industry (Hossain, 2022). These systems have therefore been used to mitigate the excessive nutrient and chemical load resulting from intensive aquaculture farms, as well as to maximise the use of limited space (Neori *et al.*, 2004; Sara, 2007).

These consist of circular production systems in which species at different trophic levels — such as fish, shellfish, and algae — are produced simultaneously and strategically to minimize the environmental impact of aquaculture (Checa *et al.*, 2024). These species are classified into three groups: fed species, which are provided with external feed such as fish and shrimp; organic extractive species, such as filter feeders or deposit feeders that feed on organic waste (polychaetes, sea cucumbers); and inorganic extractive species, such as algae or aquatic plants (Troell *et al.*, 2009) . Combining different trophic levels in an IMTA means that uneaten feed and nutrient losses will be recaptured by other organisms and converted into valuable products, such as biomass that is rich in essential fatty acids and harvestable seafood (Checa *et al.*, 2024).

The IMTA concept is more commonly applied in land-based aquaculture, and most studies have been focused on this area (Troell *et al.*, 2009). However, there are also many examples of IMTA in offshore aquaculture, although most of these are in relatively calm waters (Buck *et al.*, 2018; Troell *et al.*, 2009).

In land-based aquaculture, the extractive species is placed either in the same tank as the fed species or in a separate tank containing water rich in organic matter, while in offshore aquaculture, the extractive species is kept either on the seabed or suspended around the cages of the fed species. There are also decoupled IMTA systems, in which waste from one fed species is collected and preserved before being supplied to extractive species in another location.

1.4. Polychaete *Hediste diversicolor*

1.4.1. *Hediste diversicolor* biology and ecology

1.4.1.1 Distribution and brief description

The common ragworm (*H. diversicolor* O.F. Müller, 1776) (Figure 1.2) is a polychaete annelid belonging to the Nereididae family and the Phyllodocida order. First described by Müller in 1776, this species has several common names in the different places it inhabits, including Serradela and minhoca nacional in Portugal. It is widely distributed in the North Atlantic and can be observed along the north coast of the American continent, the European coast, and in the Mediterranean, Caspian, and Black Seas (Scaps, 2002; Virgilio & Abbiati, 2004).

This annelid has a highly variable body size but in typical natural conditions, adults grow to between 6 and 12 cm in length (Scaps, 2002). Its reversible proboscis is quite characteristic (Figure 1.3), and the oral and maxillary rings of this proboscis have paragnaths (small chitinous denticles) (Scaps, 2002). Behind these structures is a subtriangular prostomium (dorsal plate relative to the mouth), where its four eyes, two large palps, and two small frontal antennae are located (Scaps, 2002). Following the prostomium is the peristomium, the first true segment. Unlike the other body segments, which have parapodia (locomotive appendages), the peristomium has four tentacular cirri (Scaps, 2002).



Figure 1.2- Two *Hediste diversicolor* individuals.

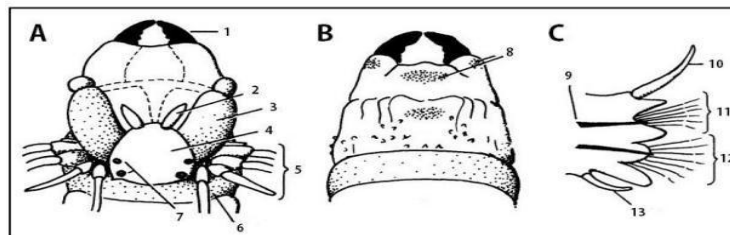


Figure 1.3 – Representative morphology of the polychaete *Hediste diversicolor*. A and B - head with everted proboscis (dorsal and ventral positions, respectively); C - parapod; 1- mandibles; 2- frontal antennae; 3- palpus; 4- prostomium; 5- tentacular cirri; 6- peristomium; 7- eyes; 8- paragnaths; 9- aculeus; 10- dorsal cirrus; 11- notopodium; 12- neuropodium; 13- ventral cirrus (Taken from Bagarrão, 2013; originally adapted from Blake & Ruff, 2007).

1.4.1.2. Habitat and ecological role

This species of polychaete is highly adaptable, as it can tolerate variations in salinity and temperature and is resistant to low oxygen levels (Fidalgo e Costa *et al.*, 2006). Due to its adaptability, it is found in both marine and brackish waters (Virgilio & Abbiati, 2004). It has a benthic lifestyle, inhabiting various substrates such as sandy mud, but also gravel, clay, and even turf, where they build U- or Y-shaped burrows that can reach a depth of about 30 cm (Kristensen & Mikkelsen, 2003; Scaps, 2002). The depth of the galleries mainly increases in line with the size of the individual, and in nature it also varies according to the water temperature (Scaps, 2002).

In nature it lives in densities that can vary from 35 to 3700 individuals per square meter (Durou *et al.*, 2008). This species has a major influence on the distribution and fate of matter and oxygen at the water-sediment interface and within the sediment due to its high population density and the various active bioturbation processes it carries out, such as the construction of biogenic structures, irrigation of its galleries, production of faecal pellets and sediment homogenization (Duport *et al.*, 2006).

As sediment is a major source of chemicals introduced into the aquatic environment by humans, benthic species lives near these compounds and are therefore particularly susceptible to chemical stress (Durou *et al.*, 2008). For that reason, this species is highly adaptable, even in contaminated environments, as it can develop resistance to toxins by producing enzymes or proteins that detoxify them (Pedro *et al.*, 2022). Nevertheless, this defence mechanism uses up energy that would normally be used for growth, maintenance and reproduction. (Pedro *et al.*, 2022).

Thus, the *H. diversicolor* species is highly important for the functioning of the ecosystems it inhabits. Individuals affect the biogeochemical cycles of nutrients and contaminants in the substrate and represent an important food source for various species of fish, crustaceans and birds (Durou *et al.*, 2008; Fidalgo e Costa, 2001).

1.4.1.3. Feeding habits

This species is also highly adaptable in terms of its feeding methods, employing strategies ranging from detritivory, herbivory, carnivory and filtration feeding. When it comes to detritivorous feeding, *H. diversicolor* employs two strategies (Fidalgo & Costa *et al.*, 2006; Scaps, 2002).

The first consists of crawling on the sediment surface, prospecting for food and ingesting it immediately (Scaps, 2002). The second involves capturing food through mucous secretions located on either side of the worm's body. When the worm retreats into the burrow, it brings the mucous back, leaving a food/mucous pellet near the burrow, then it ingests the pellet progressively within its burrow (Scaps, 2002).

Then there is the filtration-feeding method, which occurs when the concentration of suspended phytoplankton is very high, individuals release a mucus net into the gallery and perform undulating

movements at its base (Riisgård & Larsen, 2010). These movements encourage continuous water flow, enabling phytoplankton suspended in the water column to be sucked into the burrow. (Bagarrão, 2013; Fidalgo e Costa *et al.*, 2006; Riisgård & Larsen, 2010). This net is then collected and swallowed by individuals who feed on the particles captured by it (Riisgård & Larsen, 2010).

As well as the strategies described above, members of the *H. diversicolor* species are still able to behave in a herbivorous way, feeding on a variety of algae, and in a carnivorous way, feeding on various small species that make up the benthic fauna (Bagarrão, 2013; Fidalgo e Costa *et al.*, 2006; Riisgård & Larsen, 2010).

1.4.1.4. Reproduction and life cycle

This species of ragworms is gonochoristic (Rangel, 2008) and has a semelparous reproduction pattern meaning that it only reproduces once in their lifetime and then it dies (Bagarrão, 2013; Scaps, 2002). Sexual maturation is temperature-dependent, and spawning is synchronised with the lunar cycle, typically occurring during a full or new moon (Rangel, 2008). This takes place when individuals are between one and two years old (Scaps, 2002).

During the maturation process, differences begin to appear between sexually mature males and females. Those that have not yet reached maturity are reddish-brown in colour, while sexually mature males are bright grass-green (due to the white mass of sperm they store in their coelom), and females are a much darker shade of green (Scaps, 2002)

Unlike other species of Nereididae, which undergo metamorphosis to transform into the typical heteronereid epitokous form (a specialised pelagic reproductive form), *H. diversicolor* remains atokous throughout its life (Aberson *et al.*, 2011; Scaps, 2002). The females spawn inside the gallery, while the males release their gametes into the water near the female's gallery. The females then increase their ventilatory activity — the same activity they use to filter phytoplankton — to bring the sperm into their galleries and fertilise the oocytes (Bagarrão, 2013; Scaps, 2002). The fertilized oocytes remain inside the burrow and, during incubation, the trochophore larvae (semi-pelagic) and post-larvae remain underneath the female's body for at least ten days (Scaps, 2002).

They are protected during this period and feed on the mucus and microbial biofilm found on the burrow walls (Bagarrão, 2013; Bartels-Hardege & Zeeck, 1990). This goes on until they reach the 7 or 8 segments stage, and the female dies. Around this phase larvae become completely benthic having a 3-setiger erpochaete stage (Scaps, 2002). When the larvae reach the six-setiger stage, they begin to dig burrows. Finally, when they reach the twelve- to fifteen-setiger stage, their feeding and territorial behaviours closely resemble those of adults and they are considered juveniles (Scaps, 2002).

1.4.1.5. *Hediste diversicolor* aquaculture

As we observed in earlier sections, this species is highly resilient to ecological variations, thrives in high numbers in its natural habitat, grows rapidly, and possesses a diverse diet. In addition, this species has economic value in several countries, where it is sold as live bait for recreational fishing, and it has also been used as supplementary feed for some species of fish and crustaceans in aquaculture production (Cole *et al.*, 2018; Pombo *et al.*, 2020; Rasines *et al.*, 2023), and it has the potential to be used as a complement to for finishing diets of farmed marine organisms (as a source of essential fatty acids) (Marques *et al.*, 2018).

These characteristics make this species ideal for aquaculture production: it does not require much technology or maintenance, it grows quickly, it eats almost anything, and there is already a market for it. On top of it, there is already a great deal of information available on its production conditions, due to the number of scientific articles written on this species, encompassing specifics such as production densities, categories of feed and substrate depth, the induction of sexual maturation, and others (Nesto *et al.*, 2012; Santos *et al.*, 2016; Wang *et al.*, 2019b).

1.4.2. Utilization of *Hediste diversicolor* in waste treatment

H. diversicolor is a species used in a wide variety of scientific experiments by researchers from different fields, because it's abundant, easy to maintain, resistant to various pollutants and plays a very important role in the ecosystem.

However, this polychaete has particularly distinguished itself in the field of bioremediation, especially of aquaculture waste. This is because it is a species of low trophic level that can digest

various types of food, including waste and bacteria (Wang *et al.*, 2019a), making it ideal for feeding on different types of waste. This bioremediation is usually carried out through Integrated Multi-trophic Aquaculture (IMTA), either decoupled or coupled. Several authors have already tested various types of waste, including sea urchin waste, smolt and post-smolt salmon waste, and biogas production waste (Anglade *et al.*, 2023; Santos *et al.*, 2025; Wang *et al.*, 2019a).

Galasso *et al.* (2020) estimated that, in the case of an aquaculture facility producing 20 tonnes of sea bass *Dicentrarchus labrax* (Linnaeus, 1758), it would be possible to reduce waste produced by around 2–9% with high densities of *H. diversicolor* (3,700 ind./m²). In other words, producing these polychaetes alongside other species in IMTA will improve the production system's water quality, reduce effluent production and transform waste products into valuable polychaete biomass containing high levels of essential fatty acids and proteins (Marques *et al.*, 2018), making aquaculture more sustainable.

1.5. Aims of the study

This study's objective was to evaluate the potential for using the polychaete *H. diversicolor* to convert waste (more specifically, agricultural waste—wet olive pomace—and mussel production waste) into valuable biomass. Two trials were carried out to answer this question, each focusing on a different waste product: wet olive pomace and mussel waste. The two trials were respectively:

The first trial involved testing the incorporation of wet olive pomace into experimental diets (without animal protein) for *H. diversicolor*, to determine the effect of varying amounts of wet olive pomace on their survival and growth.

The second trial involved developing an integrated multitrophic aquaculture system incorporating *M. edulis* mussels and testing the compatibility of producing these two trophic levels together in order to understand whether it is possible to grow this species of polychaete from the waste of this mussel.

In both trials, the effectiveness of the different treatments was evaluated by calculating survival and growth parameters (survival rate, final weight, SGR, AGR and final biomass). Both topics

researched in each trial are relatively unexplored in the current literature and focused on creating more environmentally friendly and efficient waste disposal methods.

2. Materials and methods

2.1. Reproduction for obtaining juveniles

2.1.1. Preparation of substrate and breeding tanks

The first step in obtaining juveniles for the experimental trial, was to collect sand that was used as sediment for the polychaetes, as this type of substrate is one of the most used by other authors (Costa, 1999; Santos *et al.*, 2025). The sand was collected in Cova da Alfarroba beach (Peniche), and was subsequently transported to the Cetemares building (MARE-IPLeiriria) where it was subjected to a sifting process using two sieves with decrescent apertures (2 and 0.5 mm respectively) to remove the larger particles, such as pieces of shells and stones, and to obtain only sand with a particle size of less than 500 μm or 1 to 2 phi (Krumbein phi scale) (Costa, 1999). This sand was prepared in large quantities so that there would be enough to use in the experimental trial.

The substrate was disinfected using a sodium hypochlorite and water solution (approximately 1 mL per L of water) (Ferreira, 2009), accompanied by sporadic stirring over a period of two days. This was followed by five days of strong aeration and frequent water exchange, which facilitated the evaporation of the sodium hypochlorite and removed most of the remaining detritus.

The broodstock was obtained from a local fishing shop by buying 3 boxes of bait (*H. Diversicolor*) and selecting the 60 individuals that looked the healthiest. These broodstock were weighed (wet weight) using a precision scale (Sartorius TE1245; Sartorius Corp., Gottingen, Germany; ± 0.1 mg) and transferred to five rectangular 50 L tanks (40*40*40 cm) (Figure 2.1), each with a 10 centimeter layer of substrate (Santos *et al.*, 2025), at a 88 ind./m² (Santos *et al.*, 2016).

This resulted in 14 polychaetes that were conditioned for one month and fed daily with ground Biomar Introplus MT 1.5 mm feed. The daily amount of feed started at 3% of their wet weight, and

that value was increased by 3% each day compared to the previous day, this method was adapted from Rasines *et al.*, 2023.



Figure 2.1- 50 L flat-bottom fiberglass tanks used for *Hediste diversicolor* reproduction and growth of larva and juveniles.

2.1.2. Spawning induction and juvenile selection

Following the acclimation period, spawning was induced by thermal shock (5.0 ± 0.5 °C) (Santos *et al.*, 2016), and the first juveniles were observed in the substrate after 20 days (Figure 2.2). Juveniles were fed ad libitum for 60 days, meaning that a small amount of food (Biomar Introplus MT 1.5 mm) was always available throughout the surface of the substrate. Following this rearing period, juveniles were selected by siphoning small patches of sand from the tanks onto a tray and individuals of similar size (± 20 mg) were selected using a plastic Pasteur pipette.

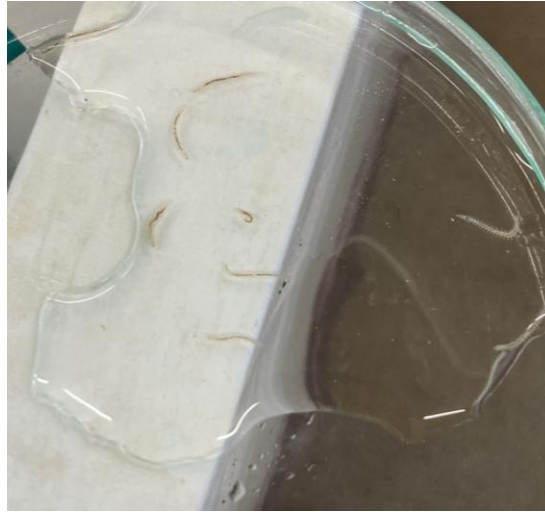


Figure 2.2- Hatchery reared juvenile *Hediste diversicolor* individuals in a petri dish.

2.2. Nutritional trial with wet olive pomace diets

2.2.1. Ingredient preparation

To analyze the possibility of the polychaete *H. diversicolor* feeding on wet olive pomace, four treatments which consisted in different diets, were prepared. These were made with strictly plant-based ingredients, in line with the aim of using waste from the agricultural industry. They were respectively:

-Wet olive pomace (provided by Cooperativa Agrícola de Ervedal e Figueira e Barros, C.R.L.) (Figure 2.3) as one of the sources of lipids; -Fresh carrots as the source of beta-carotenes (Pingo doce); -Pea protein (Isuari) served as a source of protein; - Cooked maize (Pingo Doce), which is a very rich source of carbohydrates; -Rapeseed oil (Byodo Rapsöl), as a source of lipids; - Agar-Agar powder (Algamar ®).



Figure 2.3- Wet olive pomace after some time in the laboratory oven.

To make the wet olive pomace easier to use in the diets and store, it was placed in thin layers in plastic trays and dehydrated in a Binder FD 115 oven at 50 °C for 48 hours to reduce lipid oxidation (Conte *et al.*, 2020; Zhuang *et al.*, 2022). The maize used was ordinary cooked maize, and it was purchased at a supermarket.

To preserve the carrots and increase their shelf life, they were first grated with the skin on and placed in plastic trays. They were then dehydrated in an oven at 50 °C for 12 hours (Demiray & Tulek, 2017). The dehydrated wet olive pomace and carrots were stored in the feed room, which is located in the Cetemares building and has temperature and humidity control, to ensure they were preserved in the best possible conditions. The remaining ingredients were stored in their original packaging without requiring any further treatment.

2.2.2. Preparation of the experimental diets

The 4 jellified diets were designed to be isoproteic and composed roughly of the same ingredients. This planning was done using the proximal compositions on the labels of each ingredient or, in the case of unlabelled ingredients, with information available in the literature.

Two diets were formulated with a lipid content of 6% and two with a content of 12%. Additionally, the source of the lipids differed within each group (wet olive pomace and rapeseed oil). The four

diets used were: Diet 1 (6% of lipids, with wet olive pomace); Diet 2 (6% of lipids, without wet olive pomace); Diet 3 (12% of lipids, with wet olive pomace); Diet 4 (12% of lipids, without wet olive pomace). The diets description and composition is described in the Table II.I.

Table II.I- Feed ingredients and respective stipulated proximal composition (% DM) of the different diets. Diet 1 (6% of lipids, with wet olive pomace); Diet 2 (6% of lipids, without wet olive pomace); Diet 3 (12% of lipids, with wet olive pomace); Diet 4 (12% of lipids, without wet olive pomace).

Feed ingredients (%):	Dietary treatments			
	Diet 1	Diet 2	Diet 3	Diet 4
Dehydrated carrot	5	5	5	5
Pea protein	24.5	24.5	24.5	24.5
Corn	32	67.5	0	60.5
Wet olive pomace	38	0	64.5	0
Rapeseed oil	0.5	3	6	10
Total	100	100	100	100
Stipulated proximate composition:				
Crude fat (% DM)	6.02	6.09	12.45	12.37
Crude protein (% DM)	22.25	22.45	22.31	22.25
Carbohydrates (% DM)	40.44	13.89	57.73	13.05
Total calories (Kcal/100 g)	387.94	392.42	417.6	424.14

These diets were prepared weekly throughout the trial and stored in the fridge. To prepare them, the ingredients were first weighed with the same Sartorius TE1245 precision scale mentioned before (± 0.1 mg) and crushed together in a mortar. A solution of salt water and agar was then prepared in glass goblet, heated until it boiled and then mixed with the dry feed to give it a gelatinous consistency.

The amount of salt water and agar solution (0.4 g of agar to 100 mL of water) used was 2.58 mL per 1 gram of feed (the smallest amount for which the desired consistency was found, by trial and error). Once solidified, the jellified feed was broken into pieces and stored in plastic jars.

2.2.3. Growth trial and systems

The tanks employed had a volume of 60 L, and 5 cm of substrate (which had been previously prepared, as described in the section 2.1.1. of this work) (Narciso & da Fonseca, 2000; Nesto *et*

al., 2012; Santos *et al.*, 2025). These tanks were organised in recirculating aquaculture systems (RAS) (Figure 2.4 and 2.5), with one sump tank for every three polychaete tanks, equipped with mechanical (polyester fabric) and biological (bioballs) filtration, into which the water fell by gravity. A TMC Iberia Reef Pump 10000 DC was used to pump the water back into the tanks at a flow rate of 2.4 L per minute. This meant that the tanks' water volume was renewed multiple times per day. A total of 12 tanks were used, with three tanks (1 system) per diet (Figure 2.4).

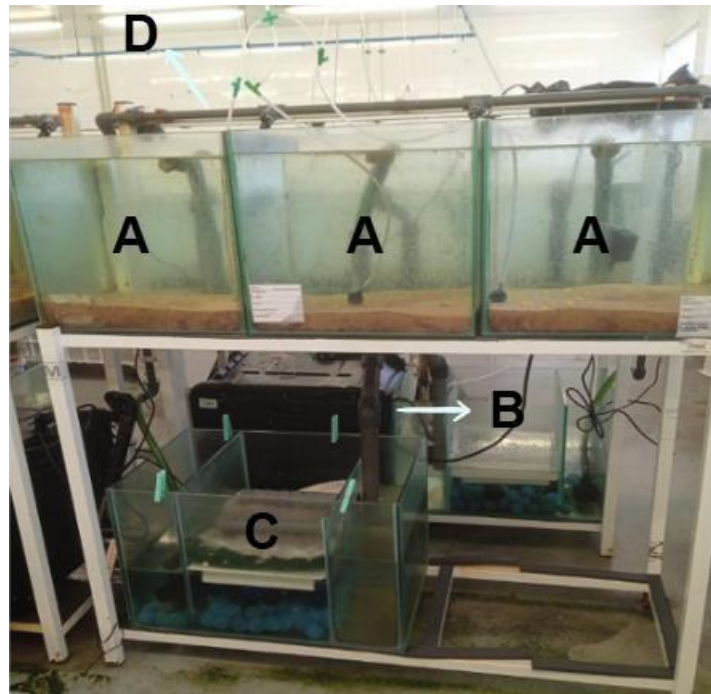


Figure 2.4- Recirculating aquaculture systems used in the trial (A- 60 L tanks; B- Outlet water pipe; C- SUMP tank; D- Inlet water pipe).

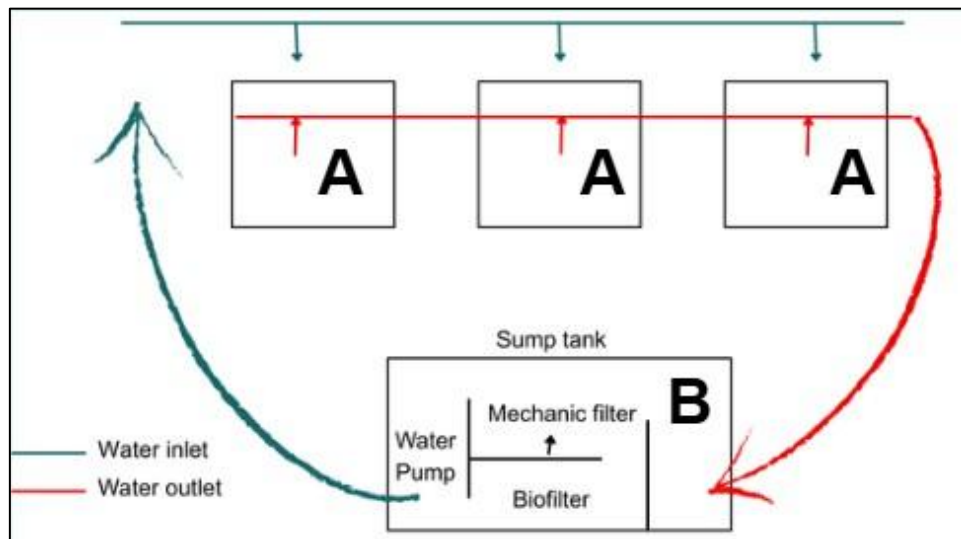


Figure 2.5- Graphical representation showing how the employed RAS function (A- 60 L tanks; B- SUMP tank).

A total of 864 juvenile polychaetes (mean weight: 23.27 ± 0.86 mg) were selected for the experimental trial and divided among 12 tanks at a density of 450 ind./m² (Gilbert *et al.*, 2021; Nesto *et al.*, 2012), meaning that each tank contained 72 polychaetes. Different diets were provided daily, starting at 3% of the total polychaete biomass, and every day the amount of food was increased by 3% compared to the previous feeding. This feeding method was adapted from Rasines *et al.*, 2023. The trial lasted 45 days, which was deemed necessary to avoid extending beyond the maturation period of the polychaetes and thereby risking their reproduction and subsequent mortality.

Water quality parameters were measured every two days with a multiparametric probe (YSI Professional Plus). The average values of temperature, dissolved oxygen, salinity and pH, throughout the trial were respectively 17.5 ± 0.44 °C, 7.48 ± 0.22 mg/L, 35.1 ± 0.63 and 8.36 ± 0.10 , respectively. Nitrogen compounds (ammonia, nitrites and nitrates) were measured weekly with measurement kits (API Mars fishcare Kits), averaging respectively 0.15 ± 0.03 mg/L, 0.8 ± 0.04 mg/L and 11.3 ± 3.53 mg/L, respectively, throughout the trial.

2.2.4. Growth and survival parameters

At the end of the experiment, all the individuals were collected by siphoning the substrate in each tank through 2 mm and 1 mm sieves (Figure 2.6). The polychaetes in each tank were then counted

and weighed together in a Petri dish (± 0.1 mg) to determine the number of living and the total biomass per tank. This was done after the excess water had been removed from the individuals with a paper towel, with the aid of a Sartorius TE1245 precision scale. The survival rate (SR), specific growth rate (SGR) and absolute growth rate (AGR) were calculated as follows:

$$SR(\%) = \frac{N^{\circ} \text{ of individuals at the end of the trial}}{N^{\circ} \text{ of individuals at the start of the trial}} \times 100$$

$$SGR (\% \text{ day}^{-1}) = \frac{(\ln(W \text{ final}) - \ln(W \text{ initial}))}{t} \times 100$$

$$AGR (\text{mg day}^{-1}) = \frac{(\ln(\text{final } W) - \ln(\text{initial } W))}{N^{\circ} \text{ of trial days}}$$

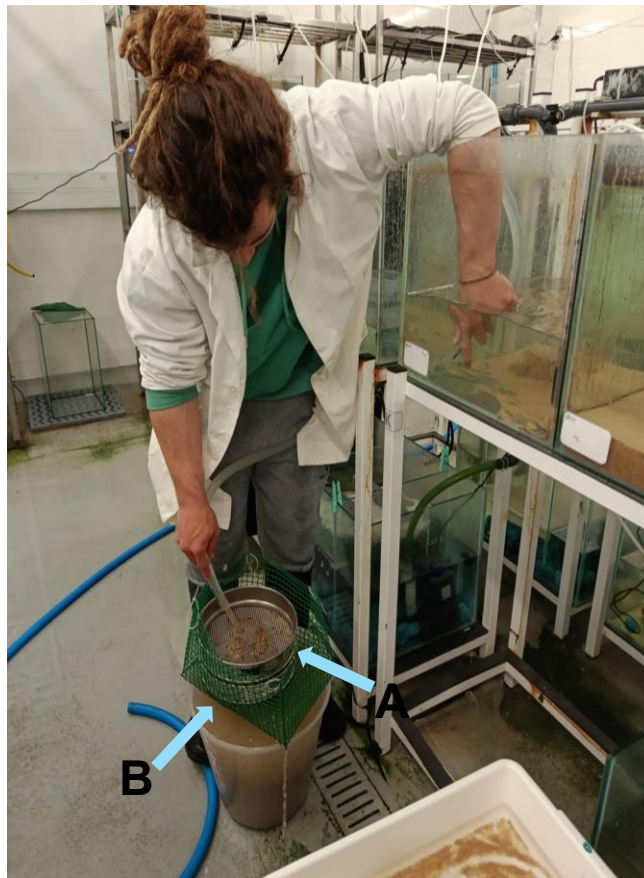


Figure 2.6- Sampling with 2 mm (A) and 1 mm (B) sieves in progress.

2.3. *Mytilus edulis* waste trial

2.3.1. Harvesting and conditioning the mussels

The individuals were captured by manual harvesting in Ponte Velha de Peniche (coordinates: 39.359492, -9.377611) and taken to the Cetemares building, where they were cleaned and sorted. During cleaning, the mussels were first washed in salt water to remove any debris or organisms accidentally transported during capture. Then, the bico filaments were cut to separate and wash the individuals more effectively.

Finally, encrusting organisms were removed from the valves using a knife. The mussels that were too small for the trial were also sorted out. Those selected for use were placed in plastic net baskets, inside a 250 L RAS system consisting of a 200 L rectangular plastic vat with flat bottom, and a 50 L SUMP tank with a mechanic filter and bioballs. This RAS system operated via an air lift, with partial water changes (30% of the total volume) being carried out daily, and the mussels were left for four days without food.

2.3.2. Mussel sampling, cultivation system and experimental trial

During the four-day fasting period, 16 mussels (mean weight: 18.65 ± 5.74 g) were weighed with a precision scale (Kern PCB 2500-2) and distributed in each tank (144 individuals in total) to ensure an even density of 5 kg/m³.

A total of nine tanks were used (the same 60 L tanks used before), and three treatments were tested (three tanks per treatment). The systems and tanks used in this trial were the same as those used in the wet olive pomace trial, with two differences: the addition of skimmers (Bubble-magus) with 300 L/hour skimmer pumps (Sp1000 Bubble-magus) in the sump, specifically in the first compartment. And the addition of plastic baskets to suspend the mussels above the polychaetes (Figure 2.7).



Figure 2.7- A- SUMP tank setup used in this trial (C-Skimmer); B- Plastic basket with mussels.

The mussels were fed the respective amount of food each day depending on their treatment, and the systems were switched off for three hours after the food was distributed so that the individuals could filter out all the food. This diet consisted of a mixture of two species of live microalgae, a diatom and a green alga with a balanced nutritional profile: *Chaetoceros gracillis* (Pantocsek 1892) and *Tetraselmis chuii* (Butcher, 1959) (Harbach & Palm, 2020; Martínez-Pita *et al.*, 2016). In all treatments, the diets contained the same number of cells of each species of microalgae, but the total number of cells varied.

The total number of cells to be fed to each mussel in Treatment 1 (the one with the least amount of food) was adapted from Santos *et al.* (2023), in which individuals were fed a mixture of *Dunaliella tertiolecta* (Butcher 1959), *Chaetoceros calcitrans* (Takano 1968), and *T. chuii*. As the objective of the trial was to cultivate *H. diversicolor* specimens, this value was increased to 1×10^7 cells/day/animal, with even greater quantities of microalgae employed for treatments 2 and 3. All treatments consisted of a 1:1 ratio of *C. gracilis* and *T. chuii* cells in varying quantities of total feed. In summary, treatment 1 consisted of a diet of 1×10^7 cells per mussel; treatment 2, of 5×10^7 cells per mussel; and treatment 3, of 20×10^7 cells per mussel.

The polychaete substrate (sand) was obtained in the same way as described in the section 2.1.1 of this work, with 5 cm used per tank (Narciso & da Fonseca, 2000; Nesto *et al.*, 2012; Santos *et al.*, 2025). However, the polychaete density used in this trial was 300 ind./m² (Nesto *et al.*, 2012; Santos *et al.*, 2025; Wang *et al.*, 2019b) resulting in 48 polychaetes per tank (mean weight: 23.57

± 3.76 mg) which adds up to 432 individuals in total. This reduction in polychaete density was due to lower reproductive success, i.e. fewer juveniles were produced for this trial. The *H. diversicolor* individuals were obtained in the same way as described in chapter 2.1. of this work.

This test also lasted 45 days and, as in the last test, water quality parameters were measured every two days with the same equipment used for this purpose in chapter 2.2.3. (multiparametric probe and nitrogen compounds measurement kits). The average values of temperature, dissolved oxygen, salinity and pH throughout the test were respectively, 20.17 ± 1.26 °C, 7.05 ± 0.29 mg/L, 34.89 ± 0.33 (salinity) and 8.47 ± 0.08 (pH). As in the last test, nitrogen compounds (nitrates, nitrites and ammonia) were also measured weekly, averaging respectively 0.15 ± 0.00 mg/L, 0.43 ± 0.41 mg/L and 14.55 ± 13.85 mg/L, respectively, throughout the trial.

2.3.3. Diet preparation

To provide the mussels with food daily, four 6-litre and two 10-litre cultures (in volumetric flask and carboys) of each microalgae species (*T. chuii* and *C. gracilis*) were prepared prior to the start of the experimental trial. First, smaller cultures were inoculated into 1-litre flasks, with both microalgae species having 1 mL of Nutribloom® (Necton S.A., Portugal) medium per litre of seawater, and *C. gracillis*, being a diatom, also required 1 mL per litre of silicates (Necton S.A., Portugal).

After the concentration of these cultures increased, scale-ups were made into 6 L flasks/10 L carboys (Figure 2.8). The seawater that was utilized came from the borehole of the Cetemares building, it was then autoclaved (Uniclave 88, A. J. Costa Lda, Portugal), or in the case of the carboys, sterilised with sodium hypochlorite (1 mL per litre of water). After 24 hours, this substance was neutralised with an equal amount of sodium thiosulfate (Ferreira, 2009).



Figure 2.8- Microalgae cultures in 6 L flasks (A) and 10 L carboys (B).

As previously explained, the diet consisted of an equal number of *T. chuii* and *C. gracillis* cells (1:1). To prepare it, the number of cells in the cultures was counted daily to determine the required volume for each tank. These counts were obtained using a Neubauer chamber and an analogue counter. After counting, the concentration of the cultures was determined using a simple rule of three:

$$\text{Cell concentration per mL} = \frac{n^{\circ} \text{ of counted cells} \times 1000 (\mu\text{L})}{\text{Volume of counted squares} (\mu\text{L})}$$

The cell concentration of each culture was entered into a previously prepared Excel matrix, which subsequently calculated the volume of culture required from each species (with the formula described below) for each treatment/tank, based on the number of cells per individual and the number of individuals.

$$\begin{aligned} & \text{Volume of microalgae culture needed for each tank (L)} = \\ & = \left(\frac{\text{Desired cell concentration}(\text{cell mL}^{-1})}{\text{Cell concentration of the cultivates on the specific day} (\text{cell mL}^{-1})} \right) \\ & \quad \times N^{\circ} \text{ of mussels per tank} \end{aligned}$$

The calculated volumes were then measured using graduated cylinders and transferred into numbered goblets, which were subsequently provided to the mussels.

Every day, after the required volume had been removed from the cultures, the same amount was replenished with autoclaved seawater. As mentioned above, Nutribloom® medium was added to both species, while silicates were additionally supplied to *C. gracillis*. The health of the cultures was monitored daily to detect possible contamination or reduced microalgal growth, and new cultures were initiated when necessary to replace the older ones.

2.3.4. Growth and survival parameters

At the end of the experiment, the substrate in each tank was siphoned through a 2 mm sieve to collect all the individuals. The polychaetes from each tank were then counted and weighed together in a Petri dish (± 0.1 mg), following the same procedure as in the previous trial. The survival rate (SR), specific growth rate (SGR), and the absolute growth rate (AGR) were then calculated as previously explained in chapter 2.2.4.

The mussels were weighed, and three individuals from each tank were selected for condition index determination. The 27 selected mussels were dissected, having the soft tissue separated from the valves and then placed into pre-weighed and numbered crucibles. The crucibles with soft tissue and valves were dried in an oven (Binder FD 115) at 90 °C for 24 hours, after which they were reweighed. The condition index was then calculated using the following formula (Pantea, 2020):

$$CI = \frac{\text{Dry flesh weight (mg)}}{\text{Shell weight (mg)}} \times 100$$

2.4. Statistics of both trials

To verify the existence of statistical differences in the growth and survival parameters of polychaetes between the different treatments in both trials, a one-factor analysis of variance (ANOVA) was performed. First, the Shapiro-Wilk normality test and Levene's homogeneity of variances test were used to verify that the assumptions inherent to ANOVA (data normality and

homogeneity of variances) were met. If these assumptions were not met, a non-parametric Kruskal–Wallis test was applied instead.

If statistical differences were found following these analyses, multiple comparison tests were applied. Specifically, the Tukey test was used if the assumptions of ANOVA were met; otherwise, a Mann–Whitney U test with Bonferroni correction was used for Kruskal–Wallis tests. These calculations were performed using IBM SPSS Statistics 29.0.1.0 software (IBM Corporation, Armonk, New York, U.S.). All results were considered statistically significant at a 5% significance level (p -value < 0.05) and are presented as “mean \pm standard deviation”.

3. Results

3.1. Results from the trial with wet olive pomace diets

The survival rate (Table III.I) was very similar for diets 2 (98.60 ± 1.4 %), 4 (98.60 ± 2.42 %), and 1 (94.47 ± 4.99 %), but was significantly lower in diet 3 (35.20 ± 5.24 %) compared with the other treatments (ANOVA, $F(3, 8) = 192.285$, p -value < 0.05). So, individuals fed with diet 3 exhibited remarkably higher mortality.

Table III.I- Survival and growth parameters – survival rate (%), Specific growth rate (%) and Absolute growth rate (mg/day) - of *Hediste diversicolor* individuals fed different diets in the wet olive pomace trial. Diet 1: 6% of lipids, with wet olive pomace; Diet 2: 6% of lipids, without wet olive pomace; Diet 3: 12% of lipids, with wet olive pomace; Diet 4: 12% of lipids, without wet olive pomace. Results that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

	Dietary treatments:			
	Diet 1	Diet 2	Diet 3	Diet 4
Survival rate (%)	94.47 ± 4.99^a	98.60 ± 1.4^a	35.20 ± 5.24^b	98.60 ± 2.42^a
Specific growth rate (SGR) (%)	1.68 ± 0.13^a	3.55 ± 0.23^b	0.04 ± 0.68^c	2.84 ± 0.28^b
Absolute growth rate (AGR) (mg/day)	$0.05 \pm 8.5 \times 10^{18}a$	0.11 ± 0.01^b	0.02 ± 0.01^c	0.08 ± 0.01^d

The results showed that the average weight per polychaete (Figure 3.1) was highest in treatment 2 (Diet 2: 6% of lipids, without wet olive pomace), with a mean of 121.04 ± 12.48 mg, followed by treatment 4 (Diet 4: 12% of lipids, without wet olive pomace), with 91.81 ± 11.04 mg.

In contrast, individuals in the dietary treatments that included wet olive pomace - diet 1 (6% of lipids) and diet 3 (12% of lipids) - had lower average weights compared with those in treatments without wet olive pomace (49.29 \pm 2.84 mg and 24.56 \pm 7.44 mg, respectively). Statistically significant differences were observed between every dietary treatment except between diets 1 (6% of lipids, with wet olive pomace), and 3 (12% of lipids, with wet olive pomace) (ANOVA, $F(3, 8) = 65.145$, p -value < 0.05). Figure 3.2 shows a visual comparison of polychaetes at the end of the trial between two treatments.

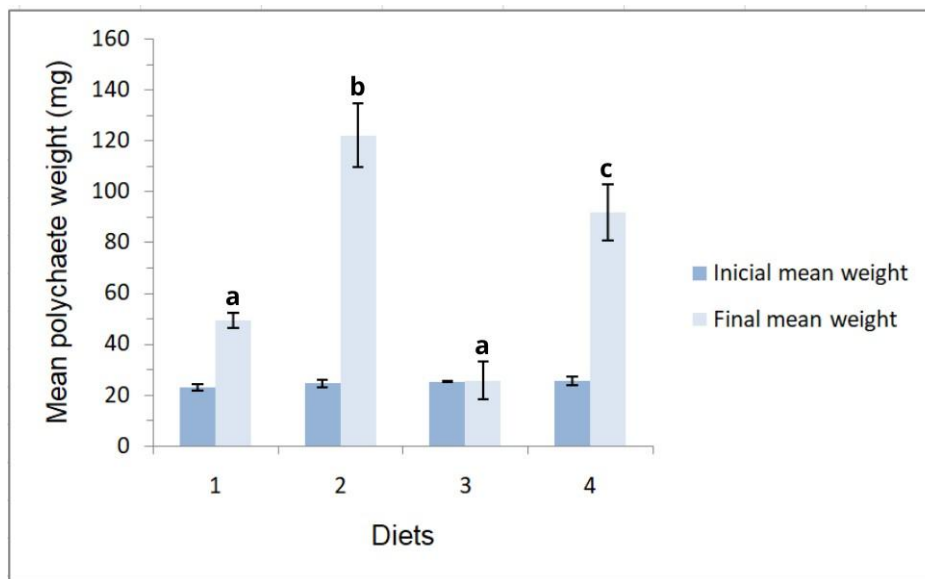


Figure 3.1- Bar chart of initial mean weights with final mean weights (mg) between treatments. Diet 1: 6% of lipids, with wet olive pomace; Diet 2: 6% of lipids, without wet olive pomace; Diet 3: 12% of lipids, wit wet olive pomace; Diet 4: 12% of lipids, without wet olive pomace. Bars that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

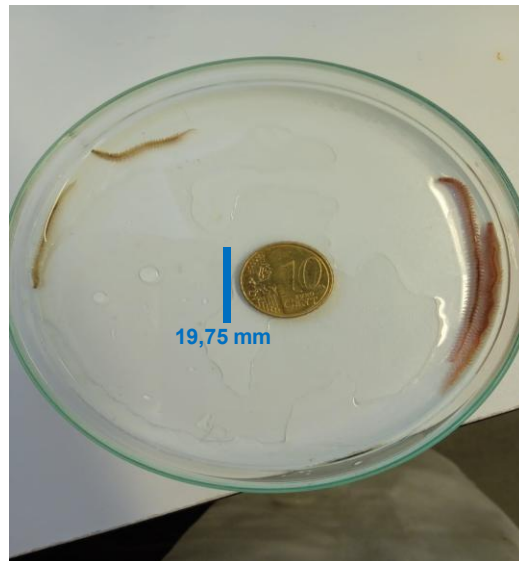


Figure 3.2- *Hediste diversicolor* individuals fed diet 2 (6% of lipids, without wet olive pomace) (polychaetes on the left) and diet 4 (12% of lipids, without wet olive pomace) (polychaetes on the right).

The results for final biomass (Figure 3.3) per treatment were broadly consistent with those for final average weight. Polychaetes fed with diets 2 (8.67 ± 0.97 g) and 4 (6.43 ± 0.93 g) exhibited significantly higher final biomasses than treatments 1 (3.36 ± 0.34 g) and 3 (0.64 ± 0.2 g).

Diet 3, which contained the highest percentage of wet olive pomace in its composition, showed a reduction in biomass relative to the beginning of the trial. Among the dietary treatments, the only statistical differences found were between diet 2 - 6% of lipids, without wet olive pomace - and diets 1 and 3 - 6 and 12% of lipids respectively, both with wet olive pomace (Kruskal-Wallis, $H(3) = 10.385$, $p\text{-value} = 0.016$).

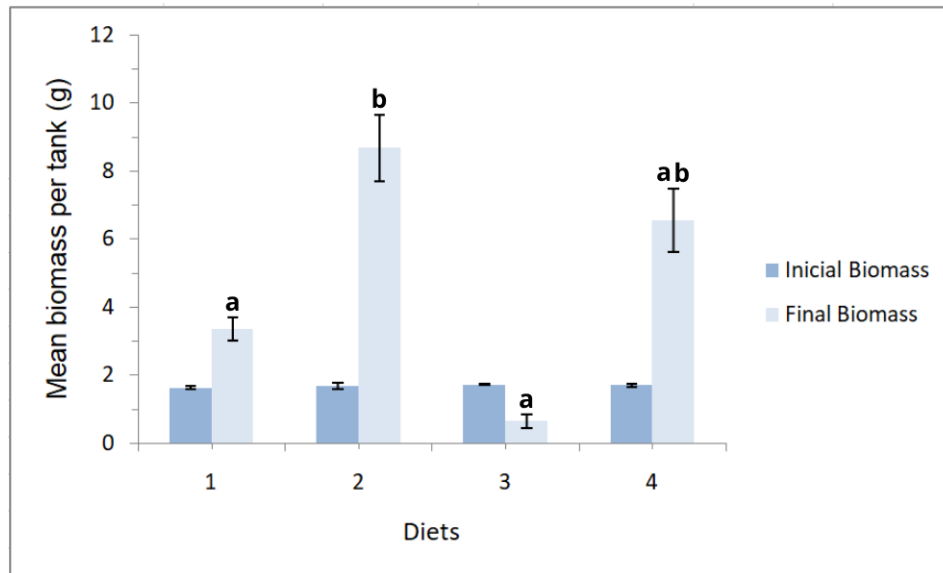


Figure 3.3- Bar chart of the initial and final biomass (g) per tank, between treatments. Diet 1: 6% of lipids, with wet olive pomace; Diet 2: 6% of lipids, without wet olive pomace; Diet 3: 12% of lipids, with wet olive pomace; Diet 4: 12% of lipids, without wet olive pomace. Bars that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

The SGR and AGR (Table III.I) followed the same trend as the other parameters, being higher in treatments without wet olive pomace than in those with it. Polychaetes fed with diet 2 recorded the highest SGR ($3.55 \pm 0.23\%/day$), followed by polychaetes fed with diets 4 ($2.84 \pm 0.28\%/day$), 1 ($1.68 \pm 0.13\%/day$), and 3 ($0.04 \pm 0.68\%/day$). All dietary treatments, with the exception of diet 2 and 4, differed significantly from one another (ANOVA, $F(3, 8) = 48.591$, p -value < 0.05).

The highest AGR was also observed in polychaetes fed with diet 2 (0.11 ± 0.01 mg/day), followed by diets 4 (0.08 ± 0.01 mg/day) and 1 ($0.05 \pm 8.5 \times 10^{-18}$ mg/day), with polychaetes fed with diet 3 (0.02 ± 0.01 mg/day) exhibiting the lowest value. Statistically significant differences in this parameter were observed between every dietary treatment (ANOVA, $F(3, 8) = 72.143$, p -value < 0.05). Analyzing these two growth parameters (SGR and AGR) reveals that individuals fed diet 3 (12% of lipids, with wet olive pomace) showed virtually no growth.

3.2. Results from the *Mytilus edulis* waste trial

3.2.1. *Hediste diversicolor*

In terms of survival rate (Table III.II), this parameter showed the least variation among treatments. No statistically significant differences were detected between treatments (ANOVA, $F(2, 6) = 8.171$, p -value = 0.019). Nevertheless, the results followed the same trends as the other parameters, with polychaetes in treatment 3 showing the highest survival rate (SR = $97.91 \pm 3.60\%$), followed by treatments 2 and 1 (SR = $89.58 \pm 2.08\%$ and SR = $86.11 \pm 4.81\%$, respectively).

Table III.II- Survival and growth parameters – survival rate (%), Specific growth rate (%) and Absolute growth rate (mg/day) - of *Hediste diversicolor* individuals in the different treatments of the mussel waste trial. Treatment 1: 1×10^6 cells per mussel; Treatment 2: 5×10^6 cells per mussel; Treatment 3: 20×10^7 cells per mussel. Results that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

	Dietary treatments:		
	Treatment 1	Treatment 2	Treatment 3
Survival rate (%)	86.11 ± 4.81^a	89.58 ± 2.8^a	97.91 ± 3.60^a
Specific growth rate (SGR) (%)	2.22 ± 0.89^a	4.81 ± 0.41^b	7.65 ± 0.48^c
Absolute growth rate (AGR) (mg/day)	0.06 ± 0.02^a	0.20 ± 0.03^b	0.71 ± 0.14^c

The results of the second test (IMTA of polychaetes with mussels) showed that polychaetes in treatment 3 (where mussels received more food) achieved a much higher final average weight (Figure 3.4 and 3.5) of 777.24 ± 148.49 mg, compared with those in treatments 1 and 2 (66.08 ± 24.99 mg and 202.66 ± 32.62 mg, respectively).

Polychaete growth across treatments was proportional to the amount of food provided to the mussels. The average polychaete weight in treatment 3 was significantly higher than in treatment 1 (ANOVA, $F(2, 6) = 53.993$, p -value < 0.05), whereas in treatment 2 showed no differences compared to the remaining treatments.



Figure 3.4- *Hediste diversicolor* individuals belonging to one tank in each treatment (1- first treatment with 1×10^6 cells per mussel; 2- second treatment with 5×10^6 cells per mussel; 3- third treatment with 20×10^7 cells per mussel).

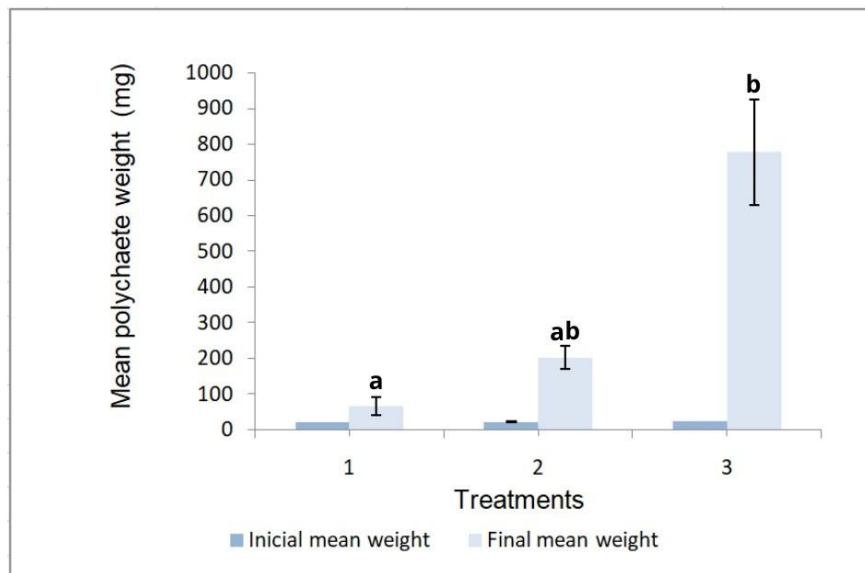


Figure 3.5- Bar chart of the initial mean weight and the final mean weight (mg), between treatments. Treatment 1: 1×10^6 cells per mussel; Treatment 2: 5×10^6 cells per mussel; Treatment 3: 20×10^7 cells per mussel. Bars that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

The final biomass per tank (Figure 3.6) followed a trend similar to that of the average final weight, being proportional to the amount of food provided to the mussels in each treatment. Treatment 3 exhibited the highest final polychaete biomass (11.55 ± 1.47 g), followed by treatments 2 and 1 (2.72 ± 0.26 g and 1.16 ± 0.29 g, respectively), with the latter showing almost no increase from

the initial biomass (1.09 ± 0.01 g). Statistically significant differences were observed only between treatments 1 and 3 (Kruskal–Wallis test, $H(2) = 7.200$, $p = 0.027$).

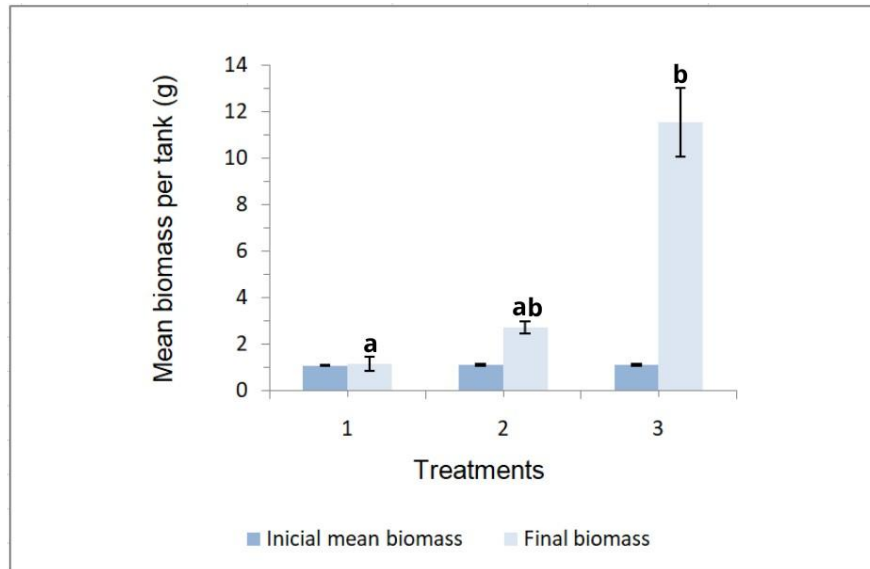


Figure 3.6- Bar chart of the initial mean biomass and the final mean biomass per tank (g), between treatments. Treatment 1: 1×10^6 cells per mussel; Treatment 2: 5×10^6 cells per mussel; Treatment 3: 20×10^7 cells per mussel. Bars that do not share a letter are significantly different (p -value < 0.05). Results are represented as the mean \pm standard deviation.

For the specific growth rate (SGR) (Table III.II) and absolute growth rate (AGR) (Table III.II), similar trends were obtained. Treatment 3 produced polychaetes with the highest SGR ($7.65 \pm 0.48\%$) and AGR (0.71 ± 0.14 mg/day), followed by treatment 2 (SGR = $4.81 \pm 0.41\%$; AGR = 0.20 ± 0.03 mg/day). Treatment 1 produced polychaetes with the lowest growth rates, which were statistically lower than those observed in the other two treatments (SGR = $2.22 \pm 0.89\%$; AGR = 0.06 ± 0.02 mg/day) (p -value < 0.05).

Statistically significant differences in SGR were detected between all treatments (ANOVA, $F(2, 8) = 54.868$, p -value < 0.05). For AGR, polychaetes in treatment 1 differed significantly from the ones in the other two treatments (ANOVA, $F(2, 8) = 44.521$, p -value < 0.001), whereas treatments 2 and 3 did not differ significantly from one another.

3.2.2. *Mytilus edulis* parameters

The survival rates for treatments 1, 2 and 3 were respectively $79.17 \pm 3.61\%$, $89.58 \pm 7.22\%$ and $89.58 \pm 7.22\%$ (Table III.III). However, no statistically significant differences were detected among the treatments (ANOVA, $F(2, 23) = 2.778$, $p\text{-value} = 0.141$). Therefore, it can be concluded that the different amounts of food provided to the mussels did not have a significant effect on their survival ($p\text{-value} > 0.05$).

Table III.III- Survival rate (%), mean initial and final weights (g), and condition index of *Mytilus edulis* individuals in the different treatments with different amounts of food in the mussel waste trial. Treatment 1: 1×10^6 cells per mussel; Treatment 2: 5×10^6 cells per mussel; Treatment 3: 20×10^7 cells per mussel. Results that do not share a letter are significantly different ($p\text{-value} < 0.05$). Results are represented as the mean \pm standard deviation.

Treatments:	Survival rate (%)	Mean initial weight (g)	Mean final weight (g)	Condition index (CI)
1	79.17 ± 3.61^a	18.62 ± 5.74^a	18.49 ± 6.21^a	7.21 ± 1.07^a
2	89.58 ± 7.22^a	18.79 ± 5.93^a	18.91 ± 6.1^a	7.34 ± 0.86^a
3	89.58 ± 0.02^a	18.61 ± 5.75^a	19.00 ± 6.02^a	8.95 ± 1.80^b

M. edulis mean weight comparing the beginning and end of the trial showed no statistically significant differences for weight (see Table III.III) (ANOVA, $F(1, 266) = 0.34$, $p\text{-value} = 0.853$). Similarly, comparisons between mussels from different treatments at the end of the trial, revealed no statistically significant differences in final mean weight (ANOVA, $F(2, 265) = 0.058$, $p\text{-value} = 0.944$).

Condition index (CI) values were highest in treatment 3 (CI = 8.95 ± 1.8), followed by treatment 2 (CI = 7.34 ± 0.86), and lowest in treatment 1 (CI = 7.21 ± 1.07) (Table III.III). While CI appeared to increase with the amount of food provided to the mussels, the only statistically significant differences found were in treatment 3 comparatively to treatments 1 and 2 (ANOVA ($F(2, 23) = 2.778$, $p\text{-value} = 0.141$)).

4. Discussion

4.1. Trial with wet olive pomace diets

It is difficult to directly compare the results of this study with those in the literature, as most research incorporating wet olive pomace into diets has been conducted with insects or fish (Ramzy *et al.*, 2022; Yildirim & Guroy, 2015). To date, there are no studies directly assessing marine invertebrates. Furthermore, many studies examining olive mill waste in marine or aquatic animal diets use forms of the waste different from that used in this trial, which consisted solely of wet pomace dehydrated after two-phase extraction. Other studies have used olive pomace meal, olive pomace (obtained through three-phase extraction) or olive pomace oil (Nasopoulou *et al.*, 2011; Yildirim & Guroy, 2015).

The results show that diet 3 (12% lipids, 6% of which originated from wet olive pomace) was the only diet to cause a statistically significant reduction in the polychaetes survival rate, at $35.20 \pm 5.24\%$ ($p < 0.05$). Compared with survival rates reported in other studies that incorporated wet olive pomace or other olive oil extraction by-products, the survival rate in treatment 3 is significantly lower. For example, Nasopoulou *et al.* (2011) tested the incorporation of different olive oil production by-products in diets for *Sparus aurata* and *Dicentrarchus labrax*, and the lowest survival rate obtained in their trial was 86.4%.

This outcome may be related to the relatively high proportion of wet pomace in diet 3, representing approximately 64% of the diet. In contrast, other studies have incorporated lower percentages of olive mill waste, such as 10%, 20% or 30% (Nasopoulou *et al.*, 2011), or even smaller amounts around 0.1%, 0.2% or 0.3% (Hassan & Abedalhammed, 2025).

The low survival may also be attributable to the nature of wet olive pomace used, which was obtained directly via double-phase extraction and therefore contained a substantial amount of olive mill wastewater (OMW), a highly toxic by-product for aquatic organisms (Elnabris, 2014). In the trial, the waste was only dehydrated at 50 °C for 48 hours, a process that may have been insufficient to eliminate the toxicity, potentially contributing to the observed mortality.

In terms of growth, polychaetes fed diet 2 (6% lipids, no wet olive pomace) exhibited the best growth parameters, followed by polychaetes fed diet 4 (12% lipids, no wet olive pomace). Only in some parameters were observed significant differences between the polychaetes fed these two diets (p -value > 0.05). These results suggest that individuals *H. diversicolor* does not appear to rely heavily on dietary lipid content for growth. This is supported by the findings of Santos *et al.* (2016) and Wang *et al.* (2019a), who demonstrated a positive correlation between dietary protein content and specific growth rate (SGR) in *H. diversicolor* (Wang *et al.*, 2019a).

The table in attachment I was compiled using the growth parameters of *H. diversicolor* obtained by various authors, facilitating subsequent comparison without requiring readers to consult the original articles for important details such as cultivation density and temperature.

When comparing the growth parameters (mean weight, SGR, and AGR) of polychaetes fed diets 2 and 4 with those reported in the literature, some results are similar (Wang *et al.*, 2019b; Rasines *et al.*, 2023; Santos *et al.*, 2025) (Attachment I). However, a lot of studies investigating the growth of this polychaete employed different parameters, such as animal density and substrate height (Nesto *et al.*, 2012; Rasines *et al.*, 2023). These findings demonstrate that, although less efficiently, *H. diversicolor* can be cultured on diets composed entirely of plant-based ingredients. Nevertheless, several authors have achieved superior growth results using commercial feeds (Nesto *et al.*, 2012; Santos *et al.*, 2016).

Conversely, polychaetes fed diet 3 exhibited the lowest survival rate and the poorest performance across all growth parameters. The final mean weight in this treatment (25.56 ± 7.44 mg) was very similar to the initial mean weight (25.22 ± 0.31 mg). Statistically significant differences observed for all these parameters compared with polychaetes fed with other diets (p -value < 0.05). The lack of growth and high mortality in this treatment are most likely attributable to the toxicity of olive mill wastewater present in the wet olive pomace used as an ingredient (Elnabris, 2014).

Finally, regarding polychaetes fed with diet 1, although growth parameters were lower than those of diets 2 and 4 (which did not contain wet olive pomace), with statistically significant differences in mean weight and SGR, they were comparable to values reported in other studies (Santos *et al.*, 2025; Wang *et al.*, 2019a). This is particularly evident in the results of Wang *et al.* (2019a), in

which the SGR was 0.7% for polychaetes fed on biogas production waste - lower than the SGR observed in diet 1 in this trial (1.68%).

Based on these results, it can be concluded that the inclusion of wet olive pomace as an ingredient in diets is potentially toxic to *H. diversicolor*. When included in large quantities (as in diet 3, where it represented 64% of the feed — see table II.I), it caused high mortality. Even at lower levels (as in diet 1, representing 38% of the feed — see table II.I), it resulted in reduced growth compared with diets without wet olive pomace.

To confirm the potential toxicity of wet olive pomace for this species, it is important to analyse the proximate composition of the prepared diets to ensure they meet the nutritional needs of *H. diversicolor*. This analysis was intended in the present trial, but insufficient resources prevented the necessary biochemical analyses. However, samples of all four diets (1, 2, 3 and 4) have been freeze-dried for potential future analysis.

Furthermore, although growth of polychaetes fed diet 1 was statistically lower (p -value < 0.05) compared with diets 2 and 4, the polychaetes still exhibited some biomass increase during the trial (3.36 ± 0.34 g), albeit less than half of that obtained in the tank with the highest growth, tank 2 (8.67 ± 0.97 g).

H. diversicolor assimilates fatty acids deposited in sediments extremely efficiently, a process that is crucial for the flow of lipids through the food chain (Bradshaw *et al.*, 1990). As a result, the fatty acid profile and quantity in the biomass of this polychaete are greatly influenced by diet (Santos *et al.*, 2025). Wet olive pomace is rich in fatty acids and other bioactive lipid compounds (Nunes *et al.*, 2018). For this reason, it would be valuable to analyse the proximal composition and fatty acid profile of polychaetes fed different diets (1, 2, 3 and 4), as well as the wet olive pomace itself. Freeze-dried samples of both the diets and the polychaetes from the various treatments are already available for such futures biochemical and fatty acid analyses.

If polychaetes fed diet 1 are found to contain nutritionally important fatty acids, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), this wet olive pomace bioremediation strategy could still be considered successful. Although polychaete growth on diets containing wet olive pomace was lower than on diets without this waste, biomass accumulation still occurred. If

this biomass is rich in high-value fatty acids, like EPA and DHA, then waste material with little economic value is effectively transformed into a valuable product, albeit through a less efficient process compared with growth rates reported in most studies.

In future research, it would be valuable to explore alternative processing methods for wet olive pomace, such as fermentation, prior to its incorporation into experimental diets. For example, Campani *et al.* (2017) added polyphenol-metabolising fungi to olive mill waste and subsequently assessed the toxicity of both the processed and the unprocessed waste in red worms (*Eisenia fetida*).

Campani *et al.* (2017) found that processing olive mill waste reduced its toxicity. Mortality in *E. fetida* reached 80% in the treatment containing the highest amount of processed agricultural waste, whereas no mortality occurred in treatments with lower percentages of processed waste.

Since this trial aimed to bioremediate waste by incorporating large quantities into polychaetes diets, it would also be informative to test the effects of adding small amounts of wet olive pomace to control diets. This approach could help determine the maximum quantity of waste that can be incorporated without negatively affecting polychaete growth, and whether it might confer immunological or physiological benefits. Nasopoulou *et al.* (2011), for instance, replaced 8% of fish oil in a feed with olive pomace and found that the biological activity and cardioprotective properties of *Spaurus aurata* were enhanced without negatively affecting growth or survival.

4.2. *Mytilus edulis* waste trial

In this trial, polychaete survival exceeded 85% in all treatments, consistent with the high survival rates reported in other studies on rearing this species with different diets (Anglade *et al.*, 2023; Nesto *et al.*, 2012). Survival rate is a key indicator of overall system viability in integrated aquaculture (Grosso *et al.*, 2021).

Higher survival rates were observed in treatments with greater quantities of mussel food: treatment 3 ($97.91 \pm 3.60\%$), treatment 2 ($89.58 \pm 2.08\%$) and treatment 1 ($86.11 \pm 4.81\%$). However, this

apparent proportional increase in survival with the amount of food was not statistically significant (p -value < 0.05).

Regarding growth parameters (mean weight, SGR and AGR), all of them were statistically higher in polychaetes from treatment 3 (Figure 3.5 and table III.III) compared with treatment 1, which received the lowest food quantity (66.08 ± 29.99 mg; $2.22 \pm 0.89\%$; 0.06 ± 0.02 mg) (p -value < 0.05). Polychaetes in treatment 2 yielded intermediate values, showing significant differences only for some treatments and specific parameters. Overall, growth parameters increased proportionally with the amount of food provided to the mussels, similar to survival. Treatment 3 produced a final polychaete biomass of 11.55 ± 1.47 g, approximately ten times the initial biomass of 1.13 ± 0.03 g.

Like many other filter-feeding bivalves, mussels expel pseudofaeces, which are portions of filtered organic matter rejected during pre-ingestive selection and expelled before digestion (Gosling, 2008). Furthermore, not all the matter digested by mussels is completely decomposed, so their faeces also contain nutritional value that can be exploited by detritivorous organisms (Broekhoven *et al.*, 2015).

As mentioned in the introduction, *H. diversicolor* can feed through multiple mechanisms. In an IMTA system with mussels, for example, they ingest bivalve faeces and pseudofaeces, and may also feed by filtration when phytoplankton concentrations are high (e.g. when mussel food supply is abundant) (Scaps, 2002). Thus, it is logical that treatments involving larger quantities of food resulted in greater polychaete growth, as more faeces, pseudofaeces and microalgae were available. Polychaetes grew rapidly across all treatments, suggesting that they can successfully cultivate in environments with ample organic matter or food.

The growth parameters (SGR and AGR) obtained in polychaetes from treatments 2 and 3 in this trial are comparable (Attachment I) to those reported in other studies using different food sources (Rasines *et al.*, 2023; Santos *et al.*, 2025; Wang *et al.*, 2019a; Wang *et al.*, 2019b). Polychaetes from treatment 3, which received the highest food quantity, exhibited an SGR of $7.65\% \pm 0.48\%$ and an AGR of 0.71 mg/day. These values exceed most reported in the literature (Nesto *et al.*, 2012; Rasines *et al.*, 2023; Santos *et al.*, 2016; Santos *et al.*, 2025; Wang, *et al.*, 2019a; Wang, *et al.*, 2019b), even in trials where polychaetes were provided with high protein fish feed.

This result demonstrates that mussel production waste is an effective feed for *H. diversicolor*. Aquacultured mussel nurseries usually occur on land, normally in fiberglass tanks (FAO, 2009). Conducting trials in such environments could help determine the feasibility of co-cultivating mussels and *H. diversicolor* in IMTA, given that mussel waste has proven to be an excellent food source for *H. diversicolor*.

However, cultivating *H. diversicolor* in offshore or deeper waters through IMTA systems presents additional challenges, as this species is benthic. To enable such trials, submerged structures would need to be installed to maintain the *H. diversicolor* beneath the mussel farms.

In future, research could also investigate whether polychaetes produced in IMTA with bivalves accumulate long-chain polyunsaturated fatty acids, to evaluate their potential use as ingredients in the diets of farmed marine organisms (Monteiro *et al.*, 2024). As previously stated, the fatty acid composition of polychaete biomass is greatly influenced by diet (Santos *et al.*, 2025), and various species of microalgae are rich in important fatty acids.

Regarding mussel survival, it was higher in treatments 2 and 3 (Table III.III), at $97.91 \pm 3.60\%$ and $89.58 \pm 2.08\%$ respectively. However, no statistically significant differences were found between treatments (p -value > 0.05). Therefore, it can be concluded that the different amounts of food provided did not significantly affect mussel survival.

There was little variation between the final and initial mean weights and no statistically significant differences were observed (p -value > 0.05). The purpose of this trial was not to promote growth in *M. edulis* species but to ensure that the mussels remained healthy over the relatively short experimental period.

As for condition index (CI) the highest values were obtained in treatment 3, followed by treatment 2, and lowest in treatment 1 (Table III.III). This demonstrates a positive relationship between CI and the amount of food provided. However, the only statistically significant differences were observed between treatment 3, and the rest of the treatments, so this direct relationship is not significant (p -value > 0.05). The condition index is a useful measure of physiological quality of mussels, reflecting the availability of food in their production environment (Babarro *et al.*, 2020).

In natural environments, the amount of food available to mussels varies greatly with season and weather, and CI fluctuates accordingly. Condition indices below 10 typically indicate low energy reserves (Babarro *et al.*, 2020). In a study by Babarro *et al.* (2020), the CI of *Mytilus galloprovincialis* individuals produced in the Arousa Estuary, Galicia was calculated monthly over 18 months. The lowest CI observed was around 11 during winter, despite good food availability.

At the end of the trial, all treatments exhibited a condition index below 10, which suggests that the quantity of feed provided, or the feeding method employed, was not appropriate. Although the condition index was not measured at the start of the trial, it can be assumed that it remained unchanged, as the average weight of the individuals showed minimal variation throughout the trial. Future trials could investigate alternative feeding strategies, such as maintaining a continuous flow of microalgae to the tanks.

5. Conclusion

5.1. Conclusion of the trial with wet olive pomace diets

The first trial concluded that wet olive pomace, a by-product of two-phase olive oil extraction, is not ideal for feeding and fattening of *H. diversicolor*, particularly when it constitutes a large proportion of the diet. Since survival rate, specific growth rate (SGR), absolute growth rate (AGR), and final mean weight of individuals fed diet 3 (containing the highest percentage of wet olive pomace) were statistically lower than those of individuals fed the other diets (p-value > 0.05). Furthermore, in diet 1 (6% lipids, with wet olive pomace), survival was similar to that in the other treatments (diets 2 and 4), but growth parameters were lower, with SGR significantly reduced (p-value < 0.05).

The trial also demonstrated that *H. diversicolor* can be successfully cultured on a strictly plant-based diet, as demonstrated by polychaetes fed with diets 2 and 4, which had growth parameters comparable to those reported in previous studies (Rasines *et al.*, 2023; Santos *et al.*, 2025; Wang *et al.*, 2019a). Future trials could focus on optimising plant-based diets for this species to maximise growth without animal protein. Similarly, diets composed solely of different types of agricultural

waste could be tested. Additionally, biochemical analyses of both diets would be valuable to determine their proximal composition and assess their suitability for *H. diversicolor*. Analysing the polychaetes themselves could reveal whether differences in diet influence the proximal composition of individuals, particularly regarding their fatty acid profile. This would clarify the effect of dietary lipid content on polychaete biomass quality. As discussed, future trials could also evaluate the use of pre-processed wet olive pomace - such as fermentation - before inclusion in diets. Alternatively, smaller amounts of this residue could be tested to determine whether it provides immunological or physiological benefits to polychaetes, as suggested by other studies.

In conclusion, although incorporating wet olive pomace into the diets in this trial resulted in slower polychaete growth compared with other diets described in the literature, this approach should not be dismissed. If polychaetes fed wet olive pomace accumulate significant quantities of high-value fatty acids, such as EPA and DHA, this strategy still converts a low-value waste product into a more valuable biomass, albeit less efficiently than other feeding methods.

5.2. Conclusion of the *Mytilus edulis* waste trial

In the second trial, it was concluded that the polychaete *H. diversicolor* can feed successfully on waste produced by *M. edulis* mussels, and that these two species can be sustainably produced in an IMTA system. Survival rates of the polychaetes exceeded 85% in all treatments, with no statistically significant differences between treatments. In addition to high survival, treatment 3 produced growth parameters comparable to the best reported in the literature for this species (Santos *et al.*, 2016; Nesto *et al.*, 2012). The SGR of this treatment ($7.65 \pm 0.48\%$) is potentially among the highest published, with final biomass 10 times greater than the initial biomass. Polychaete growth was observed to vary according to the amount of food provided to the mussels. Despite the lower growth observed in treatments with reduced bivalve feeding, treatment 2 still exhibited growth parameters comparable to those reported by other researchers (Rasines *et al.*, 2023; Santos *et al.*, 2025; Wang *et al.*, 2019b). These results demonstrate that mussel production waste is an effective feed for *H. diversicolor*, as even in lower quantities of waste – corresponding to reduced bivalve feeding – can support substantial polychaete growth.

The condition index (CI) at the end of the trial was similar across treatments, with statistically significant differences observed (p -value < 0.05) in only one of the treatments (treatment 3). All CI values were below 10, which is considered low in aquaculture systems (Babarro *et al.*, 2020), showing that the mussels were not in optimal physiological condition. Future trials could explore IMTA systems using a feeding method that maintains a constant flow of microalgae into the tanks throughout the day. This is particularly pertinent given that polychaetes were able to survive and grow effectively in environments with higher levels of waste, as evidenced by their low mortality rates and enhanced growth in treatments with increased food availability for mussels.

Additionally, it would be interesting to test an IMTA system with these two species under real-world aquaculture conditions to assess the feasibility of co-cultivation. Finally, a biochemical analysis and determining the fatty acid profiles of polychaetes produced in such a system would provide insight into whether they accumulate long-chain polyunsaturated fatty acids suitable for use in feed production.

6. Bibliography

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Attachments

Attachment I- Comparison table of *Hediste diversicolor* growth parameters - Mean final weight (mg); Specific growth rate (%/day); AGR (mg/day) - from different articles in the bibliography. Diet 1- 6% of lipids, with wet olive pomace; Diet 2- 6% of lipids, without wet olive pomace. Results are represented as mean.

Citations	Rearing temperature °C	Polychaete density	Type of feed	Rearing period	Mean final weight (mg)	SGR (%/day)	AGR (mg/day)
Trial with wet olive pomace diets (this trial)	17.5 ± 0.44	450 ind m ⁻²	Diet 1	45 days	49.29	1.68	0.05
			Diet 2	45 days	121.99	3.55	0.11
(Wang et al., 2019b)	19 ± 1	275 ind m ⁻²	Fish feed	30 days	-	2.5	-
(Santos et al., 2016)	25 ± 1.0	170 ind m ⁻²	Seabream dry feed (Aquagold)	60 days	890	6.49	-
(Wang, 2019a)	-	-	Solid Biogas Digestate	30 days	-	0.7	-
(Santos et al., 2025)	18.66 ± 1.76	270 ind m ⁻²	Sea Urchin Waste	50 days	140.2	1.6	-
(Rasines et al., 2023)	18.9 ± 1	1000 ind m ⁻²	Sole weaning feed pellets	70 days	-	3.47	0.7
(Nesto et al., 2012)	19 ± 1 °	1000 ind m ⁻²	Larviva-Biomar®	56 days	920	6.35	-