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Ragworms can Utilise Chitosan-Treated Aquaculture Wastewater, Enabling Sustainable Recycling and Production of Aquafeed Ingredients

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Supervised by

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Declaration

I declare that this thesis has been completed solely by Blair A. Mackie at the University of Edinburgh, under the supervision of Dr Andrea J.C. Semião and Dr Jonathan Hughes. Reference is given where other sources are used. This work has not been submitted for any other degree or personal qualification.

Blair Mackie

July 2024

Abstract

Aquaculture facilities are highly dependent on sources of high-quality ingredients from conventional capture fisheries (fish meal and fish oil), which have stagnated in production in recent decades. In addition, wastewater from aquaculture facilities requires specific treatment and disposal. Both issues are possibly limiting the potential of the aquaculture industry. An approach to increase resource efficiency in aquaculture is to utilise nutrient-dense wastewater from aquaculture to grow biomass that could be returned to and applied in aquaculture systems as high-quality ingredients by inclusion in aquafeeds.

It has been shown in previous research that ragworms (*Hediste diversicolor*) can recycle nutrients found in aquaculture wastewater with good growth rates, as well as favourable nutritional profiles that are suitable for many species of marine animals that are typically reared in aquaculture systems. However, the aquaculture wastewater must be treated in some way before it can be fed to ragworms as they cannot be cultured in the wastewater directly. One way to do this that has not been investigated before is by coagulating and flocculating the aquaculture wastewater using chitosan before feeding it to ragworms. Chitosan is a natural biopolymer derived from chitin, which is a major component of the exoskeletons of crustaceans and insects. Chitosan is generally accepted as non-toxic and has many other benefits over metal-based coagulants (e.g., alum) including biodegradability and environmental friendliness.

Therefore, the objective of this thesis was to investigate technical aspects related to utilising ragworms to recycle nutrients contained in aquaculture wastewater that have been coagulated and flocculated by chitosan. To do this, growth rates, mortality rates and the nutritional suitability of ragworms as a feed resource for fish species reared in aquaculture after being fed chitosan coagulated and flocculated aquaculture wastewater were assessed. A series of cultivation experiments were carried out on juvenile ragworms in the laboratory. The results indicated that ragworms can successfully be cultivated on aquaculture wastewater that has been coagulated and flocculated by chitosan. In addition, the ragworms had nutritional compositions (amino acid and fatty acid profiles) that met the requirements for a variety of fish species. However, it was determined that a high concentration of chitosan

in aquaculture wastewater can have detrimental effects on ragworms when it is fed to them. Furthermore, aquaculture wastewater can contain toxic contaminants, such as heavy metals, which chitosan was demonstrated to decrease ragworm exposure to toxic aluminium when feeding.

Ultimately, it was demonstrated that ragworms can efficiently utilise and incorporate nutrients contained within chitosan-coagulated and flocculated aquaculture wastewater to produce aquafeed that is high in protein and contains essential fatty acids and amino acids.

Lay Summary

Aquaculture, a rapidly expanding sector responsible for producing substantial quantities of fish, faces significant sustainability challenges. The industry's heavy reliance on wild-caught fish for high-quality feed ingredients is unsustainable, as the increasing demand for fish far outstrips the available supply. Additionally, aquaculture generates large volumes of polluted water, necessitating effective water treatment solutions.

Ragworms, a type of marine worm, offer a promising dual-purpose solution: they can serve as a nutritious food source (alternative feed ingredient) for farmed fish and assist in the clean-up of polluted water. These worms can thrive in contaminated environments, making them ideal for such applications. Furthermore, chitosan, a natural biopolymer, has shown efficacy in removing pollutants from water, thus creating a cleaner environment for ragworms to consume and thrive.

The project explored the feasibility of using chitosan-treated aquaculture wastewater to develop ragworm nutritional contents. The findings demonstrated that ragworms can effectively develop in such conditions, suggesting a novel, sustainable method for producing fish feed while simultaneously addressing water pollution issues. This innovative approach has the potential to significantly enhance the environmental sustainability of the aquaculture industry, providing a dual benefit of waste reduction and efficient production of a high-quality ingredient in fish food.

Acknowledgements

Currently, I am writing the final few parts of my PhD thesis. I believe it has addressed my initial objectives, bringing huge relief. However, completing the project would not have been possible without the guidance and help of many people over the last four years. I hope to demonstrate my genuine appreciation and gratitude toward them in the following few paragraphs.

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Thank you to MOWI® Anglesey who kindly provided aquaculture wastewater samples which were vital for the experiments in this thesis. Thank you to Dr Stephen Craig of Seabait Ltd who supplied high-quality ragworms which were also essential for the experiments in this thesis.

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Thank you to my family for being there and for their support, especially Mum and Dad, who are genuinely selfless and outstanding. I hope you enjoy the happiest years of your lives in retirement. Please never stop asking me, 'what is it you do again?'

Finally, thank you to Nicola for your never-ending and unconditional love, patience, encouragement, and support. I love you.

Contents

Abbreviations

AA	Amino Acid
ALA	Alanine Acid
ANOVA	Analysis of Variance
ARA	Arachidonic Acid
ARG	Arginine Acid
ASP	Aspartic Acid
AW + C	Aquaculture Wastewater and Chitosan
AWCE	Centrifuged Aquaculture Wastewater
AWCH	Chitosan Coagulated Aquaculture Wastewater
AWFD	Freeze-Dried Aquaculture Wastewater
AWSE	Settled Aquaculture Wastewater
BALA	Beta-Alanine Acid
C	Carbon
CF	Commercial Feed
DD	Degree of Deacetylation
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DW	Dry Weight
EAA	Essential Amino Acid
EDTA	Ethylenediaminetetraacetic Acid
EPA	Eicosapentaenoic Acid
EPSRC	Engineering and Physical Sciences Research Council

FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FAO	Food and Agriculture Organisation
GABA	Gamma-Aminobutyric Acid
GC	Gas Chromatography
GCMS	Gas Chromatography-Mass Spectrometry
GLU	Glutamic Acid
GLY	Glycine Acid
GRAS	Generally Recognised as Safe
H	Hydrogen
HIS	Histidine Acid
HPLC	High-Performance Liquid Chromatography
HUFA	Highly Unsaturated Fatty Acid
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectroscopy
ILEU	Isoleucine Acid
IMTA	Integrated Multi Trophic Aquaculture
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acid
LEAA	Limiting Essential Amino Acid
LEU	Leucine Acid
LOD	Limit of Detection
LYS	Lysine Acid
MET	Methionine Acid
MS	Mass Spectrometry
MUFA	Monounsaturated Fatty Acid

MW	Molecular Weight
N	Nitrogen
NEAA	Non-Essential Amino Acid
NERC	Natural Environment Research Council
NIST	National Institute of Standards and Technology
NMP	National Marine Plan
NTU	Nephelometric Turbidity Units
ORN	Ornithine Acid
PHE	Phenylalanine Acid
PUFA	Polyunsaturated Fatty Acid
SD	Standard Deviation
SER	Serine Acid
SFA	Saturated Fatty Acid
SGR	Specific Growth Rate
SOFIA	State of World Fisheries and Aquaculture
SRM	Standard Reference Material
THR	Threonine Acid
TOM	Total Organic Matter
TYR	Tyrosine Acid
UN	United Nations
VAL	Valine Acid
WW	Wet Weight

Chapter 1

Introduction

In 2020, the global aquaculture industry produced an all-time high of 122.6 million tonnes of fish and related products at a value of USD 281.5 billion (FAO, 2022). This number includes the production of 87.5 million tonnes of aquatic animals (FAO, 2022). Approximately 11% of total fish production was used to create commercial aquaculture feeds (aquafeeds) to produce most aquacultured species (FAO, 2022). The United Nations (UN) Food and Agriculture Organisation (FAO) estimates that aquatic animal output from aquaculture will grow by 14% by 2030 to keep up with the increased worldwide demand for seafood (FAO, 2022) due to the predicted global population rise to 9.7 billion people by 2050 (United Nations, 2017). However, sources of fish meal and fish oil (high-quality ingredients in aquafeed) are becoming increasingly scarce (Tacon and Metian, 2015; FAO, 2020; Jerónimo *et al.*, 2021) and, therefore, the long-term supply of seafood to market is under threat due to overfishing, improper management of essential fish stocks and unsustainable aquaculture practices.

Furthermore, factors related to climate change, such as rising temperatures, rising sea levels and severe weather, also pose significant sustainability concerns for the aquaculture industry (Maulu *et al.*, 2021). Additionally, aquaculture effluents have been discovered to pollute natural water bodies, for example, by eutrophication (Folke, Kautsky and Troell, 1994). Therefore, climate-resilient sustainable aquaculture and wastewater treatment innovation is required to grow the industry responsibly.

The diverse group of marine annelids known as polychaetes are resilient and abundant animals most found living in brackish estuaries where freshwater meets the sea. Polychaetes can survive in various contaminated environments and salinities (Amaral, Morgado and Salvador, 1998; Scaps, 2002; Galloway *et al.*, 2010). Specifically, the omnivorous polychaete *Hediste diversicolor* (ragworm) utilises multiple feeding mechanisms depending on the food source available, including scavenging, deposit-feeding, and filter-feeding. They also consume detritus (Kuhl and Oglesby, 1979), bioremediating the waters they naturally inhabit. In addition, they have a natural affinity for coprophagous feeding, meaning they have excellent production potential when raised on aquaculture wastewater streams (Riisgård, 1994; Vedel, Andersen and Riisgard, 1994; Scaps, 2002; Wang *et al.*, 2019; Jerónimo *et al.*, 2021).

Additionally, polychaetes have been shown to bioremediate industrial waste effluents, resulting in a greater than one-third reduction in suspended solids (Giangrande *et al.*, 2005;

Costa, Oliveira and Da Fonseca, 2006; A. Bischoff, Fink and Waller, 2009; Palmer, 2010; Stabili *et al.*, 2010; Brown, Eddy and Plaud, 2011; Yearsley *et al.*, 2011; Palmer *et al.*, 2014). Furthermore, polychaetes are routinely used as aquafeed ingredients in Penaeid shrimp hatcheries and finfish aquaculture because they provide a nutrient-rich source of proteins and lipids, including essential amino acids and fatty acids (Lytle, Lytle and Ogle, 1990; Luis and Passos, 1995; Olive, 1999; Thanh Binh *et al.*, 2008; A. Bischoff, Fink and Waller, 2009) with a positive impact on production and the viability of future progeny (Scaps, 2002). Recently, polychaetes have also been incorporated into pet food, sold in the ornamental aquarium market (Parthiban *et al.*, 2006) and used in the pharmaceutical industry (Rousselot *et al.*, 2006). Therefore, because ragworms can simultaneously supply high-quality ingredients to be included in aquafeeds, and can bioremediate aquaculture wastewater, they are excellent candidates to augment the methods currently used to produce aquafeed.

Particulate matter is the primary food composition for polychaetes (Scaps, 2002). However, many pollutants in industrial effluents are dissolved, with their removal often involving hazardous chemicals, which is costly and energy-intensive. For instance, water treatment facilities utilise aluminium or iron-based chemicals to coagulate dissolved pollutants in freshwater into particulate form, producing large amounts of sludge that must be disposed of in landfills. Every year, 270 thousand tonnes of dry sludge are disposed of in the UK alone (Ofwat, 2015). However, this could be partly avoided by utilising chitosan, a non-toxic and biodegradable biopolymer derived from waste shellfish by-product, to coagulate and flocculate the dissolved pollutants in wastewater into larger particles. The ragworms can then be fed the chitosan-coagulated aquaculture wastewater. Chitosan has demonstrated promising results in coagulating freshwater pollutants such as algae and dissolved organic matter (Bratskaya, Avramenko and Sukhoverkhov, 2002; Divakaran and Pillai, 2002) and, more importantly, effluents from aquaculture (Chung, Li and Chen, 2005; Renault *et al.*, 2009).

Therefore, this project focuses on achieving a Circular Economy by reutilising waste to create value in an integrated multitrophic aquaculture system (IMTA), as presented in the simplified form below in Figure 1.1, through:

1. Resource recovery by producing chitosan from crustacean exoskeleton waste (Pennosan®);

2. Resource recovery of valuable components (proteins, lipids etc.) through coagulation of aquaculture wastewater using chitosan;

3. Feeding the chitosan-coagulated aquaculture wastewater to ragworms; and

4. Resource recovery of ragworm biomass, which has a marketable value (Olive, 1999) and can be used as a component in aquafeeds (A. Bischoff, Fink and Waller, 2009).

Figure 1.1 – Chitosan coagulation and ragworm bioremediation for aquafeed production

The key objectives of this thesis are as follows:

- To evaluate the impact of chitosan on ragworms, compare ragworms fed aquaculture wastewater with varying concentrations of chitosan to those fed wastewater without chitosan. This will reveal chitosan's effects on key factors such as growth, mortality,

and nutritional composition, which are crucial for their inclusion as ingredients in aquafeeds (Chapter 4).

- To understand the suitability of ragworms fed aquaculture wastewater treated with chitosan for inclusion as ingredients in aquafeed, comparing their growth, mortality, and nutritional composition to ragworms fed wastewater treated by other methods. This comparison will determine the effectiveness of chitosan-treated wastewater in supporting ragworm development (Chapter 5).

- To evaluate aluminium accumulation in ragworms from aquaculture wastewater-derived feeds, feed them aquaculture wastewater with varying aluminium concentrations over different periods (with and without chitosan). This study will examine how aluminium transfers to ragworms, from aquaculture wastewater with high aluminium levels, and the potential risk of aluminium accumulation in fish fed ragworm-derived aquafeeds in aquaculture systems (Chapter 6).

Chapter 2

Aquaculture, Ragworms, Chitosan and Literature Review

2.1 Research Area and Relevance

Aquaculture is relevant to the UK, particularly since the publication of the 2022 UK Government Food Strategy policy paper outlining plans for at least £65 million allocated for the seafood industry, including aquaculture expansion (Department for Environment Food & Rural Affairs, 2022).

Furthermore, the aquaculture industry is significant to the UK Government's objective of reducing waste generation. This project investigates a novel sustainable methodology for recovering valuable resources from aquaculture wastewater in the form of sludge, turning it into an alternative aquafeed ingredient in the form of ragworms while concurrently lowering the amount of energy needed for waste disposal and preventing environmental damage. Aquaculture is the central area of focus for the methodology, which can pave the way to obtain value from other waste streams and promote the productivity and clean growth of the seafood sector. Therefore, this thesis fits in perfectly with the funding priorities stated by the Natural Environment Research Council (NERC), such as "Resource Recovery from Waste", "Pollution, Waste and Resources", and "Resource Efficiency", as well as by the Engineering and Physical Sciences Research Council (EPSRC) such as "Manufacturing the Future" and "Living with Environmental Change".

Moreover, Scotland is the world's third-largest producer of farmed Atlantic salmon (Kenyon and Davies, 2018), with a Gross Value Added total of £766 million last year, which is expected to reach £1 billion in the coming years (Fishfarmingexpert, 2022). In addition, Scotland has recognised the Food and Drink industry as a significant area for economic growth. The National Maritime Plan (NMP), which outlines the strategy for promoting the sustainable economic expansion of marine sectors while considering environmental protection, was recently endorsed by the Scottish Government. As a result, the output of finfish and shellfish is expected to increase by 25 and 100%, respectively over the next few years (Marine Scotland, 2015).

Additionally, on a global level, this thesis covers several UN Sustainable Development Goals (Figure 2.1), such as Life Below Water (Goal 14), Industry Innovation and Infrastructure (Goal 9), Responsible Consumption and Production (Goal 12) and Zero Hunger (Goal 2) (United Nations, 2020). The Sustainable Development Goals are a set of 17 interrelated goals that are

intended to act as a “shared blueprint for peace and prosperity for people and the planet, now and into the future”.

Figure 2.1 – The United Nations Sustainable Development Goals
Image from (United Nations, 2020)

2.2 Global Production of Fish

The State of World Fisheries and Aquaculture (SOFIA) biennial report from the Fisheries and Aquaculture Division at the Food and Agriculture Organization (FAO) of the United Nations (UN) is an excellent source of data on global fish consumption and production (FAO, 2022). The report describes the rising per capita demand over time and the rising fish production around the globe, which is displayed in Figure 2.2 below.

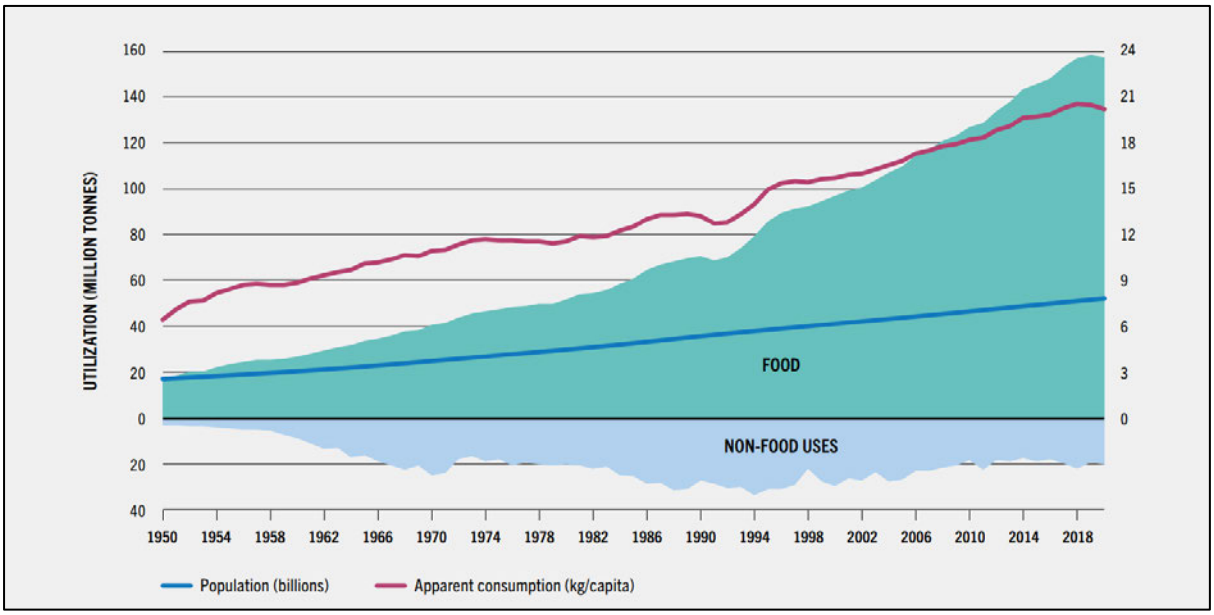

Figure 2.2 – Global fish consumption, population, and per capita food supply
Image from (FAO, 2022)

According to the report, while global fish output has constantly increased since the 1950s, conventional capture fisheries have plateaued since the 1980s. Aquaculture has since filled the void left by this deficiency, as seen in the accompanying Figure below (Figure 2.3).

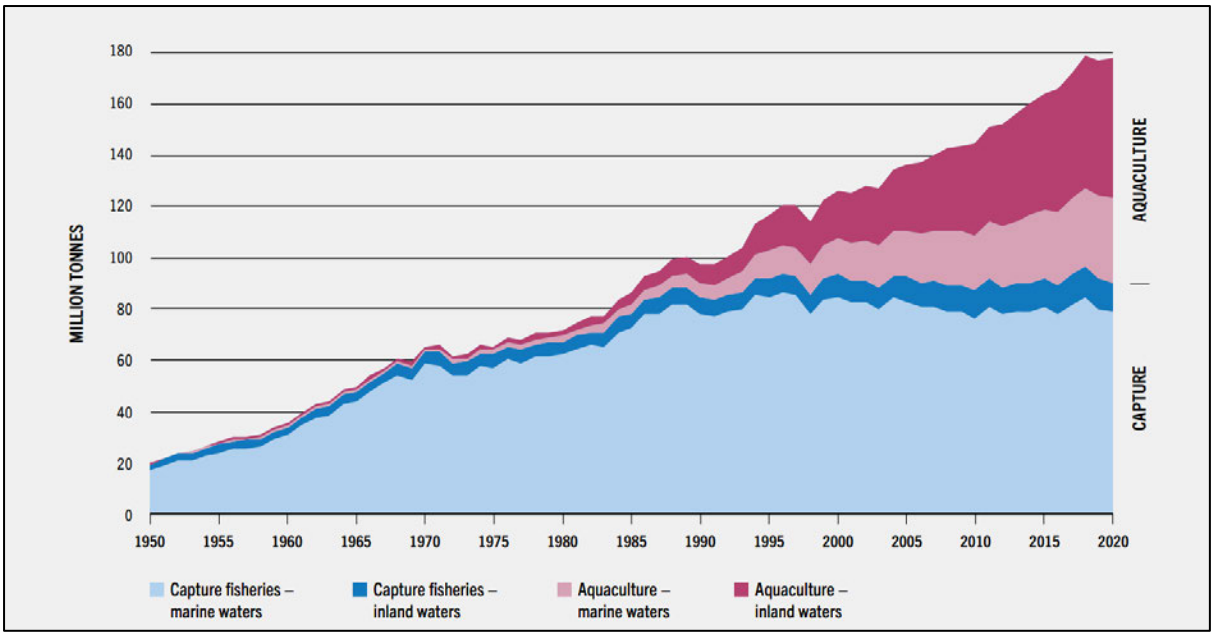

Figure 2.3 – Global fish production from conventional capture fisheries and aquaculture
Image from (FAO, 2022)

However, despite stagnating capture fishery output levels, the share that is not biologically sustainable has grown since the 1970s (Figure 2.4). This suggests that more aquaculture is required to cover increasing demand, but it must be done sustainably to help reduce overfishing.

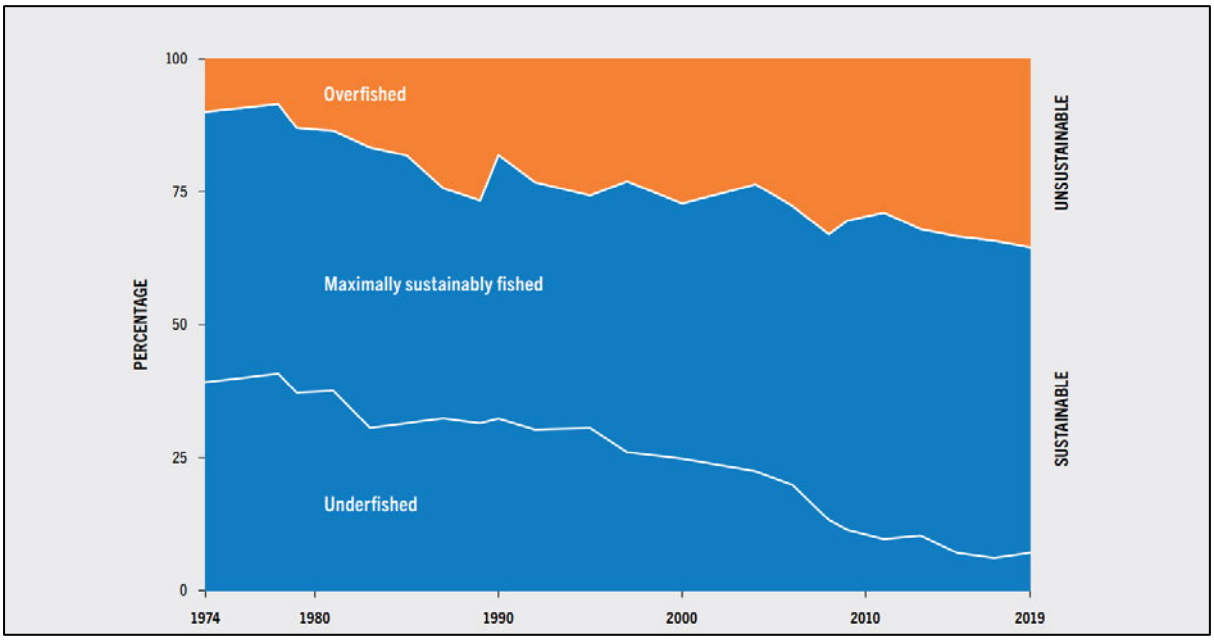

Figure 2.4 – Global sustainability of wild fish stocks since 1974
Image from (FAO, 2022)

2.2.1 Aquaculture

Aquaculture is the fastest-growing industry for producing animal food (Ottinger, Clauss and Kuenzer, 2016). It already has a significant impact on the world's food supply, and as pressure on food security mounts, its significance is predicted to increase (Bostock *et al.*, 2010). The increase in the human population and the overfishing of wild fish supplies, which has caused a plateau in fishery captures, further emphasise this (Godfray *et al.*, 2010). Sustainable aquaculture is not the sole solution for global food security, but it may be a realistic option to partly meet the need for protein sources (FAO, 2022).

Since aquaculture is not a traditional sector (in terms of intensively farmed species), several contentious concerns (other than aquafeed derived from caught fish) surround it (Diana, 2009). For example, eutrophication brought on by aquaculture wastewater consisting of uneaten food and excretory products (Qin *et al.*, 2007), the invasion or interbreeding of non-native species or escaped animals (Fleming *et al.*, 2000) and coastal erosion or deterioration

(Thampanya *et al.*, 2006). However, much research is aimed at increasing the sustainability of the aquaculture sector, including methods that maximise the value of the goods that may be produced, such as integrated multi-trophic aquaculture (IMTA) discussed in greater detail later in Section 2.3.7 - Ragworms in IMTA.

2.2.2 Aquaculture Feedstock (Fishmeal and Fish Oil) Requirement

In 2015 literature (Tacon and Metian, 2015), problems related to the need for aquaculture feedstocks were researched. The research underlines that aquaculture is now the leading consumer of non-food caught fish products (fishmeal and fish oil) and highlights the recent growth in their use reflecting the growth in aquaculture production. Additionally, the research shows that using aquafeed produced commercially is necessary for 68% of fish production. The research also describes the considerable amount of aquafeed used by the sector in recent years and the substantial anticipated increase in demand (Figure 2.5). As stated earlier, fish production through capture has plateaued; hence it is urgent to discover alternative feedstocks to produce aquafeed ingredients sustainably.

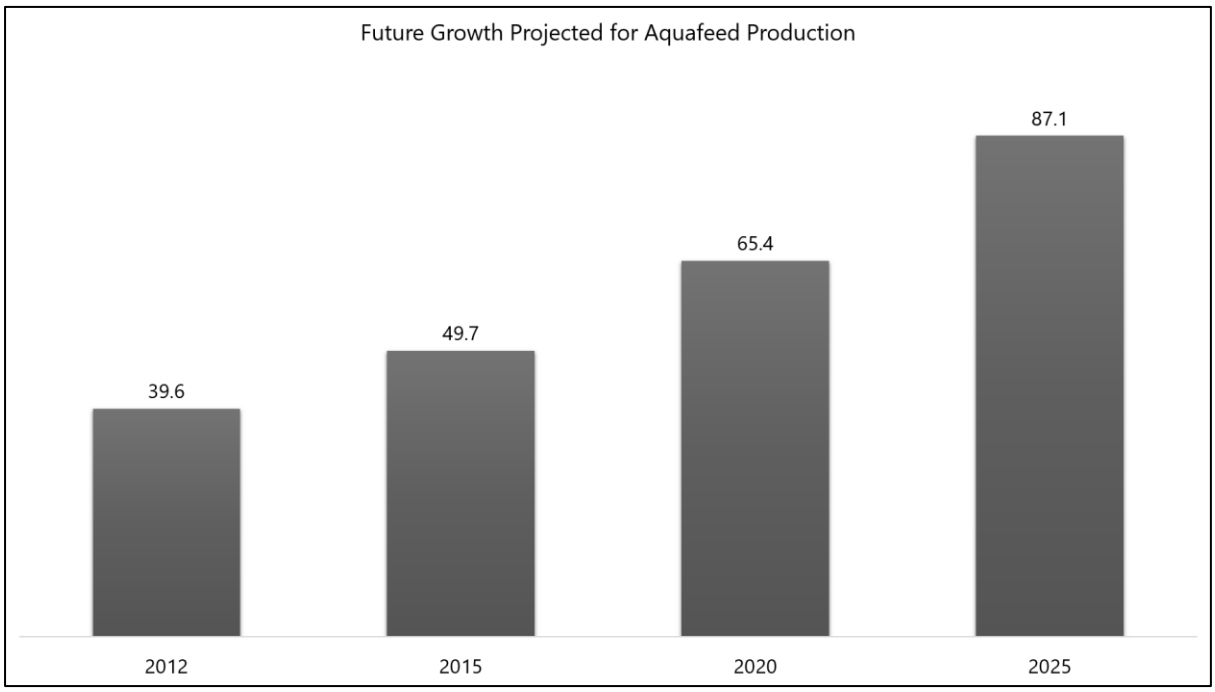

Figure 2.5 – Aquafeed demand increase 2012 – 2025
Data from (Tacon and Metian, 2015)
Units = million metric tonnes

2.2.2.1 Current and Historical Fish Meal and Fish Oil Production Levels

As of 2023, global production of fishmeal and fish oil has significantly decreased, with fishmeal production at approximately 1.738 million metric tonnes (MMT) —a 23% drop from 2022— and fish oil production down by 21% compared to the previous year (International Fishmeal and Fish Oil Organization (IFFO), 2023). These declines are largely attributed to environmental factors such as El Niño, which heavily impacted Peru's fishing activities, leading to the cancellation of fishing seasons and lower oil yields. Early 2024 data indicates continued reductions, with fishmeal and fish oil production dropping by about 27% and 30%, respectively, compared to the same period in 2023 (eFeedLink, 2024; International Fishmeal and Fish Oil Organization (IFFO), 2024).

Historically, the production levels of fishmeal and fish oil have fluctuated due to various factors, including environmental conditions, regulatory changes, and market demand. The mid-2000s to early 2020s saw increased volatility in production levels, influenced by stricter fishing regulations and sustainability efforts. For instance, fishmeal production in 2014 was about 4.8 MMT, but it experienced significant year-on-year changes due to regulatory adjustments and environmental events like El Niño. Looking ahead, while current production levels are low, there is potential for recovery if favourable conditions and sustainable practices are maintained (International Fishmeal and Fish Oil Organization (IFFO), 2023; The Fish Site, 2023).

2.2.2.2 Aquafeed Composition Over the Years

The composition of aquafeeds has evolved significantly over the years, primarily driven by the need to enhance sustainability and reduce reliance on traditional marine-based ingredients like fishmeal and fish oil. Traditionally, these feeds were predominantly composed of fishmeal and fish oil due to their high protein content and beneficial fatty acids, which are crucial for the growth and health of farmed fish (Ytrestøyl, Aas and Åsgård, 2015). However, due to concerns over the sustainability of fish stocks and the environmental impact of overfishing, there has been a shift towards incorporating more plant-based proteins and oils into aquafeeds. This transition has been supported by advancements in feed formulation

technology and a better understanding of the nutritional requirements of different aquaculture species (Aas, Åsgård and Ytrestøyl, 2022).

The shift from marine to plant-based ingredients in aquafeeds has had several impacts. For one, it has helped alleviate some of the pressure on wild fish populations, promoting more sustainable aquaculture practices. However, this change has not been without challenges. Plant-based ingredients often lack certain essential amino acids and fatty acids that are abundant in marine-based ingredients. Consequently, there have been concerns about the potential impacts on the growth rates, health, and overall nutritional quality of farmed fish (Sprague, Dick and Tocher, 2016). Furthermore, plant-based ingredients can contain anti-nutritional factors that may affect the digestion and absorption of nutrients, necessitating additional processing or supplementation to ensure feed efficacy.

Currently, other ingredients being explored and used in aquaculture include insect meals, algae, and single-cell proteins. These alternatives offer promising nutritional profiles and are more sustainable compared to traditional fishmeal and fish oil. However, they come with their own set of issues. For instance, the production of insect meal is still in its infancy and faces scalability challenges. Algae and single-cell proteins, while nutritionally rich, can be expensive to produce on a large scale. Additionally, there are regulatory and consumer acceptance hurdles that need to be addressed before these ingredients can be widely adopted in aquafeeds (Aas, Åsgård and Ytrestøyl, 2022).

2.2.2.3 Aquafeed Demand

Aquafeed demand has significantly increased over recent years, with global aquaculture feed usage reaching approximately 60 MMT in 2018. This demand is expected to double by 2050 to meet the rising need for seafood driven by population growth and increasing protein consumption. Despite this growth, the usage of fishmeal has remained stable at around 5 MMT since 2005, and fish oil usage has also been consistent at approximately 0.8 MMT. This stability highlights the industry's efforts to incorporate alternative protein and oil sources to reduce reliance on traditional marine-based ingredients due to sustainability concerns (Ytrestøyl, Aas and Åsgård, 2015; Aas, Åsgård and Ytrestøyl, 2022).

2.2.3 Aquaculture Wastewater

Water that is released from aquaculture facilities, such as fish farms, shrimp farms and other aquatic farms is referred to as aquaculture wastewater. Depending on the kind of aquaculture system in operation, the species being raised and the management techniques employed, the precise composition of aquaculture effluent can change.

In general, a wide range of pollutants, such as nutrients, organic matter, suspended solids, dissolved oxygen, antibiotics, and chemicals can be found in aquaculture wastewater. If not effectively controlled, these pollutants may have detrimental effects on the environment and potentially human health too. Aquaculture wastewater has several associated issues, including eutrophication, disease transmission, antibiotic resistance, water quality impacts and biodiversity impacts:

For example, nitrogen and phosphorous are two nutrients typically found in excess in aquaculture wastewater that can cause eutrophication of the receiving water bodies (Figure 2.6). Fish fatalities, oxygen depletion and harmful algal blooms (HABs) are three effects of eutrophication which can have a devastating impact on aquatic environments (Smith and Schindler, 2009). To a less extreme extent, aquaculture wastewater discharge can result in a decline in water quality where the aquaculture facility is located, which may impact water resources required elsewhere, such as water for drinking and irrigation (Zhang *et al.*, 2020).

Moreover, pathogens from aquaculture wastewater can spread to wild populations and result in disease outbreaks in other aquatic animals (Iber and Kasan, 2021). The pathogens include bacteria, viruses, and other parasites, which can also pose a risk to human health. For example, *Vibrio parahaemolyticus* is often found to affect seafood such as squid, mackerel, tuna, and sardines.

Furthermore, antibiotics are frequently used in aquaculture operations to both prevent and treat fish disease. Yet, using antibiotics excessively can result in the growth of germs that are resistant to them, endangering human health (Pepi and Focardi, 2021). Additionally, aquaculture wastewater may contain other chemicals, such as pesticides and disinfectants. If these chemicals are not adequately managed, they can be damaging to the environment and human health.

Finally, aquaculture wastewater has the potential to harm local biodiversity by destroying habitats and introducing non-native species (Diana, 2009). Furthermore, due to the high organic matter concentration in aquaculture effluent, dissolved oxygen levels can be low, which can cause fish deaths and other detrimental effects on aquatic ecosystems. In addition, fish faeces and uneaten feed are examples of organic solid particles commonly referred to as suspended solids that can impair aquatic life and diminish water quality.

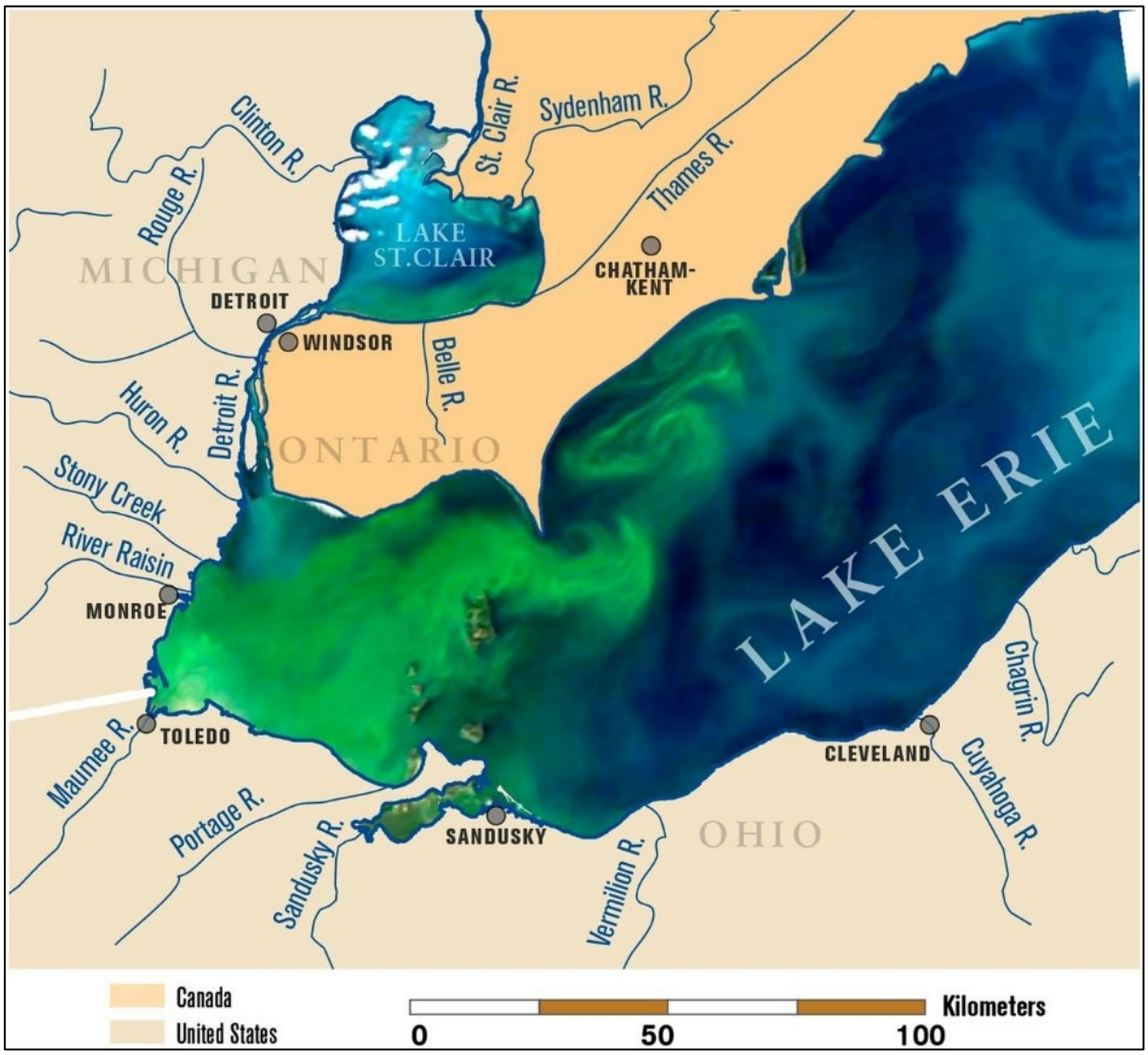

Figure 2.6 – Satellite image of eutrophication in Lake Erie, USA (03/09/2011)
Image from (Michalak *et al.*, 2013)

2.3 Hediste diversicolor (Ragworm)

2.3.1 Ragworm Physiology

Hediste diversicolor (O.F. Müller, 1776) (binomial name), more commonly referred to as ragworms or harbour ragworms, is a member of the Nereidae family (Order *Phyllodocida*, Class *Polychaeta*, Phylum *Annelida*, and Kingdom *Animalia*). In addition, controversially, its name varies in the literature, with some publications referring to ragworms as *Nereis diversicolor* (O.F. Müller 1776) or *Nereis* (*Hediste*) *diversicolor* (O.F. Müller, 1776). In the interest of clarity, they will be referred to as *Hediste diversicolor* or ragworms throughout this thesis.

In the wild, ragworm body lengths can reach up to 15 cm, but typically they are between 6 and 12 cm at maturity. Ragworms are segmented, with 90 to 120 segments, each with a pair of parapodia on either side containing bristled chaetae. Appendages on the head or posterior end of the body are visible, consisting of two antennae, two palps and four pairs of tentacles called cirri. They have four eyes and have chemosensory nuchal organs. The mouth is located beneath the prostomium and contains a pharynx which becomes everted when feeding. The eversible pharynx contains small conical chitinous teeth (paragnaths) on the proboscis, which capture food particles. The ragworm uses its appendages to sense its environment and neighbouring worms. The parapodia are used to aid in movement, primarily crawling and swimming.

The colour of *H*. *diversicolor* varies, hence the name. Mature ragworms turn green approaching and during spawning. Otherwise, they are a deep red-orange-brown colour. They are easily identifiable by a prominent dorsal red blood vessel running their body length (Figure 2.7).

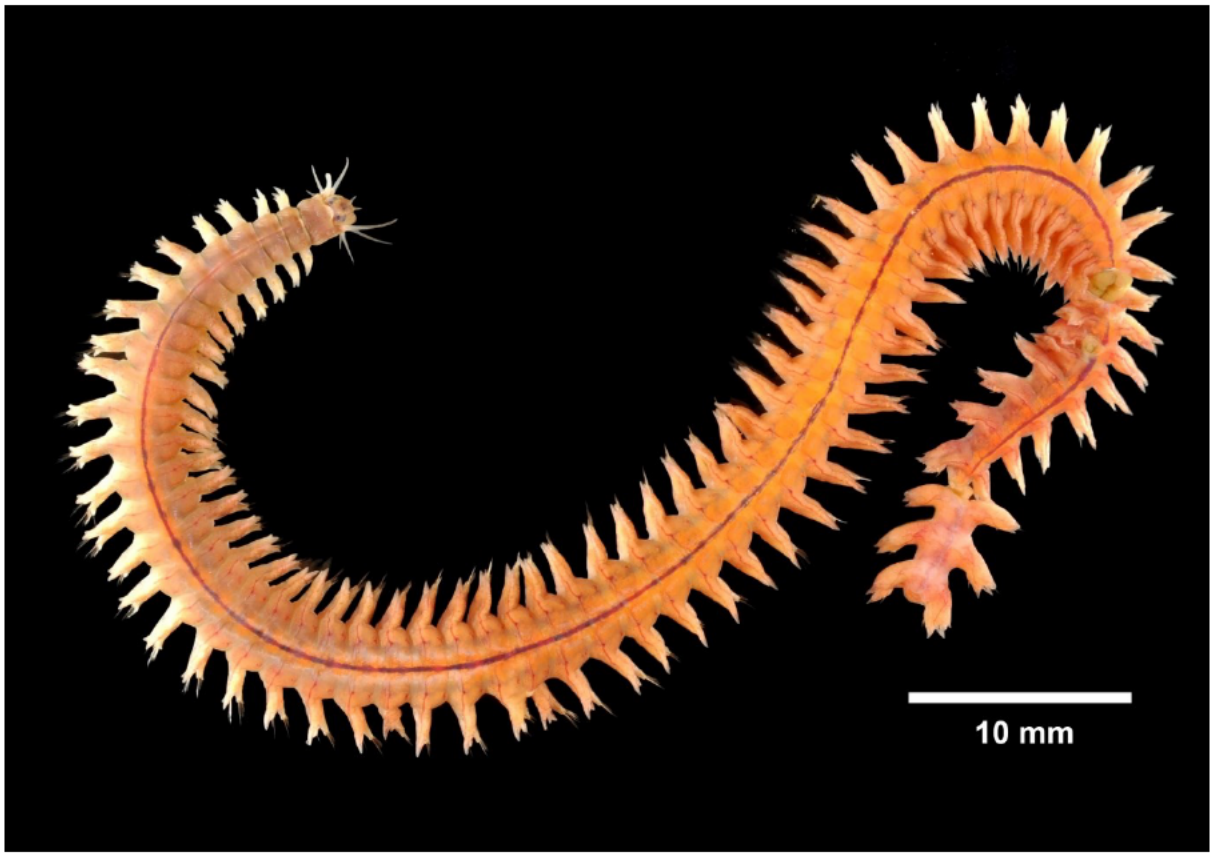

Figure 2.7 – *Hediste diversicolor* (ragworm)
(GBIF.org (29 September 2019) GBIF Occurrence Download https://doi.org/10.15468/dl.lfzpyg)

2.3.2 Ragworm Ecology

Hediste diversicolor is an estuarine polychaete found in intertidal temperate shallow marine soft-bottom coastal habitats. They are widely distributed throughout northwest Europe, on the Baltic Sea, North Sea, and along Atlantic coasts to the Mediterranean. They prefer weak tidal strengths with a natural shelter to wave exposure.

Ragworms are a euryhaline species enduring between <18 and 40 ppt salinity. Research has observed individuals residing near the upstream limit of estuaries frequently encountering salinities of less than 0.5 ppt (Smith, 1956). Ragworms behave like brackish water animals in predominately marine settings and may be in the least salinized area of accessible ground. Due to competition in the form of interspecific aggressions, the distribution of ragworms in high-salinity environments decreases (Kristensen, 1988). Ragworms only sustain high population densities in marginal habitats where the fitness of stronger competitors like *Nereis virens* (king ragworm) is reduced (Kristensen, 1988).

The ragworm population typically has a maximum density in the centre of an estuary, with density falling both toward the head and mouth of the estuary. Research has also found that areas with the most significant salinity changes had the highest populations of ragworms (Smith, 1956). The worm population density changes depending on the life cycle stage and region. Juveniles may exist above 100,000 individuals per square metre, whereas adults have been observed in densities of around 1000 individuals per square metre (Chambers and Milne, 1975).

Ragworms in the wild spend most of their time under the sediment in U or J-shaped mucous burrows up to 20 cm in depth. However, they are sometimes seen crawling on the surface if scavenging for food, making them more open to predation by birds and fish.

Burrow openings are 1 to 2 mm in soft mud. As the worm seeks food around its burrow, the posterior end of the body is secured in the burrow, and the front section of its body creates short, shallow depressions that radiate outward from the aperture. Burrow depths deepen as body length increases (Esselink and Zwarts, 1989).

Ragworms are particularly vulnerable to predation by birds and fish while eating near the mud's surface. Thus, the burrow serves as a crucial place of safety. The depth of ragworm burrows also changes with the season (Esselink and Zwarts, 1989). Wintertime is when the most extensive burrows are found, and it is possible that deeper burrowing is an adaptation to avoid the cold. As a result of ragworms' ability to withdraw from most predators, such as curlews and oystercatchers, the average depth of burrows is around 15 cm. Although each burrow has a well-defined boundary, an individual's territory does not extend outside of the burrow (Scaps, 2002).

2.3.3 Ragworm Lifecycle

Ragworms are monotelic (Olive and Garwood, 1981), gonochoristic (dioecious), and lifelong atokous (Scaps, 2002). The sexes are not visually discernible in the summer or autumn since they are both orange-red-brown. Females predominate in each population but vary depending on the location (Dales, 1950). Ragworm populations have a sex ratio that is highly skewed toward females. For example, ratios of 4.6:1 female to male have been observed in the wild (Olive and Garwood, 1981).

Researchers speculated that ragworms were hermaphrodites with parthenogenetic reproduction (Dales, 1950). However, it is now known that ragworms are not hermaphrodites and do not exhibit epitoky or swarming behaviour associated with sexual reproduction in closely related species (*Nereis succinea* and *Nereis virens*).

With maturation, colour distinctions between the sexes become pronounced. Following a period of cold winter temperatures, an early spring temperature spike triggers maturation and spawning. The male turns a vivid green colour. The female, in comparison, has a deeper shade of green that may not be present on the ventral side, and orange-red brown pigments may still be discernible.

Females at least 6 months old or around 7 cm in length may be the first to show coelomic germ cells. The coelom, surrounded by a loose mass of heterogeneous corpuscles that the eggs progressively dislodge, is where the eggs develop.

The female ragworm becomes exceptionally brittle due to histolysis of the muscle layers by phagocytes, which allows the eggs to be released upon rupturing the body wall, accomplished by thrashing within the burrow. Additionally, ragworms are synchronous to the lunar cycle, which causes spawning to occur during new or full moon phases typically.

When mature females are outside their burrows, mature males crawl about and release sperm through their nephridia. It is not required that the sexes interact directly. However, pheromones play a crucial role in coordinating activities, including mate finding and the timing of gamete release and spawning at the population level during the final stages of reproduction. Due to the low male prevalence in ragworms, it has been hypothesised that there may be a chemical signal the opposite sex might pick up on (Dales, 1950). Later observations confirm this as males exclusively discharged sperm outside burrows occupied by adult females (Bartels-Hardege and Zeeck, 1990).

Following the discharge of sperm, there is a spike in activity as the female, and those in nearby burrows undertake vigorous ventilation manoeuvres to pull sperm into their tunnel. Like eating, they may also use the proboscis to carry sperm into the burrow. Fertilised eggs are kept in the females' protective burrows. Within minutes of spawning, both sexes perish.

Ragworms are often said to mature between the ages of one and three. In terms of spawning times, populations seem to exhibit regional peculiarities. Spawning may last a little time in the spring or all summer. For example, ragworms in the Thames Estuary (UK) reached maturity a year after spawning, with some living up to 18 months (Dales, 1950). Ragworms from the Severn Estuary (UK) reportedly developed quickly in the spring and spawned at two years of age (Mettam, Santhanam and M.C.S, 1982). In the Blyth Estuary (UK), females were two years old before eggs started to show, indicating that they most likely spawned in their third year (Olive and Garwood, 1981). However, it was also found that spermatogenesis only took around six months to complete. Because of this, it was hypothesised that a variable age of maturity might result from either the population's genetic polymorphism or environmental factors (Olive and Garwood, 1981). Two spawning periods have also been observed in a ragworm population in the Ythan Estuary (UK), with the first occurring between January and March and the second between June and August (Chambers and Milne, 1975).

2.3.4 Ragworm Feeding Mechanisms

Ragworms are omnivorous and use a variety of feeding strategies, including active scavenging, filter-feeding on suspended particles and deposit-feeding on materials in and on the sediment. The sensory organs on the head of ragworms, including the palps, cirri, and eversible pharynx, are used for feeding. Ragworms have a remarkable capacity to meet their metabolic needs from a diet of phytoplankton like a normal obligate filter-feeder, which makes it stand out from the similar *Nereis virens* (king ragworm) (Nielsen *et al.*, 1995).

Harley first described filter feeding in 1950 (Harley, 1950). Undulating body motions drag a funnel-shaped net of thin mucous threads across the burrow, driving a water stream through the net (Fauchald and Jumars, 1979). When the net is complete, all the gathered particles are devoured (Fauchald and Jumars, 1979). Ragworms are an underappreciated essential component in the management of phytoplankton in shallow environments (Riisgard, 1991). Ragworms can be considered suspension feeders when there are enough algal cells in the water. However, it is uncertain how much they use their capacity to survive on suspended food particles in nature (Riisgard, 1991).

Two primary forms of food prospecting behaviour are observed in ragworms while deposit feeding (Esnault, Retière and Lambert, 1990). One involves the ragworm searching for food by crawling over the sediment surface, grabbing it with its teeth and instantly eating it. In the second, the ragworm leaves mucous strings on the sediment surface on either side of its body. The mucous is then transported and formed into a pellet as the ragworms withdraw back into their burrow. The pellet can either be devoured immediately or saved for later consumption (Esnault, Retière and Lambert, 1990).

Other research has observed juvenile ragworms picking up detritus from the sediment surface and storing it in their burrow (Olivier *et al.*, 1995). The juvenile ragworms water their burrows to maintain an aerobic environment to encourage bacterial development, which speeds up the breakdown of plant detritus (gardening).

Ragworms also have the propensity to consume bacteria, highlighted by the discovery of bacteriolytic activity in their digestive tract (Lucas and Bertru, 1997).

2.3.5 Ragworm Aquaculture

Since it first appeared in the 1980s, polychaete aquaculture has become a more significant subset of the aquaculture sector. Numerous farms have been established in the UK and internationally; they mainly culture *Nereis virens* (king ragworm) and *Hediste diversicolor* (ragworm).

Two key reasons drive polychaete aquaculture. In addition to being utilised as feed in other aquaculture systems, the worms can be used as live bait for sea fishing. Many live polychaetes are imported from the USA and the Far East into Europe. Due to the demand, there are sometimes more imported non-native polychaetes sold as bait than domestic ones (Olive, 1999).

Moreover, because polychaetes are very nutritious and relatively simple to produce, farmed polychaetes are ideal candidates for aquaculture feeds. Using polychaetes as feeds is technically viable and environmentally sustainable because several farmed finfish and crustaceans naturally consume polychaetes in the wild. In coastal areas, polychaetes are

consumed by many species of fish and crustaceans. For example, about 45% of flounder prey and 15% of sole prey were discovered to be *H. diversicolor* (Luis and Passos, 1995).

Polychaetes can be used as a larval diet and an adult fish maturation feed. Due to their high quantities of protein, lipids and long-chain polyunsaturated fatty acids (LC-PUFA), they are regarded as high-quality maturation diets (Meunpol, Meejing and Piyatiratitivorakul, 2005). A high-quality maturation diet provides optimal levels of proteins, essential fatty acids, vitamins, and minerals to support health and reproductive success. Polychaetes also include several hormone-active substances (Lytle, Lytle and Ogle, 1990).

However, there are several concerns with using wild polychaetes, including their fluctuating nutritional value and potential for disease transmission (Vijayan *et al.*, 2005). *H. diversicolor*, for instance, in the wild, has a lipid concentration that varies between 6.6 and 19.3% (Luis and Passos, 1995). However, a farm-regulated environment with controlled inputs can make their nutritional value more consistent. Moreover, polychaetes in the wild are bioaccumulators of heavy metals and other toxins and therefore feeding them to other species carries a risk of disease transmission (Rainbow *et al.*, 2006). Again, culturing polychaetes in a controlled farm system negates several risks related to disease and contaminant transmission.

2.3.6 Ragworms as an Aquafeed Ingredient

Lipids

In addition to ragworms' high quantities of protein, lipids and LC-PUFA, utilising ragworms as an aquafeed ingredient can help relieve the strain on wild fish populations to produce aquafeed. For example, omega-3 and omega-6 (ω-3 and ω-6) fatty acids present in considerable concentrations in polychaetes are considered crucial for developing the prawn reproductive system (Lytle, Lytle and Ogle, 1990). Particularly ω-3 fatty acids are essential for crustacean egg production (García-Alonso and CT, 2008), and ragworms have been discovered to encourage gonad development and spawning in shrimp and sole (Luis and Passos, 1995).

Most marine fish, such as salmon, mackerel, and sardines, are known for their high ω-3 content. However, many freshwater fish and some farmed fish species such as tilapia and catfish are unable to synthesize ω-3 fatty acids efficiently and rely on their diet to obtain these

essential nutrients (Young, 2009). Therefore, their daily diet must supply enough to satisfy their requirements for good health and protect them against disease. On the Canadian coast, researchers found ragworm samples with essential fatty acid concentrations of 24.4% eicosapentaenoic acid (EPA) and 3.5% docosahexaenoic acid (DHA) and a total LC-PUFA concentration of 48.8% (Copeman and Parrish, 2003). These concentrations are well above the requirements of aquafeeds. EPA and DHA are crucial for the growth, development, and overall health of farmed fish, contributing to immune function, reproductive performance, and the nutritional quality of the fish for human consumption. Furthermore, other research reportedly found that ragworms can biosynthesise certain essential fatty acids such as EPA and DHA when fed a diet that is deficient in fatty acids (Luis and Passos, 1995).

Lipids in polychaetes serve as an internal nutrition and energy source during times of inadequate feeding conditions and negative energy balance (Pocock, Marsden and Hamilton, 1971). Lipids, such as sterols and phospholipids, are among the structural lipids essential for parts of biomembranes. The excess fat from ragworms' intestines is transported via blood or coelomocytes and stored in the epidermis since marine invertebrates lack organs or adipose tissue for storing lipids.

Hormones

Ragworms have been shown to contain a variety of hormonally active compounds, making them a good diet for the development of prawn broodstock (Lytle, Lytle and Ogle, 1990; Meunpol, Meejing and Piyatiratitivorakul, 2005). Hormones are crucial for prawns as they regulate growth, moulting and reproduction, and immune responses, which are vital for maintaining healthy and productive broodstock.

General Nutrition

A research study in 2017 (Wan, Snellgrove and Davies, 2017) compared fish fed on three different diets – fishmeal plus ragworm meal (RW + FM), fishmeal plus silkworm meal (SWP + FM) and fishmeal plus silkworm meal and ragworm meal (SWP + RW + FM). The RW + FM diet produced the highest growth rate of any group. This is noteworthy since RW + FM used less

fishmeal and fish oil than the control group, indicating that adding ragworm as a supplement to feedstocks could reduce the need for fishmeal and fish oil.

Another research study in 2016 (Kals *et al.*, 2016) demonstrated the advantages of using ragworms as a feed for intensive aquaculture. In intensive production environments, sole (*Solea solea*) frequently experiences mild anaemia. Ragworms functioned better as feedstock than industrial pellets in raising the levels of haemoglobin and haematocrit in the soles, reducing anaemia. It is unclear whether this results from increased food consumption or the worms' high nutritional value. However, it still implies that, regarding fish health, using ragworms as a feedstock is preferable to using commercial feed pellets in this scenario.

Lastly, research in 2014 considered ragworms' nutritional makeup when raised in sand filters. According to the study, larger worms grown in low density have a greater lipid content and, more importantly, a nutritional profile suitable for shrimp and fish broodstock (Palmer *et al.*, 2014).

2.3.7 Ragworms in Integrated Multitrophic Aquaculture

Integrated multitrophic aquaculture (IMTA) can be considered the cooperative cultivation of aquatic organisms from different trophic levels (Troell *et al.*, 2009). The underlying idea is that organisms at a lower trophic level recycle waste materials or uneaten food from the primary cultivated species. For example, macroalgae attached to lines in water can assimilate soluble nutrients and polychaetes filter-feed particle debris that falls to the bottom. At the same time, finfish are cultured in a cage nearby (Figure 2.8). In addition to reducing waste, the synergism may also result in creating a secondary economically viable product (e.g., ragworms as an alternate aquafeed ingredient).

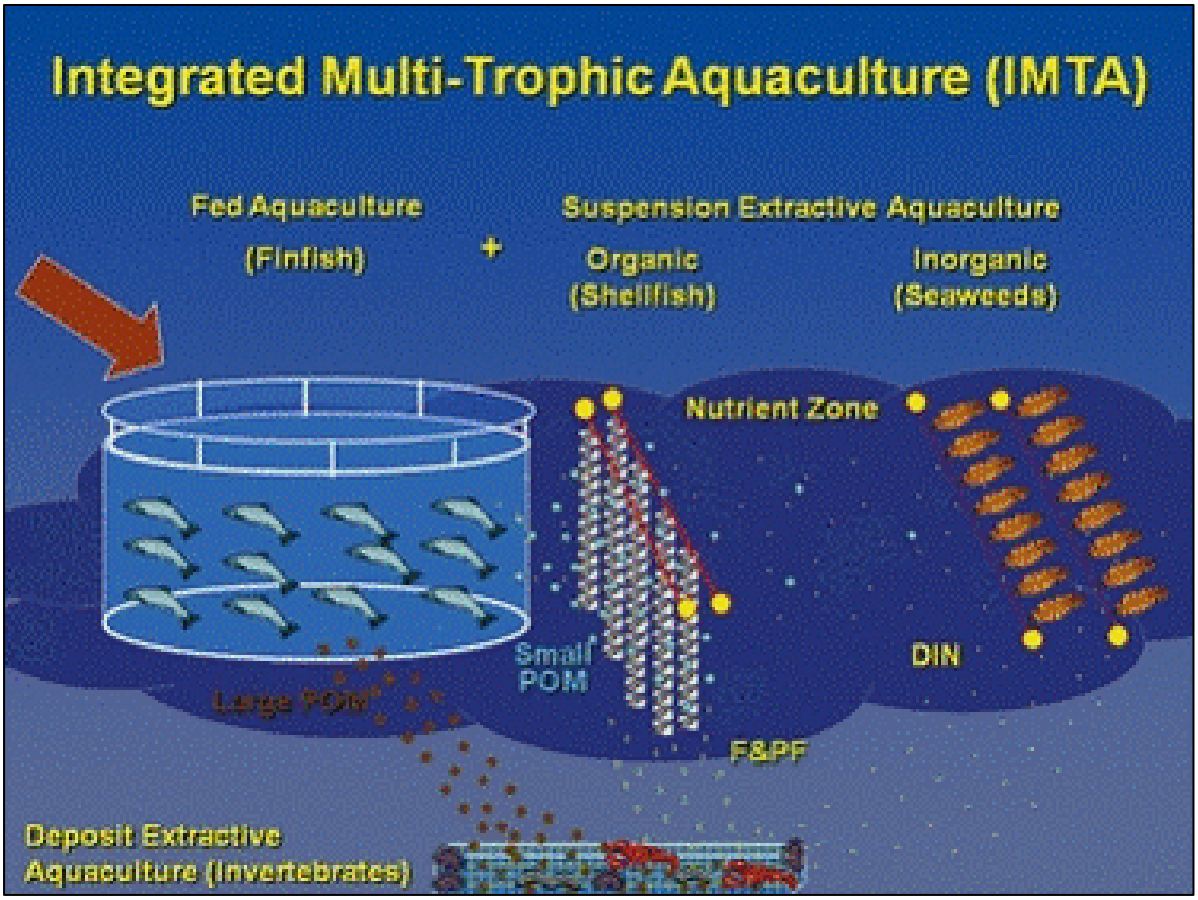

Figure 2.8 – An illustrative diagram of integrated multi-trophic aquaculture (IMTA)
Includes rearing fish, cleaning debris by invertebrates and extracting dissolved nutrients with seaweed.
Image from (Chopin *et al.*, 2012)

Numerous polychaetes, including *H. diversicolor*, are beneficial in reducing pollution because they eat the organic material in the sediment and enhance oxygen levels via burrowing. In research culturing ragworms in an IMTA system, they were found to have better fatty acid profiles (enhanced composition and balance of essential fatty acids) than their wild counterparts, making them more appropriate for inclusion in the diets of fish and shrimp maturation feeds (Marques *et al.*, 2019).

The relevance to this project is the potential for ragworms to be cultivated as part of IMTA in a closed recirculating aquaculture system (RAS). In other words, not only performed in an open-water (cage) system like the Figure above. The ragworms can capture internal pollutants for their growth while subsequently producing an alternative ingredient for inclusion into aquafeeds.

2.4 Chitosan

Chitosan is an important part of this project as it is used to process aquaculture wastewater before being fed to ragworms.

Chitin is the most prevalent amino polysaccharide polymer found in nature and the substance that provides crustaceans, insects, and fungi cell walls with their strength. Chitin is the most abundant natural biopolymer on earth, apart from cellulose (No and Meyers, 2000). Chitin may be deacetylated chemically or enzymatically to produce the well-known derivative, chitosan. Shrimp and crab shells, a plentiful by-product of the food processing industry, are the primary natural sources of chitin (Elieh-Ali-Komi and Hamblin, 2016). Although chitosan has various uses most commonly biomedical applications (Younes and Rinaudo, 2015), its usage as a coagulant is what makes it pertinent to this project.

For example, in a study focusing on the treatment of aquaculture wastewater, chitosan was used to coagulate and remove organic compounds and inorganic nutrients effectively. Chitosan achieved its highest removal efficiencies with the following percentages: turbidity at 90%, suspended solids at 61%, chemical oxygen demand at 69.7%, ammonia at 89.2%, and phosphate at 95.6% (Chung, 2006).

“Chitosan” often refers to a group of polymers produced by various degrees of chitin deacetylation. Chitin and chitosan differ in acetylation degree, representing the distinction between the two. Chitosan is the product’s name when the degree of deacetylation (DD) is higher than 50% (molar percentage), and can readily dissolve in acidic aqueous solutions (Roberts, 1992; Rinaudo, 2006). In addition to the removal of acetyl groups, changes in chitosan's molecular weight (MW) during deacetylation indicate that depolymerisation has taken place.

Using enzymatic preparations (Aiba, 1994) or chemical procedures (No and Meyers, 1995), chitin may be transformed into chitosan. Because they are inexpensive and well-suited for mass production, chemical procedures are frequently used to prepare chitosan for commercial use (No and Meyers, 1995).

The effectiveness of chitosan can be enhanced through chemical modifications such as grafting with polyethylene glycol to improve its solubility and biocompatibility (Casettari *et al.*, 2012).

2.4.1 Making Chitosan

The procedure for making chitosan from marine sources of chitin is described by Younes & Rinaudo (2015) (Younes and Rinaudo, 2015). Firstly, chitin is extracted from shells using acid to dissolve calcium carbonate, and then other proteins are dissolved in an alkaline solution. Then, chitin can be deacetylated in several ways to create chitosan. This entails switching out β–(1-4)-N-acetyl-D-glucosamine groups for β-(1-4)-D-glucosamine groups. The deacetylation process is typically carried out by placing the chitin in a hot solution of concentrated sodium hydroxide, which typically yields chitosan with a deacetylation level between 85 and 99%. Various adaptations can then be made to increase the effectiveness of the chitosan for a particular purpose. The molecular structures of chitin and chitosan can be seen below in Figures 2.9 and 2.10.

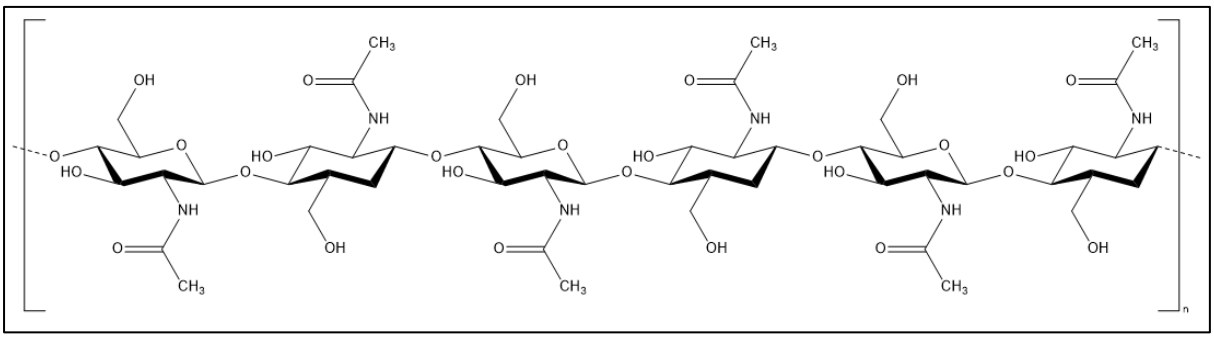

Figure 2.9 – Chitin
Three repeat monomers of the chitin polymer.
Figure created using ChemDraw®

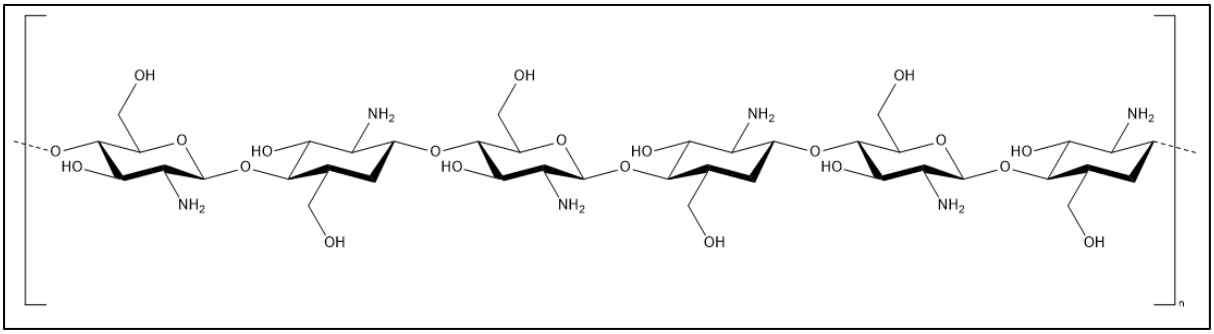

Figure 2.10 – Chitosan (3 repeat monomers of the polymer)
Three repeat monomers of the chitosan polymer. Notice the absence of acetyl groups along the polymer chain creating binding sites.
Figure created using ChemDraw®

Depending on the chitin source and the desired chitosan qualities, the specifics of the procedure may change. To guarantee consistency, quality, and purity in chitosan production for commercial sale or laboratory applications, production often requires specific tools and highly controlled procedures. A flow diagram outlining the general steps for making chitosan is shown below in Figure 2.11.

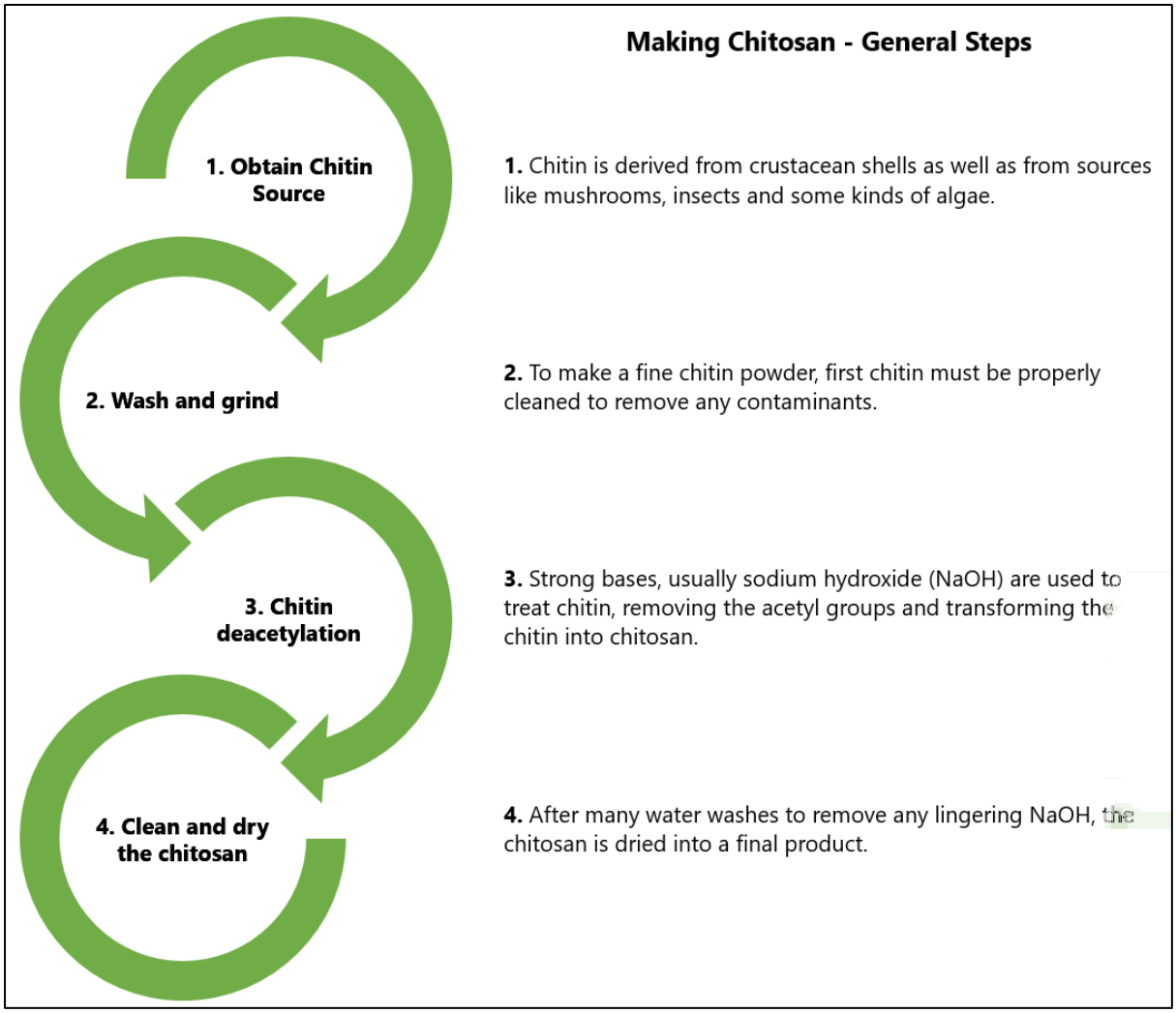

Figure 2.11 – General steps required to make chitosan

2.4.2 Coagulation and Flocculation

The removal of suspended particles and contaminants from water can be accomplished using coagulation and flocculation. To destabilise and aggregate the suspended particles, coagulation includes the addition of a coagulant such as alum or ferric chloride. To encourage the creation of larger, heavier flocs that are easier to remove, flocculation entails gently agitating the water.

According to one study, coagulation and flocculation are highly effective at removing turbidity, chemical oxygen demand (COD) and total suspended solids (TSS) from urban wastewater (Barros *et al.*, 2022). Another study investigated the ability to remove toxic algae from natural waters with a conventional coagulant. The study found that 99.9% of the cells

could be removed with their optimised process (Drikas *et al.*, 2001). Furthermore, the researchers found no additional release of toxins from the algae in the treated water.

Coagulation and flocculation are significant steps in the water-treatment process that can greatly raise the standard of water. However, factors like pH, temperature, and the type of contaminants in the water must be taken into consideration when attempting to coagulate and flocculate (described in subsequent sections).

2.4.3 Chitosan Coagulation and Flocculation Mechanism

Chitosan is a natural biopolymer that may be used as a water coagulant in place of aluminium salts and artificial polymers to prevent harmful effects on human health (Soros *et al.*, 2019). Chitosan is unlike other industrial polymers because it is a highly molecular-weighted cationic polymer, which is beneficial in normally anionic wastewater (Bellich *et al.*, 2016). However, more importantly, chitosan is GRAS (generally recognised as safe) (Bellich *et al.*, 2016) meaning chitosan can potentially be used to coagulate and flocculate aquaculture wastewater and fed to ragworms without toxicity (which ragworms can then be used as an alternative aquafeed ingredient). This is discussed in greater detail in Section 2.4.7 - Chitosan Toxicity in Animals.

As described above, coagulation and flocculation are processes by which colloids are brought together with the aid of a coagulant to create bigger particles known as flocs. However, the mechanism by which chitosan functions differs from other commonly inorganic and organic coagulants. Lichtfouse *et al*., (2019) (Lichtfouse *et al.*, 2019) summarise the mechanisms by which chitosan works (Figure 2.12).

Chitosan chains initially create micro-flocs during the coagulation process in wastewater, which destabilises suspended colloid particles. Micro-flocs are subsequently combined in the flocculation process, which calls for mixing to cause clumping and enable solid removal in later steps. Charge neutralisation and bridging processes are primarily responsible for chitosan coagulation and flocculation. Additionally, chitosan coagulation and flocculation may be influenced by electrostatic patching, sweeping, adsorption, entrapment, complexation, chelation, and precipitation.

Charge neutralisation refers to the interaction of chitosan and colloids which attract each other because they are oppositely charged (a). By bridging between individual colloids, chitosan chains aggregate them and by generating chitosan-colloid complexes, more colloids adsorb onto chitosan chains in this phenomenon (b). Intercolloidal bridging occurs when the loops and tails of the adsorbed chitosan chains protrude and connect to other chitosan-colloid complexes in the aqueous solution. The interaction of highly charged chitosan chains with negatively charged colloids causes electrostatic patching (c). In other words, if chitosan and colloid charges are in opposition, a chitosan-colloid patch then binds other colloids. The sweeping process (d) may be summarised as chitosan forming a bulky precipitate that enmeshes colloids. Following these mechanisms, colloids may then settle out or combine with the precipitate to form a floc.

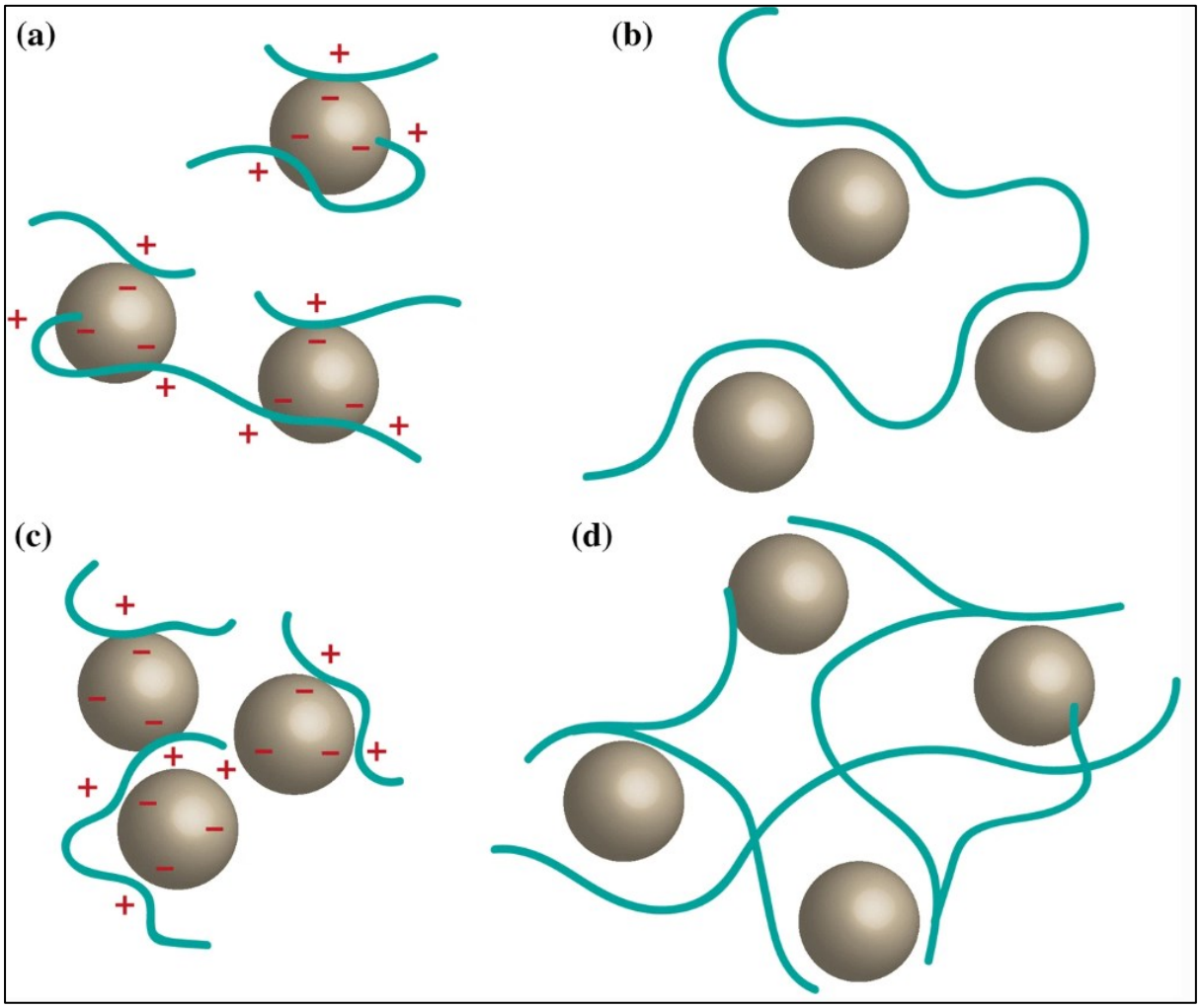

Figure 2.12 – Chitosan coagulation and flocculation mechanism
Green lines represent chitosan. Red plus and minus signs indicate charge. Spheres represent colloids. (**a**) = charge neutralisation; (**b**) = intercolloidal bridging; (**c**) = electrostatic patching and (**d**) = sweeping.
Adapted from (Lichtfouse *et al.*, 2019)

Chitosan's ability to act optimally as a coagulant depends on the dose and the efficiency follows a curve (Chung, Li and Chen, 2005; Chung, 2006). In other words, the efficiency of chitosan will decline if the dosage passes a certain point (the optimum dose). While charge neutralisation is not the main mechanism by which coagulation occurs, using too much chitosan can result in a reversal of the surface charge of the coagulated particles, causing the colloids to repel one another again, thereby rendering the chitosan ineffective.

In addition to dosage, other characteristics must be recognised for the optimal treatment of wastewater with chitosan. For example, an understanding of the MW, degree of deacetylation (DD) and the inclusion of functional groups along the polymer chain is necessary to identify the best chitosan polymer for wastewater (Soros *et al.*, 2019)

Chitosan has been used for the reduction of contaminants in a variety of wastewaters other than aquaculture (Chi and Cheng, 2006; Rizzo *et al.*, 2008; Renault *et al.*, 2009; Soros *et al.*, 2019) with the added benefit that chitosan can remove turbidity at low doses (e.g. 1 – 10 mg/L) (Divakaran and Sivasankara Pillai, 2001; Rizzo *et al.*, 2008; Soros *et al.*, 2019).

2.4.4 Chitosan and Molecular Weight

According to Chung, Li and Chen, 2005, higher molecular weight (MW) chitosans result in a higher peak value for turbidity reduction and a lower optimal dose (Figure 2.13). Higher MW chitosan has longer polymer chains and therefore more effective interparticle bridging requiring a lower dose than chitosans with lower MW (Chung, Li and Chen, 2005).

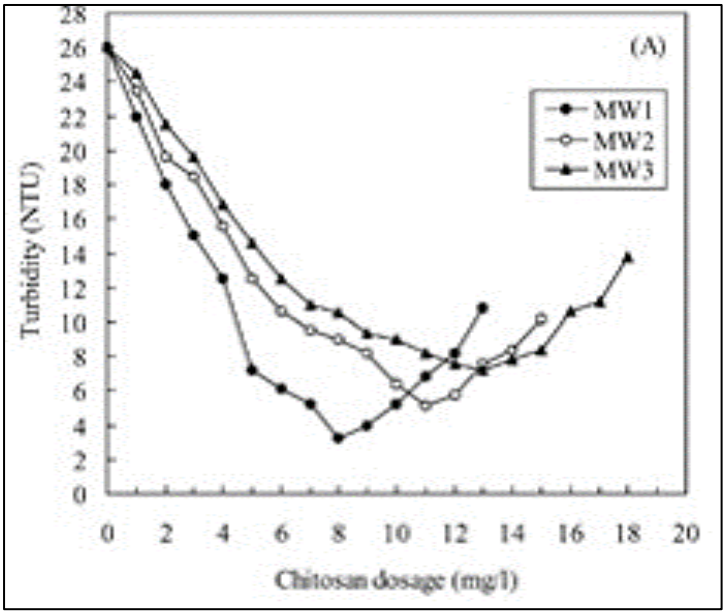

Figure 2.13 – Remaining turbidity of aquaculture wastewater following chitosan coagulation and flocculation at various doses and molecular weights
Initial turbidity was 26 ± 1.53 nephelometric turbidity units (NTU) (n = 3). The MW of MW1, MW2 and MW3 were 3.62×10^6, 4.73×10^4, and 6.21×10^3, respectively.
Adapted from (Chung, Li and Chen, 2005)

2.4.5 Chitosan and Degree of Deacetylation

The degree of deacetylation (DD) of chitosan is another crucial chitosan parameter. As explained in Section 2.4 - Chitosan, the degree of chitin (molar percentage) that has been deacetylated within the polymer chain is referred to as DD. According to Chung (2006), chitosan DD has a substantial impact on how efficiently chitosan functions as a coagulant. Chung's investigation examined the removal of turbidity, SS, COD, and other contaminants by chitosans of various DD at various concentrations. It was concluded that higher DD results in a lower optimum dose and higher turbidity removal (Figure 2.14).

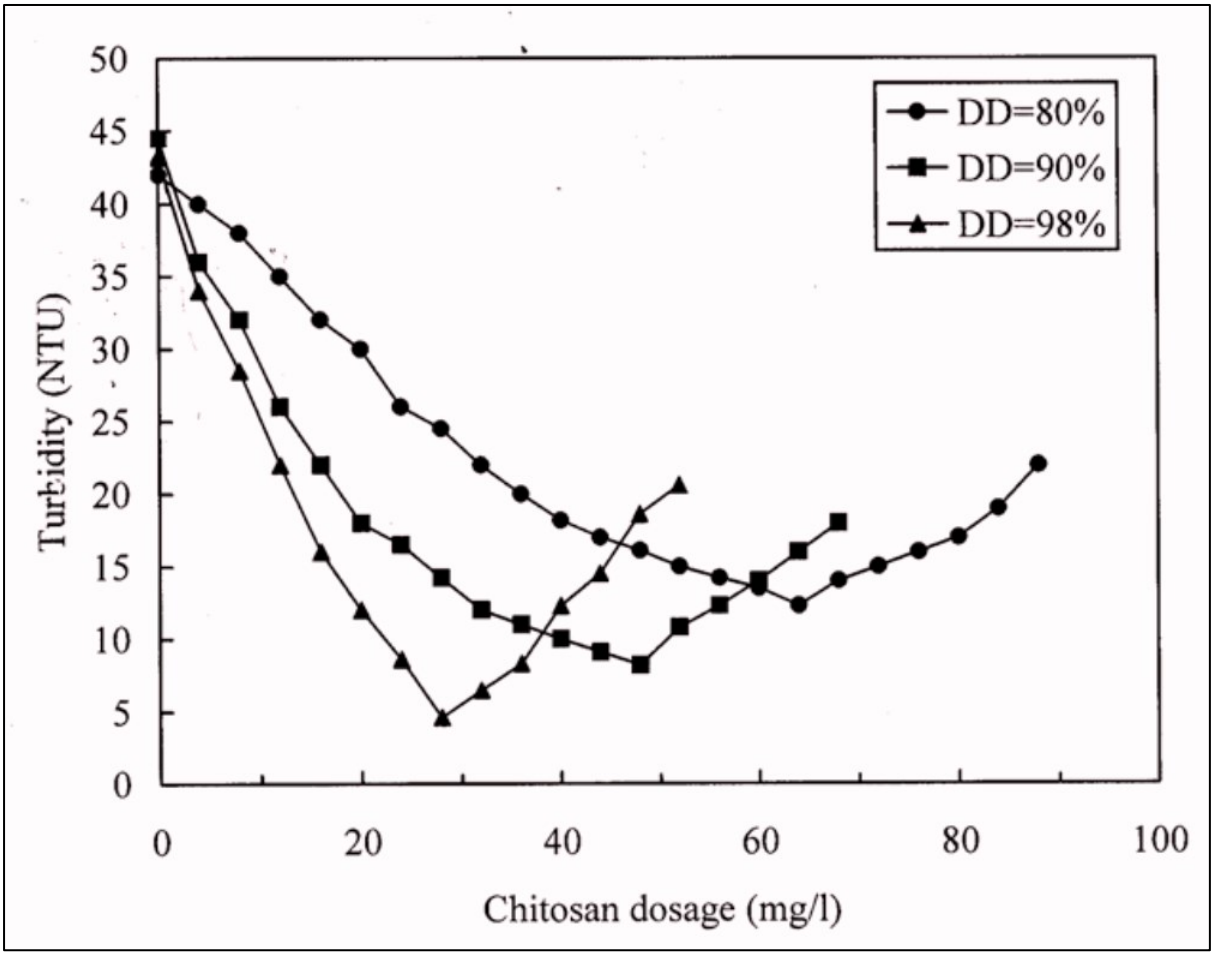

Figure 2.14 – Effects of chitosan concentration and degree of deacetylation (DD) level on the elimination of turbidity in aquaculture wastewater
Initial turbidity was between 42.0 and 44.5 nephelometric turbidity units (NTU).
Adapted from (Chung, 2006)

The mass of chitosan needed to capture colloids and cause a reversal of charge decreases as the fraction of the polymer chain that can attract colloids grows, explaining why a lower dose is required with chitosan of higher DD. (Huang and Chen, 1996; Chung, 2006).

2.4.6 Chitosan and pH

Like dose, chitosan is effective over a certain pH range and when the pH is too low or too high, chitosan loses its effectiveness. Which can be further complicated by the range of pH in which chitosan works also being dependent on other parameters such as the type of chitosan used. Chitosan is insoluble in water, but in acidic pH, the free amino groups along the polymer chain become protonated and chitosan becomes a soluble cationic polymer with high charge density (Shalaby, 2017).

2.4.7 Chitosan Toxicity in Animals

If chitosan is to be used to coagulate and flocculate aquaculture wastewater before feeding to ragworms, the potential toxicity of chitosan to ragworms must be considered. Chitosan is widely considered nontoxic, biocompatible, biodegradable and antimicrobial (Leonida, Ispas-Szabo and Mateescu, 2018). Furthermore, chitosan is Generally Recognised as Safe (GRAS) by The United States of America Food and Drug Administration (FDA) (USA FDA, 2019).

However, despite chitosan being GRAS and widely referenced as nontoxic (Thanou, Verhoef and Junginger, 2001), safety data sheets (SDS) from ThermoFisher Scientific and Sigma-Aldrich state that one of the uses of chitosan they advise against is its inclusion in food (Appendix A and Appendix B). This statement refers to food for human consumption rather than ragworm consumption. However, it is still important to note.

Furthermore, the SDS reference that the lethal dose (LD_{50}) for oral consumption in rats (*Rattus rattus*) is 10 g kg^{-1}, the lethal concentration (LC_{50}) in freshwater trout (*Oncorhynchus mykiss*) is 1.73 mg L^{-1}, and the half maximal effective concentration (EC_{50}) in water fleas (*Daphnia pulex*) is 13.69 mg L^{-1} (Appendix A). The SDS also refer to their knowledge that "the chemical, physical and toxicological properties (of chitosan) have not been thoroughly investigated" (Appendix B).

In addition to the SDS, the National Toxicology Program (NTP) at The United States Department of Health and Human Services carried out a technical report on the toxicity of chitosan (National Institutes of Health and US Department of Health and Human Services, 2017). The report includes details of various clinical studies on the acute toxicities of chitosan in experimental animals. According to those studies, chitosan can be hazardous to some animals when taken in excessive doses.

For example, one study concluded that chitosan's LD_{50} in the mice (*Mus musculus*) they studied was 16 g kg^{-1} (Hirano, 1996). In another 4-week study with a 1% or 5% chitosan diet, no discernible changes were observed in weight between exposed male Charles River albino rats and controls (Vahouny *et al.*, 1983). Another 21-day study found no significant changes between a control diet and a 2% or 5% chitosan diet in male Wistar rats in their growths, feed intakes, liver weights or dry faecal weights (Fukada, Kimura and Ayaki, 1991). Another study

fed chitosan to male Sprague-Dawley rats for eight weeks, and no toxicity effects were reported in the animals at doses up to 5%. However, at 10 and 15%, there were gradual growth decreases and clinical pathology changes. For example, at 15%, the animals had enlarged livers and kidneys (Landes and Bough, 1976). Moreover, body weight losses were reported in female BALB/c mice fed a 5% chitosan diet for four weeks which was strongly linked with reduced feeding rates and changes to the animals' normal gut flora (Tanaka *et al.*, 1997). Finally, in another study, a 5% chitosan diet was fed to male Charles River Japan Sprague Dawley rats for two weeks which exhibited reduced mineral absorption and bone mineral content. The rats fed the 5% chitosan diet also had lower blood levels of vitamin E compared to their counterparts fed a control diet without chitosan (Deuchi *et al.*, 1995).

Moreover, the route of exposure to chitosan in animals can have a massive variation in toxicity effects. For example, chitosan was tested for its efficacy in removing solids from a rainbow trout (*Oncorhynchus mykiss*) recirculation system (Bullock, 1993). Chitosan was introduced to the system at 1 ppm after being dissolved in 1% acetic acid. Within a few hours, a fatal toxic reaction was observed. Inspection of the injured trout revealed pale gills with an abundance of mucus and haemorrhages. However, in other research, where chitosan was added to the diet of rainbow trout at a 5% concentration, no harmful effects were observed (Siwicki, Anderson and Rumsey, 1994). In addition, similar research reported that feeding 10% chitosan diets to three species of marine fish was not negatively impacted and growth rates remained the same as controls (Kono, Matsui and Shimizu, 1987).

Overall, if chitosan at certain concentrations in feed can negatively impact animals such as trout, water fleas, rats, and mice, then it is conceivable that chitosan at certain concentrations in aquaculture wastewater-derived feed can negatively impact ragworms too. However, it is important to remember that chitosan toxicity in animals varies based on the species, dose, route of exposure, and length of exposure. Hence, before feeding chitosan to animals, it is crucial to carefully weigh the advantages and disadvantages.

2.4.8 Chitosan in Aquaculture

Chitosan has been used in aquaculture as a dietary supplement (Kono, Matsui and Shimizu, 1987), for the controlled release of vaccines and as an immunostimulant to improve

salmonids' resistance to bacterial infections (Anderson and Siwicki, 1994). In addition, the regulated release of proteins like bacterins into aquaculture systems has been achieved by combining chitosan with alginate in orally ingested microcapsules (Polk *et al.*, 1994).

More relevant to this project and described in the chapter 1 introduction, chitosan has also been tested for its efficacy as a coagulant and flocculant to remove contaminants from aquaculture wastewater. For example, chitosan has been tested as an aid to remove particulates from rainbow trout recirculation systems (Bullock, 1993). However, in academic literature, the coagulated aquaculture wastewater products have never been investigated further by feeding to ragworms to create an alternative feed for the aquaculture industry, hence this project.

2.5 Literature Review

Context

Aquaculture has emerged as a vital sector in global food production, particularly in meeting the increasing demand for fish. However, it faces critical sustainability challenges, primarily due to its reliance on wild-caught fish for high-quality ingredients and the large volumes of polluted water it generates. Addressing these issues is essential for the industry's long-term viability and environmental impact.

Aquafeed Utilisation

The utilisation of feed resources in Atlantic salmon production has been extensively studied in Norway, a leading country in aquaculture. Ytrestøyl, Aas and Åsgård (2015) conducted a comprehensive analysis of feed resource utilisation, highlighting the efficiency and challenges in the industry. Their study provided a baseline understanding of feed conversion ratios and the nutritional requirements of Atlantic salmon.

In 1990, 90% of Norwegian salmon feed ingredients were of marine origin, which decreased to around 30% by 2013, with salmon production increasing by 30% between 2010 and 2013, leading to a reduction in marine ingredients used from 544,000 to 466,000 tonnes. By 2013,

the forage fish dependency ratio for fish meal had decreased from 4.4 to 0.7, making Norwegian farmed salmon a net producer of marine protein, using 0.7 kg of marine protein to produce 1 kg of salmon protein.

Aas, Ytrestøyl and Åsgård (2019) updated these findings, presenting data for 2016, which indicated improvements in feed utilisation but also underscored the continuing dependence on fishmeal and fish oil. This update highlighted the progress made in reducing the proportion of wild-caught fish in feed, a critical step towards sustainability.

The study provides an update on Norwegian salmon farming feed utilization for 2016, reporting the production of 1,252,573 tonnes of salmon and 814,172 tonnes of fillet. In 2016, 1,627,478 tonnes of feed ingredients were used, with marine ingredients constituting 25%, plant ingredients 71%, and other ingredients 4%, showing a continued shift from marine to plant-based feed sources.

Further advancements were documented by Aas, Åsgård and Ytrestøyl (2022), who provided data for 2020, showing significant strides in incorporating alternative feed ingredients, thus reducing pressure on marine ecosystems.

This paper updates the utilisation of feed resources in Norwegian salmon farming for 2020, highlighting the use of 1,976,709 tonnes of feed ingredients to produce 1,467,655 tonnes of salmon. The feed comprised 22.4% marine ingredients, 73.1% vegetable ingredients, and 4.1% micro-ingredients, with 0.4% from single cell protein and other novel sources, and showed similar feed utilisation patterns to 2016, with slight increases in production and economic feed conversion ratio.

Alternative Aquafeed Ingredients

The search for high-quality sustainable feed alternatives has led to the exploration of various sources. Sprague, Dick and Tocher (2016) investigated the impact of sustainable feeds (e.g., vegetable-derived oils) on omega-3 fatty acid levels in farmed Atlantic salmon over a decade. Their findings revealed that while sustainable feeds could maintain adequate omega-3 levels, the challenge lies in achieving this without compromising fish growth and health.

They found replacing traditional marine ingredients in salmon diets with terrestrial alternatives has led to a significant decrease in essential fatty acids, compromising the nutritional value and requiring larger portions to meet recommended intake levels, though farmed salmon still provide more eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) than most other fish and terrestrial livestock.

Subsequent research by Sprague, Betancor and Tocher (2017) explored the use of microbial and genetically engineered oils as replacements for fish oil in aquaculture feeds. These innovative solutions showed promise in providing essential fatty acids (omega-3 long-chain polyunsaturated) while reducing the dependency on wild-caught fish, thus aligning with sustainability goals.

Bioremediation of Aquaculture Waste

Effective management of aquaculture waste is another critical aspect of sustainability. Gómez, Hurtado and Orellana (2019) explored bioremediation using the polychaete *Abarenicola pusilla* to treat organic sludge from a marine recirculating aquaculture system. Their study demonstrated the polychaete's ability to reduce organic waste, providing a potential solution for sludge management.

The study showed that at a sludge concentration of 10% and a density of 200 organisms per m^2, *A. pusilla* achieved the highest removal rates of total organic matter (85.37%; 35.77 g m^{-2} day−1), total nitrogen (33.63 mg day^{-1}), and total carbon (236.78 mg day−1). Additionally, the highest specific growth rate achieved was 3.06% day^{-1}, with a survival rate of 91.67% over the 45-day trial period, showing promise for nutrient recycling and potential use in aquaculture feeds.

Wang *et al.*, 2019 extended this research by examining the growth and nutritional composition of *Hediste diversicolor* cultivated on waste from land-based salmon smolt aquaculture. This study highlighted the dual benefits of waste reduction and the production of a valuable feed ingredient.

In a 30-day study, *H. diversicolor* reared on fish feed showed the highest Specific Growth Rate (SGR) (SGR = 0.025 d^{-1}), while those on a smolt waste and microalgae mix had the lowest (SGR

= 0.003 d^{-1}). The worms' lipid content ranged from 12% to 16%, with approximately 45% as polyunsaturated fatty acids, including significant increases in DHA content (1.5% to 4.6–7.8%). Protein content ranged from 54% to 58%, with lysine and leucine as the most abundant essential amino acids. This suggests that *H. diversicolor* can effectively recycle smolt waste, producing biomass that accounts for 8% of smolt production and reducing environmental impact.

Integrated Systems

Innovative integrated systems that combine waste treatment with the production of aquaculture feed ingredients have gained attention. Asiri and Chu (2020) developed a novel recirculating aquaculture system that enables wastewater reuse and conversion of waste into immune-stimulating fish feed. This approach not only addresses water pollution but also enhances the nutritional value of the feed, contributing to the overall sustainability of aquaculture operations.

Specifically, they developed a RAS-polyhydroxybutyrate (PHB) system enabling effective wastewater treatment, producing PHB-rich biomass for antibiotic-free, protein-rich fish feed, and significantly reducing production costs by 56% compared to conventional RAS, demonstrating its potential for sustainable aquaculture.

Novel Approach: Ragworms and Chitosan

The current project explored the feasibility of using ragworms in chitosan-treated water as a sustainable solution for both high-quality feed ingredient production and water purification. Ragworms, known for their ability to thrive in contaminated environments, offer a dual-purpose benefit. They serve as a nutritious feed ingredient for farmed fish and assist in cleaning polluted water. The study's findings demonstrated that ragworms could effectively grow while feeding on chitosan-treated water, suggesting a novel method for integrating waste management and feed ingredient production. This innovative approach, by reducing waste and providing a high-quality feed ingredient, has the potential to enhance the environmental sustainability of the aquaculture industry.

In summary, the literature highlights the critical challenges and innovative solutions in aquaculture feed utilisation and waste management. The integration of alternative feed ingredients, bioremediation, and novel systems like the use of ragworms and chitosan offers promising pathways to achieving sustainability in aquaculture. These advancements are essential for reducing the environmental impact and ensuring the long-term viability of the industry.

Chapter 3

Materials and Methods

3.1 Introduction & Ethical Statement

This chapter describes the general materials and methods used in the experiments described in this thesis. For example, how ragworm growth was determined, how water quality parameters were recorded, and how nutritional and elemental analyses were determined. In subsequent chapters' materials and methods sections, anything specific to that chapter is defined.

All experiments were conducted concerning the well-being of the ragworms, minimising stress and discomfort wherever possible. Environmental sustainability was prioritised by using only the necessary number of ragworms to obtain meaningful results. This approach ensures that the research contributes positively to scientific knowledge while safeguarding the health and welfare of the ragworms involved.

3.2 Chitosan Formulations

The chitosan was supplied dry by Pennosan®. Pennosan® is a UK-based supplier of chitin and chitosan products and formulations for a variety of industries. They are based in Pwllheli, Gwynedd in Wales.

In terms of project input, Pennosan® provided details on how to prepare chitosan formulations in the laboratory. Information relating to chitosan properties and its preparation is confidential to Pennosan®. An image of the chitosan flakes used to prepare chitosan formulations is seen in Figure 3.1.

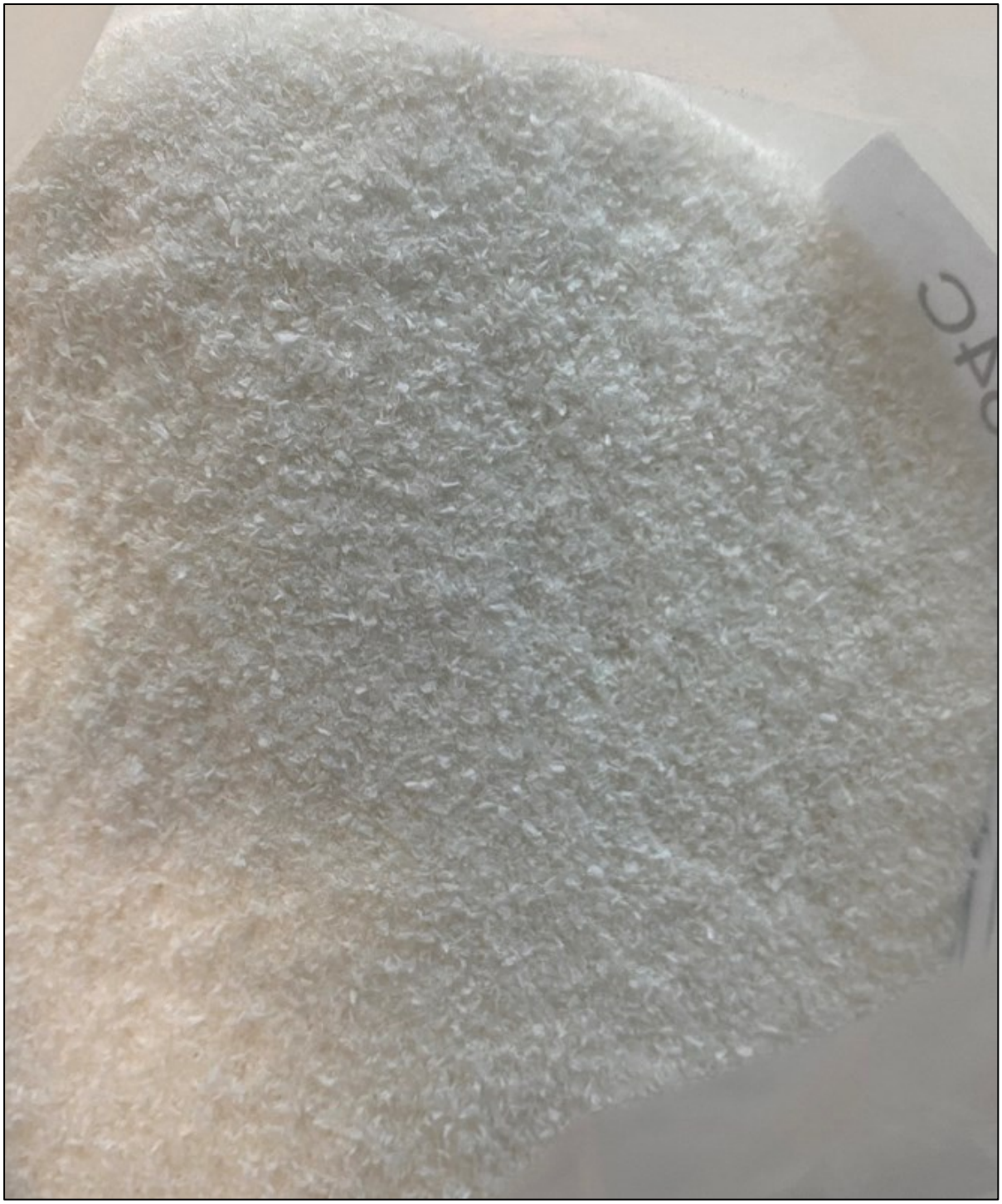

Figure 3.1 – An example of the chitosan flakes used to prepare chitosan formulations (Pennosan®)

3.3 Source of Experimental Animals and Husbandry

All ragworms used in the following chapters had been reared under natural daylight conditions at the aquaculture farm of Seabait Ltd, Northumberland, before being shipped by

courier with ice packs to the laboratories at the William Rankine Building, Thomas Bayes Road, King's Buildings, University of Edinburgh, Edinburgh, EH9 3FG (Figure 3.2).

Throughout the thesis, they were all the species *Hediste diversicolor* and referred to as ragworms in the text. Immediately upon arrival, the ragworms were separated into suitable containers and stored in a fridge to minimise their activity before acclimation to experimental conditions.

Juvenile ragworms were used, and the experiments were conducted in the spring and summer. Hence, growth rates were easier to observe. Growth rates typically decrease as animals get larger (Heip and Herman, 1979), and autumn is usually when the onset of sexual maturation occurs (Kristensen, 1988). Slower growth rates accompany sexual maturation because of seasonal variations in day length, food availability and resource partitioning (Wang *et al.*, 2019).

Figure 3.2 – Ragworms (*Hediste diversicolor*) from Seabait Ltd

3.4 Source of Aquaculture Wastewater

Aquaculture wastewater (**AW**) composition varies throughout time and may not always have the same characteristics (Palmer, 2010). The **AW** used in experiments described in chapters 4, 5 and 6 was from a cleaner fish aquaculture facility called MOWI® Anglesey (Wales). Chapter 4 was of a different batch from 5 and 6. This source was chosen because it is one of Pennosan's® contacts. Also, there is minimal literature on feeding cleaner fish **AW** to ragworm. An example of cleaner fish **AW** can be seen in Figure 3.3 below.

Figure 3.3 – Cleaner fish aquaculture wastewater from MOWI® Anglesey

The cleaner fish, Ballan wrasse (*Labrus bergylta*), are reared to remove (clean) sea lice from farmed salmon hence their commonly used name, cleaner fish. The ectoparasitic salmon louse (*Lepeophtheirus salmonis*) is the main barrier to expanding the production of the most significant finfish aquaculture product, sea-cage Atlantic salmon farming (Geitung *et al.*, 2020). One of the most efficient and cost-effective ways (Fisheries, 2020) to biologically control salmon lice without harming the welfare of the salmon is to use lice-eating cleaner fish (Imsland *et al.*, 2014; Geitung *et al.*, 2020). With an annual output of over 1.3 million tonnes, Norway is the world's most significant producer of farmed salmonids. The Norwegian government unveiled intentions in 2014 to boost salmonid output further by 2050 (Dean *et al.*, 2021). The Norwegian industry spent more than €425 million in 2015 to manage the parasite (Brooker, Skern-Mauritzen and Bron, 2018). The use of cleaner fish has drastically expanded in Norway over the past few years, going from 1.7 million cleaner fish in 2008 to over 54 million in 2017 (Fisheries, 2019). Cleaner fish supply has changed from being solely wild-caught to increasingly being hatchery-produced to meet the growing demand (Brooker *et al.*, 2018; Geitung *et al.*, 2020). With plans in Scotland to increase salmonid production (Marine Scotland, 2015), expanding cleaner aquaculture like MOWI® Anglesey is necessary to help cope with the burden of sea lice infestation (Fisheries, 2020).

The cleaner fish bred in aquaculture systems also require aquafeed and produce waste, therefore demonstrating the importance of research like this thesis which aims to address these issues through innovation and utilisation of waste streams concomitantly producing aquafeed.

The **AW** was shipped via courier to the laboratories at the William Rankine Building, Thomas Bayes Road, King's Buildings, University of Edinburgh, Edinburgh, EH9 3FG, in jerry cans, which were immediately frozen at -20°C upon arrival.

3.5 General Experimental Set-Up

Ragworms were stocked into replicates of 1 L glass beakers (145 mm x 105 mm), filled with 70 mm of aquarium sand (Pettex® Aquatic Roman Gravel, Pewter Sand) (0.5 - 1 mm grain size) and filled to the rim with artificial seawater (approximately 65 mm water depth). The artificial seawater was prepared by mixing Instant Ocean® Sea Salt at a concentration of 15 g L^{-1} in deionised water (Milli-Q® Ultrapure Water).

The sand was washed thoroughly with deionised water (Milli-Q® Ultrapure Water) and oven-dried at 105 °C for 24 hr before being added to a beaker. Bischoff (2007) claimed that coarse culture sediment could reduce ammonia levels and enhance oxygen saturation, but more mucus is needed to stabilise burrows. Therefore, fine sediments (like aquarium sand) were used for ragworm culture since producing mucus requires energy and might slow ragworm development (Bischoff, 2007).

Additionally, the aquarium sand chosen explicitly states that it is suitable for bottom feeders like ragworms. Each beaker was acid-washed, rinsed with deionised water, and then rinsed with artificial seawater before use. Each beaker was wrapped several times in dark-coloured tape up to the surface of the sand layer to help mimic the ragworms' natural environment where light does not penetrate the sediment from the sides.

The whole water volume of each glass beaker was changed every Monday, Wednesday, and Friday. The sand was rinsed thoroughly in deionised water to remove ragworm excretions, uneaten feed, and any other undesirable components before being rinsed with artificial seawater.

Constant aeration of culture water was achieved by connecting plastic tubing to an air inlet built into the laboratory wall. The plastic tubing was split via an air splitter and inserted into each beaker. The air inlet pressure is controlled with a pressure gauge built into the wall fixture. The pressure was adjusted to provide efficient dissolved oxygen (DO) concentrations while not disturbing the ragworms' feeding ability on the sediment surface. For each tube inserted into the culture water, a small plastic piece was stuffed inside to block the end of the tube. Tiny holes were punctured in the tube wall near the end of the tube using a needle, allowing air to escape and oxygenate the water. An example of the experimental setup is shown in Figure 3.4.

The photoperiod was controlled with room lights turned on between 9 am to approximately 5 pm, Monday to Friday. To avoid unwanted changes in ragworm behaviour due to unnatural light during experiments, the set-up was placed next to a window with abundant natural light to expose the ragworms to spring and summer conditions suppressing their sexual maturity (Olive, 1999). On the weekends, the beakers were only exposed to natural daylight as access to the laboratory was restricted (an unforeseen circumstance of the SARS-CoV-2 outbreak and related lockdown in 2020). The room temperature was set to approximately 20 °C. The ambient temperature of the room varied slightly by ± 1 – 3 °C on warmer and cooler days. Ragworms were acclimated for at least five days in experiment conditions and fed a basic commercial fish feed (Tetra® Pond Pellets Mini) during this time to keep them viable. Dead ragworms discovered at any time were removed immediately to limit bacterial growth but only replaced if during an acclimation period and not during an experiment to not impact results.

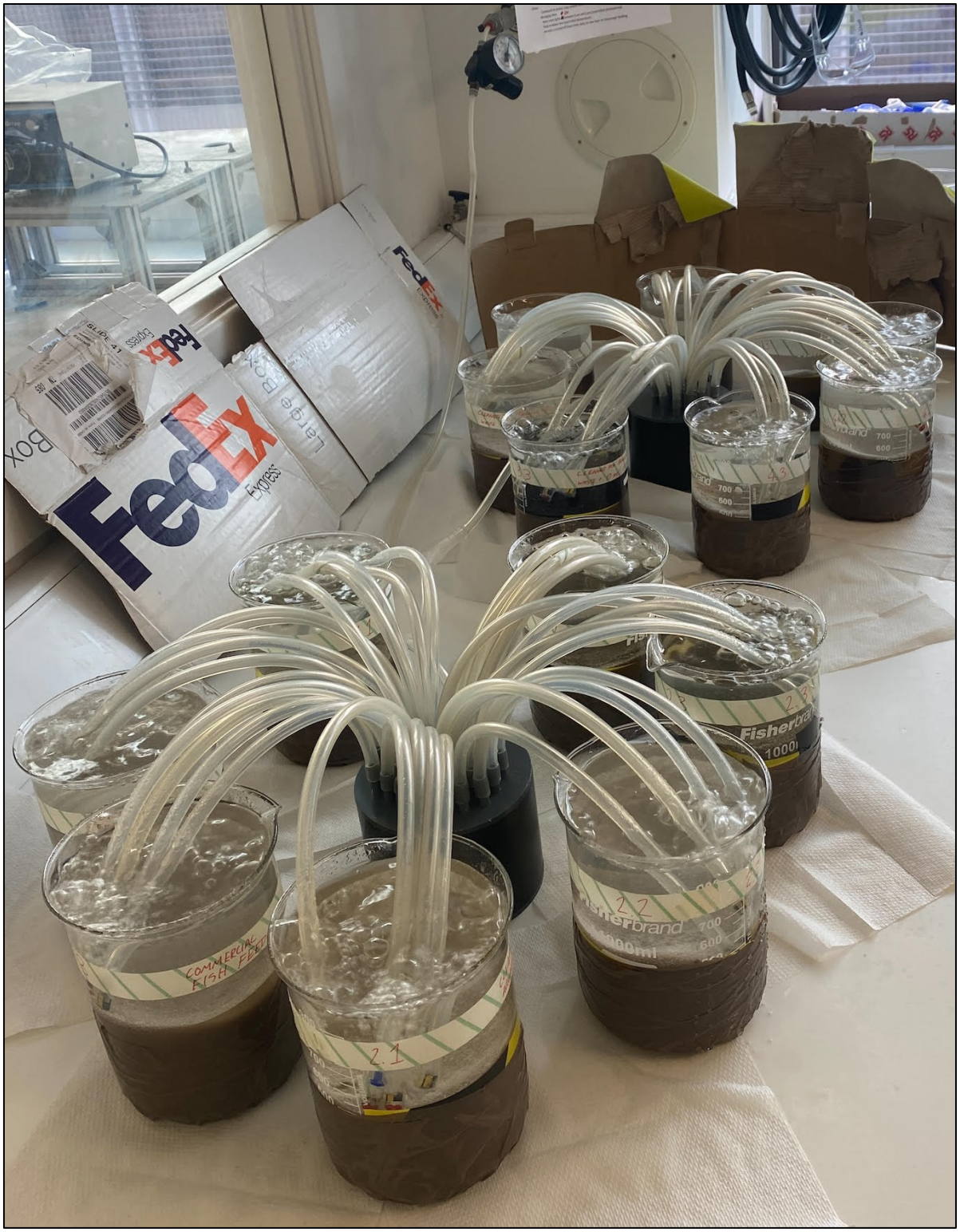

Figure 3.4 – An example of the standard experimental set-up

3.6 Water Quality Measurements

Water quality parameters, namely dissolved oxygen (DO), conductivity, pH, and temperature were monitored daily using probes connected to handheld meters. The DO was measured using the Fisherbrand™ Traceable™ Portable Dissolved Oxygen Meter. The pH was measured using a Cole-Parmer® 100-series pH/Temperature Electrode connected to a Cole-Parmer® 100-series pH and pH/Con Handheld Meter. The conductivity was measured using an Oakton® Con 6+ Handheld Conductivity Meter with Probe. Finally, the water temperature was measured using the pH and conductivity probes with built-in temperature readers and confirmed with a glass laboratory thermometer.

In addition, water turbidity was measured using a Hach® 2100N Laboratory Turbidity Meter, EPA-compliant, 240V AC. Water samples were placed in Hach® Glass Sample Cells that were acid-washed, rinsed with deionised water (Milli-Q® Ultrapure Water), dried, and wiped with silicone oil.

3.7 Determining Ragworm Weights and Growth Rates

3.7.1 Determination of Wet Weights

When determining the wet weight of ragworms, the following procedure was followed:

Ragworms were starved for 24 hours in a beaker of artificial seawater to purge gut contents and then rinsed in deionised water to remove extracellular salts and nutrients, then dried with paper towels to remove excess water before individually gravimetrically weighing on an analytical balance (Fisherbrand™ Analytical Balance FAS224) with a readout accuracy of 0.0001 g.

3.7.2 Determining Ragworm Growth Rates

Specific growth rate (SGR) was chosen over other methods of measuring growth such as the Daily Growth Coefficient (DGC) for the following reasons:

- **Comparison Across Studies**: SGR is widely used in similar scientific literature making it easier for comparison with different studies.
- **SGR is a Relative Measure**: SGR provides a relative growth rate expressed as a percentage, which is more intuitive for understanding proportional weight changes over time.
- **Small Organism Suitability**: For small organisms with relatively rapid growth rates (e.g., ragworms) SGR can offer a clear picture of growth.

The SGR (**μ**) was calculated using an adapted following formula (Jørgensen, 1990):

$$\mu = \frac{ln\,(W_t) - ln\,(W_0)}{t}$$

Where: **μ** = specific growth rate (d^{-1})

$\mathbf{W_0}$ = average weight (WW) of ragworms before the experiment (g)

$\mathbf{W_t}$ = average weight (WW) of ragworms after the experiment (g)

T = time (days)

The percentage growth per day **P** (% d^{-1}) was calculated using the following formula:

$$\boldsymbol{P} = 100 \times (\exp(\mu) - 1)$$

3.7.2.1 Adaptation to the Specific Growth Rate Equation

Specific growth rates (SGR) represent growth as a percentage change in size per unit of time, unlike instantaneous growth rates (IGR), which are logarithmic and challenging to interpret (Crane, Ogle and Shoup, 2020). Determining growth rates is a crucial indicator in aquaculture to estimate fish growth over time using an exponential function of IGR ('Age and Growth of Fishes: Principles and Techniques', 2017). For this reason, SGR calculations have trickled into research involving polychaetes (35,54). However, in literature, the calculation never considers mortalities that may happen during an experiment. When dead ragworms are not accounted for in the equation, it affects the result and, in some cases, even becomes negative when

there is growth in the ones that did not survive. Therefore, the only way the SGR calculation can be truly accurate is when ragworm mortalities occur, their weight is recorded at the time of death and included in the SGR equation. Unfortunately, weighing dead ragworms did not happen in the experiments discussed in subsequent chapters, as it is exceptionally challenging to do this at the exact time of death of a ragworm. Therefore, an adaptation was made to the equation:

$$\boldsymbol{SGR}\ (d^{-1}) = \frac{\left(\frac{\ln(Final\ Weight\ (g\ WW))}{Final\ Number\ of\ Live\ Ragworms}\right) - \left(\frac{\ln(Initial\ Weight(g\ WW))}{Initial\ Number\ of\ Live\ Ragworms}\right)}{days}$$

The adapted equation accounts for the number of ragworms at the experiment's beginning and end. Therefore, the error caused by mortalities is removed, avoiding inaccurate negative results in growth, and thus providing results more representative of reality than is obtained with the standard SGR equation described in Section 3.7.2 – Determining Ragworm Growth Rates.

SGR errors are common. The use and misuse of SGR in aquaculture literature have been investigated by Crane, Ogle & Shoup (2019), who found that only 3.3% of the papers they reviewed correctly calculated SGR. When calculating SGR, most papers multiply the IGR by 100. The correct calculation would be exponentiating the IGR, subtracting 1 and then multiplying by 100, as displayed above. Crane, Ogle & Shoup demonstrate that multiplying by 100 leads to underestimating growth rates and suggest that aquaculture experts refrain from simply multiplying by 100 when interpreting IGR to SGR (Crane, Ogle and Shoup, 2020).

3.7.3 Determining Ragworm Mortalities

Mortality resulted from the difference in the number of ragworms at the start of the experiment and the number alive at the end. Therefore, mortality was calculated using the following equation:

$$Mortality\ (\%) = \left(1 - \frac{\boldsymbol{N_t}}{\boldsymbol{N_0}}\right) \times 100$$

Where: $\mathbf{N_0}$ = the number of ragworms used in the experiment.

$\mathbf{N_t}$ = the number of surviving ragworms.

Dead ragworms were removed from experiments when they were found.

3.8 Biochemical Analyses of Ragworms and Feeds

3.8.1 Determination of Total Organic Matter Content

A slightly adapted standardised procedure (Sluiter *et al.*, 2004) was used to perform total organic matter (TOM) analysis on ball-milled, freeze-dried samples of ragworms and feeds. Glass vials were inscribed with an indelible marker, acid-washed, rinsed with deionised water (Milli-Q® Ultrapure Water) and heated for 12 hours at 500 °C in a muffle furnace (GSM 11/8, Carbolite Furnaces). The glass vials were then stored in an oven at 105 °C. In duplicate, approximately 0.06 - 1.2 g of lyophilised samples were weighed using an analytical balance into the pre-combusted glass vials. The samples were then combusted at 500 °C for 12 hours. The furnace was pre-programmed to rise to 500 °C in 35 minutes from the starting temperature. The now combusted samples were weighed once they reached room temperature. Using the following equation, TOM was calculated as the percentage of residual non-combusted material in the initial lyophilised ragworm or feed sample:

$$\%\ TOM = \frac{\boldsymbol{M2 - M1}}{\boldsymbol{DW}} \times 100$$

Where: **M2** = vial weight (g).

M1 = final weight (vial + sample) (g).

DW = initial mass of lyophilised ragworm or feed sample before combustion.

3.8.2 Determination of Carbon and Nitrogen Content

For Carbon (C) and Nitrogen (N) analysis of ragworm and feed samples, approximately 1.5 – 2.3 mg of ragworms or feed samples were each weighed in triplicate into pressed 8 x 5 mm tin capsules (Mfg.: Elemtex, Cat. X SE1003) on an analytical balance (Sartorius SC2 Ultra-Microbalance, accuracy = 0.0001 g). The samples were analysed on a Thermo Fisher Scientific FlashSmart Elemental Analyser (Serial # 2018.FLS0037), equipped with a CN/CHN Prepacked Quartz Reaction Tube (Mfg.: Elemtex, Cat. # PXR1000) and a 2 m stainless steel CN/CHN Separation Column (Mfg.: Elemental Microanalysis, Part # E3023, Lot # BN293467). The system was calibrated for C and N using four preparations, ranging from approximately 1 – 4 mg of Atropine reference standard (Mfg.: Elemental Microanalysis, Cat. # B2002, Lot # 394488). In addition, about 2.2 mg of BBOT reference standard (Mfg.: Elemtex, Cat. # RM1022, Lot # B1150) was used as the calibration check, and approximately 2.5 mg of Atropine was used as a drift standard after every 12 sample injections. The instrument specifications that were used for analysis are in Table 3.1.

Table 3.1 – Instrument specifications used for CN analysis

Instrument Specification	Setting (units)
Furnace Temperature	950 (°C)
Oven Temperature	60 (°C)
Carrier Gas (Helium) Flow	140 (ml min^{-1})
Oxygen Flow	250 (ml min^{-1})
Reference Gas (Helium) Flow	140 (ml min^{-1})
Run Time	220 (sec)
Sampling Delay	12 (sec)
Oxygen Injection End	5 (sec)
Detector	Thermal Conductivity Temperature

C and N masses (mg) were determined using the following equation:

$$C\ or\ N\ Mass\ (mg) = \frac{C\ or\ N\ Area\ of\ Peak - Intercept\ from\ Calibration\ Curve}{Slope\ from\ Calibration\ Curve}$$

Then, the C and N masses (mg) were converted to a percentage using the following equation:

$$C\ or\ N\ \% = \left(\frac{C\ or\ N\ Mass\ (mg)}{Sample\ Mass\ (mg)}\right) \times 100$$

3.8.3 Determination of Total Lipid and Fatty Acid Profiles

Total lipid content and fatty acid (FA) analysis of ragworms and feeds were extracted according to a method described by Garcia-Alonso *et al*., 2008 (García-Alonso and CT, 2008) and Bligh & Dyer, 1959 (BLIGH and DYER, 1959), with various adaptations described below.

3.8.3.1 Total Lipid Content and Fatty Acids Extraction

All glassware and metal utensils were acid-washed, rinsed with deionised water (Milli-Q® Ultrapure Water), and dried overnight at approximately 90 °C in a glassware drier. All chemicals used were laboratory grade.

Around 1 g of a frozen ragworm or feed sample was ground using a mortar and pestle until homogenous. The ground sample was then added to a 50 ml centrifuge tube (Fisherbrand™ Polypropylene) and labelled. To extract the FAs, 30 ml of a chloroform/methanol (CH_3Cl/CH_3OH) solution (2/1, v/v) was added to the centrifuge tube and vortexed for approximately 2 min using a vortexer (Vortex 3, IKA®) before centrifuging at 5000 rpm for 5 min (Eppendorf® Centrifuge 5810 R). The resulting solution was then poured into a 250 ml separating funnel (Fisherbrand™ Glass Conical Separating Funnel with PTFE Stopcock) through a filter funnel with filter paper (Whatman® Qualitative Filter Paper, Grade 1, diameter 125 mm, Cat No. 1001-125) containing approximately 5 g of oven-dried sodium sulfate (Na_2SO_4) powder. Next, another 30 ml of the chloroform/methanol solution was added to the same centrifuge tube and vortexed until homogenised before centrifuging again. Finally, the

solution was poured into the same 250 ml separating funnel through the filter funnel containing filter paper and sodium sulfate powder.

Next, 60 ml of potassium chloride (KCl) solution (8 %) was added to the separating funnel. Then, the separating funnel was stoppered, inverted, and shaken thoroughly for approximately 1 min, periodically releasing gas pressure by opening and closing the tap while inverted. The KCl washed out anything more soluble in water than chloroform and methanol; FAs are more soluble in chloroform and methanol. Once the two layers had separated, the lower chlorinated solvent layer was collected into a clean 250 ml separating funnel. Next, 60 ml of KCl (8%) and methanol solution (1/1, v/v) were added to the separating funnel. Then, the separating funnel was stoppered, inverted, and shaken thoroughly for approximately 1 min, periodically releasing gas pressure by opening and closing the tap while inverted. The KCl and methanol solution washed out anything that was not washed out in the first separation with KCl only. Including more methanol helps to pull less soluble material out of the mixture as it is less polar than water. Once the two layers had separated, the lower chlorinated solvent layer was collected into a pre-weighed 250 ml round-bottomed flask through a filter funnel containing filter paper and approximately 5 g of sodium sulfate powder.

The solvent was evaporated using a rotary evaporator (IKA® RV 10). Once the solvent had evaporated, the round-bottomed flask was dried thoroughly with paper towels. Finally, the resulting residue was weighed, which was the total lipid content.

To the round-bottomed flask, 5 ml of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (BTH) (1% in chloroform) was added. BTH is an antioxidant, preventing the deterioration of FAs. Next, the dissolved FAs were transferred to a Teflon-capped reaction vial using a Pasteur pipette. The reaction vials have a cap that releases pressure if it gets too high; this is an important safety feature when refluxing later (Figure 3.5).

Figure 3.5 – Pressure-resistant Teflon-capped reaction vial used in fatty acid extraction method

3.8.3.2 Saponification and Esterification of Fatty Acids

The solvent (BTH 1% in chloroform) was evaporated using a rotary evaporator. An adaption was required to fit the reaction vial to the rotary evaporator. First, a rubber septum was fitted over the reaction vial creating a seal. Then, a needle was pierced through the septum to allow the solvent to evaporate. The septum could then fit the adapter of the rotary evaporator (Figures 3.6 & 3.7).

Once the solvent had evaporated, 4 ml of a potassium hydroxide (KOH) solution (1 M in 95% ethanol) was added to the reaction vial. This was the saponification step, where FAs are converted into soap and alcohol by aqueous alkali (Figure 3.8). KOH was the aqueous alkali; ethanol (C_2H_5OH) was used because FAs are not soluble in water alone, so a solvent is required to keep them in the dissolved state. The reaction vial was then closed and refluxed in a heat

block for 1 hr at 100 °C (Techne Dri-Block® DB-2D). The 1 hr time and heat encourage the reaction to occur.

Figure 3.6 – 250 ml round-bottomed flask attached to rotary evaporator using manufacturer adaptors

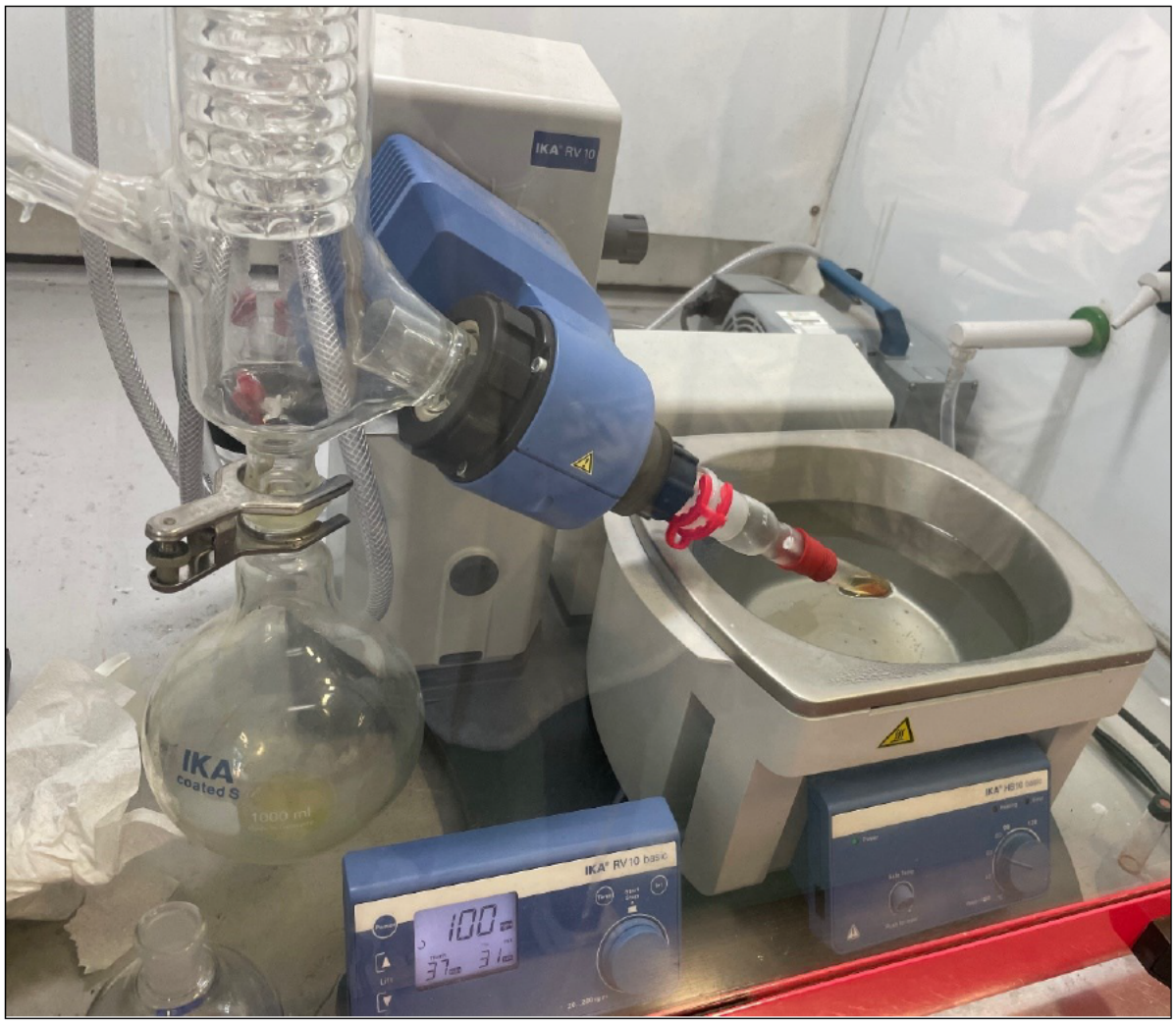

Figure 3.7 - Modified adapter to fit small reaction vials to the rotary evaporator
The red rubber septum attaches the reaction vial to the adapter. The septum was pierced with a needle to allow the solvent to evaporate.

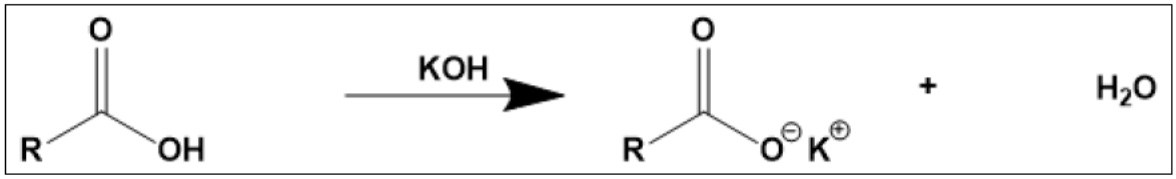

Figure 3.8 – Saponification of the fatty acids
(Figure created using ChemDraw®)

After 1 hr, the reaction vial was removed from the heat block and cooled to room temperature before adding 5 ml of deionised water (Milli-Q® Ultrapure Water). A soap-like solution was formed, indicating that the previous steps had been performed correctly. The contents of the reaction vial were then added to a 50 ml separating funnel. Then 10 ml of pharmaceutical-grade hexane (C_6H_{14}) was added to the separating funnel. The separating funnel was then stoppered, inverted, and shaken thoroughly for approximately 1 min, periodically releasing

gas pressure by opening and closing the tap while inverted. The upper solvent layer contained any extracted organics that were non-saponifiable. The lower aqueous layer contained the potassium salts of the FA from the ragworm or feed samples, as they are more soluble in water and ethanol than in hexane. The upper layer was discarded, and the lower layer was put back into the separating funnel. Separation with hexane was repeated two more times.

The hexane extraction differs from the method described by Garcia-Alonso *et al*., (2008), where the extraction was carried out with a hexanol-ether mix (1:1, v:v). When hexanol-ether was attempted in the current method, removing the solvent using the rotary evaporator was difficult, presumably because of the high boiling point. However, adapting the method to extract with hexane worked well. In addition, it did not require setting the water bath of the rotary evaporator to a temperature that would likely degrade the FAs.

The lower aqueous layer was collected into a 50 ml glass beaker. Then 2 M of hydrochloric acid (HCl) was added dropwise using a Pasteur pipette (Fisherbrand™ Soda Lime Glass Pasteur Pipette) until neutral or slightly acidic, confirmed by pH strips (Fisherbrand™ pH Indicator Paper Sticks). Adding HCl transformed the FA potassium salts into FAs (Figure 3.9).

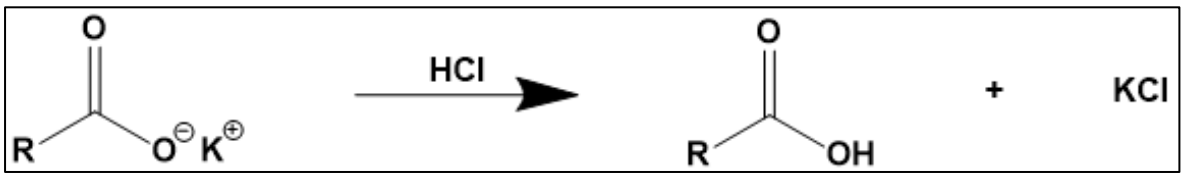

Figure 3.9 – Conversion of the fatty acid potassium salts to free fatty acids.
(Figure created using ChemDraw®)

Once the solution was neutral pH (or slightly acidic), the solution was transferred to a clean 50 mL separating funnel. To the separating funnel, 10 ml of chloroform was added before it was stoppered, inverted, and shaken thoroughly for approximately 1 min, periodically releasing the gas pressure by opening and closing the tap while inverted. Next, the lower organic layer was collected into a 250 ml round-bottomed flask through a filter funnel with filter paper and sodium sulfate powder. The upper layer containing the KCl from before was kept in the separating funnel. The chloroform extraction was repeated two more times. The solvent was then evaporated using a rotary evaporator leaving only the free FAs.

The free FAs were then dissolved in 5 ml of a 1 % sulfuric acid (H_2SO_4) in methanol solution by adding it directly to the round-bottomed flask and swirling it. The contents of the round-bottomed flask were then transferred to a clean reaction vial using a clean Pasteur pipette. The reaction vial was sealed and refluxed for 2 hrs in a heat block at 100 °C. This was the esterification step to convert the free FAs to methylated FAs; sulfuric acid was a catalyst (Figure 3.10).

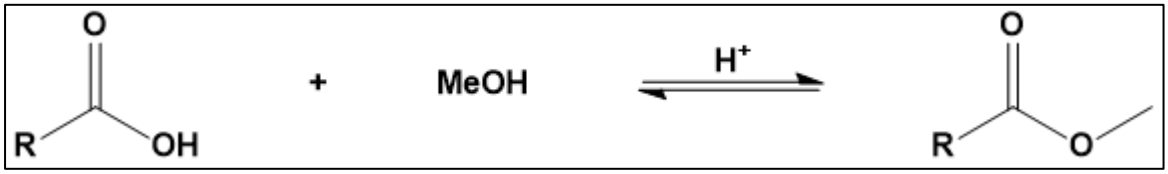

Figure 3.10 - Conversion of free fatty acids to fatty acid methyl esters (acid-catalysed esterification). (Figure created using ChemDraw®)

After 2 hrs, the reaction vial was removed from the heat block and allowed to cool to room temperature before being added to a clean 50 ml separating funnel. To the separating funnel, 5 ml of aqueous sodium chloride solution (5 % NaCl, w/v) was added. Then, 5 ml of hexane was added before the separating funnel was stoppered, inverted, and shaken thoroughly for approximately 1 min, periodically releasing gas pressure by opening and closing the tap while inverted. The upper solvent layer containing the fatty acid methyl esters (FAME) was collected into a clean reaction vial through a filter funnel containing filter paper and a small amount of sodium sulfate powder. The lower layer was kept in the separating funnel and extracted once more with hexane. The solvent was then evaporated using a rotary evaporator. Once dry of solvent, the FAMEs were re-dissolved in 1.4 ml of hexane and transferred to a GCMS vial using a clean Pasteur pipette. The GCMS vials were then labelled and frozen until GCMS analysis, if not analysed straight away.

3.8.3.3 Gas Chromatography-Mass Spectrometry Identification and Quantification of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAME) from ragworm and feed samples were analysed on a Thermo Scientific™ Trace 1300 Gas Chromatographer, with an ISQ Single Quad Mass Spectrometer and an Al/AS 1310 autosampler. One µl is injected at the inlet at 220 °C in splitless mode with a carrier flow of 1.2 ml min^{-1} and a split flow of 50 ml min^{-1}. The injection syringe is rinsed

once before and after each injection in ethanol (C_2H_6O) to avoid carry-over contamination. The GC starts at 80 °C held for 3 min, before ramping at 15 °C min^{-1} to 160 °C. Then the ramp changes to 2 °C min^{-1} until 210 °C. Then the ramp changes from 20 °C min^{-1} to 250 °C (45 min total). The transfer line and source are at 280 °C, there is a 3 min solvent delay, and the source scans 45 - 350 au (Figure 3.11).

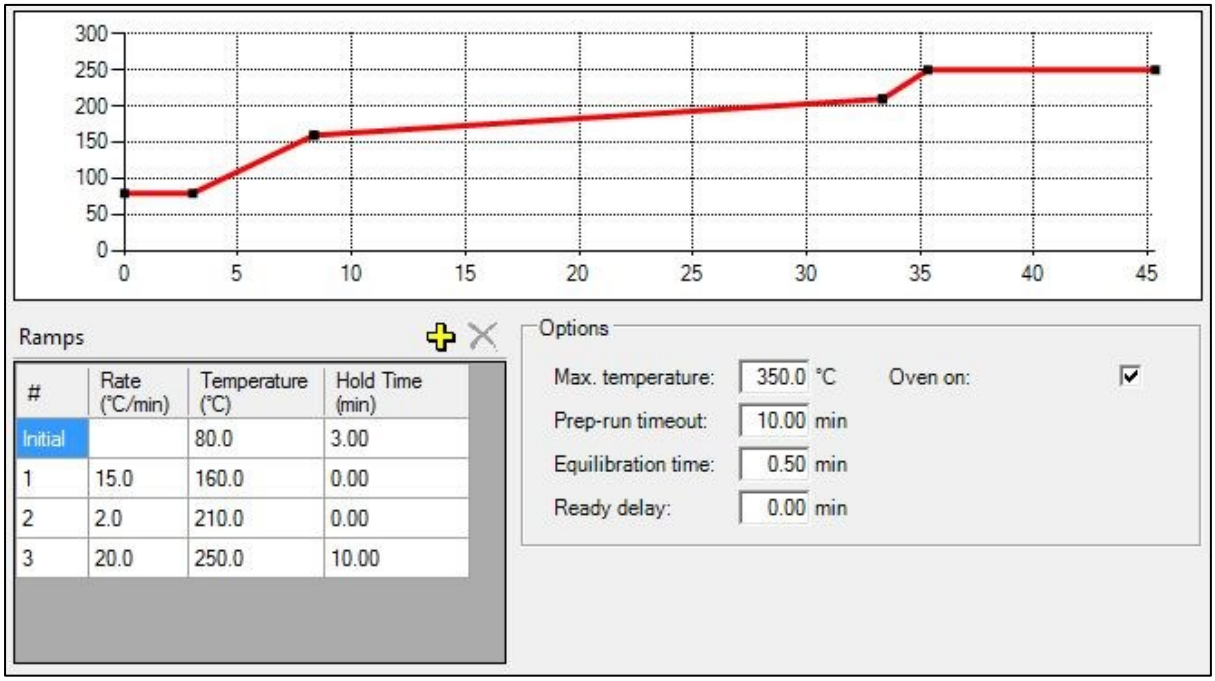

Figure 3.11 – Screenshot of the method used to separate fatty acid methyl esters

The method in Figure 3.11 was developed from methods described in two research publications (García-Alonso and CT, 2008; Jerónimo *et al.*, 2021) for determining FA composition in ragworms. Identification of FAs was made using retention time and MS spectra analysis compared with MS spectra of FAME standards (Supelco 37 Component FAME mix, ref. 47885-U, Sigma-Aldrich) and chemical databases (NIST/EPA/NIH Mass Spectral Library). The column used was a 5% phenyl phase Thermo Scientific™ TraceGOLD™ TG-5MS GC Column (30 M x 0.25 mm x 0.25 µm) (length x thickness x internal diameter).

To quantify the fatty acids (FAs), the initial plan was to use serial dilutions of the FAME standard mix for external calibration. However, this approach was not feasible due to several issues: some FAMEs present in the standard mix are absent in the animals, some FAMEs found in the animals are not included in the standard mix, and the similarity of some FAMEs complicates accurate separation and integration.

Therefore, FA quantification was achieved by calculating the peak area for each identified FA and determining its percentage of the total area of all peaks in the chromatogram. This percentage was then converted to an absolute concentration by considering the total FA content in the original extracted sample.

The GCMS column used to identify FA could not differentiate different FA with 18 carbons. FA with 18 carbons includes stearic acid (C18:0), Oleic acid, Elaidic acid and vaccenic acid (C18:1), Linoleic acid and linolelaidic acid (C18:2), α-linolenic acid and ɣ-linolenic acid (C18:3) and stearidonic acid (C18:4). Overlapping of peaks was routinely seen in samples, as is often the case with FA of the same C chain length (Supelco, 1996). Therefore, stearic acid (C18:0), oleic/elaidic acid (C18:1) and linoleic acid (C18:2) are included as they were able to be distinguished when identifying and quantifying the FA. However, the obtained concentrations likely contain some FA of other types, such as vaccenic, linolelaidic, α-linolenic, and ɣ-linolenic acids. Furthermore, isomers such as oleic and elaidic acid could not be distinguished and are quantified together.

3.8.4 Determination of Total Protein and Amino Acid Profiles

3.8.4.1 Protein Contents

Assuming that protein contains 16% nitrogen, to determine the protein content of ragworm and feed samples, the N content (Section 3.8.2 – Determination of Carbon and Nitrogen Content) was multiplied with a factor of 6.25 (Jones, 1932). Then, N percentages were converted to concentrations (mg g^{-1}) using the following equation:

$$N\ (mg\ g^{-1}) = \frac{N\ Mass\ (mg)}{Sample\ Mass\ (g)}$$

Then, total protein contents were determined using the following equation:

$$Total\ Protein\ (mg\ g^{-1}) = N\ (mg\ g^{-1}) \times 6.25$$

This calculation does not correct for non-protein nitrogen. In other words, the fraction of nitrogen in a sample that is from biological fluids and not part of proteins (e.g., urea).

3.8.4.2 Amino Acid Identification and Quantification

Amino Acids (AAs) were identified and quantified in ragworm and feed samples using a method described by Cowie & Hedges (1992) (Cowie and Hedges, 1992) with some adaptations.

3.8.4.2.1 Amino Acid Extraction and Isolation

Before the extractions, all glassware was acid-washed, rinsed with deionised water (Milli-Q® Ultrapure Water), and then combusted at 450 °C for at least 4 hr in a muffle furnace (GSM 11/8, Carbolite Furnaces). To homogenise the samples, they were freeze-dried for at least 24 hr (Modulyo® 4K Freeze-Dryer) and then ground using a mortar and pestle. Between 5.08 – 7.18 mg of ragworm and feed samples were weighed into pre-tared glass ampules. All metal utensils used when weighing were rinsed with methanol (CH_3OH) and dichloromethane (CH_2Cl_2) between samples. Glass ampules were chosen over glass vials with plastic lids to minimise contact with any plastics contaminating the samples.

Samples were hydrolysed with ultrapure 6 M hydrochloric acid (HCl), purged of air, sealed in the glass ampule, and heated to 150 °C for 70 min. Once cooled, samples were spiked with 100 µl of 3 charge-matched internal recovery standards. For acidic AAs, α-Aminoadipic acid was added; for neutral AAs, ρ-Fluorophenylalanine and basic AAs, δ-hydroxylysine. Samples were then dried of HCl using a centrifugal evaporator (JOUAN RC.10.22). When dried, samples were stored at -20 °C. When ready for analysis, samples were re-dissolved in 5 ml of deionised water, and the pH was adjusted to 9.7 – 10.5 using a weak KOH solution. They were then syringed (BD PlastiPak™ 10 ml Luer Lock) through a syringe filter (Minisart® NY25, 0.45 µm Polyamide) to remove any solids into clean vials with a plastic cap. This vial was sealed and frozen if not analysed via HPLC immediately. When samples were ready for analysis, around 0.5 ml was added to an HPLC vial.

3.8.4.2.2 High-Performance Liquid Chromatography for Identification and Quantification of Amino Acids

The High-Performance Liquid Chromatography (HPLC) instrument used to identify AAs was an Agilent Infinity 1260 with a 1260 Spectra Fluorescence Detector. The HPLC column used in the separation was an Agilent Infinitylab Poroshell 4.6 x 100 mm, 2.7 µm, with an associated guard column and in-line filter.

Each peak in the chromatogram corresponds to a different AA. The area under each peak is proportional to the amount of AA in that sample. The percentage of each AA relative to the total amount of AAs detected is calculated by dividing the area of each peak by the sum of the areas of all peaks and multiplying by 100. To convert this to absolute quantities, the known quantity of internal standard AAs was added to the sample before analysis, allowing for the calculation of each AA in the sample based on the response factor. Then, the concentrations were calculated.

There is a difference in the AA analysed in the feeds and ragworms. When analysing beta-alanine (BALA) and gamma-aminobutyric acid (GABA) in the ragworm samples, they returned tiny peaks on the computer software that were difficult to integrate and quantify with accuracy. Therefore, they are not included in the ragworm AA analysis. However, BALA and GABA are not essential amino acids (EAA) for fish (Tacon, 1987) and were not present in significant quantities in the feeds.

3.9 Statistical Analysis

Statistical analyses were carried out using Origin (Pro) (Version 2021b. OriginLab Corporation, Northampton, MA, USA). Tables and graphs were made in Microsoft Office Professional Plus 2016 Word, Excel, and PowerPoint (Microsoft Corporation, USA).

Water quality parameters, mean weights of ragworms, specific growth rates, mortality, and nutritional composition (total organic matter (TOM) content, carbon (C) content, nitrogen (N) content, protein content, lipid content, fatty acid (FA) profiles and amino acid (AA) profiles) of feeds and ragworms were tested for significant differences. All statistical analysis was performed at the 95% confidence level ($P > 0.05$).

The normal distribution of data was tested using the Kolmogorov-Smirnov test. Also, the equality of variance was analysed by the Brown-Forsythe test. Also, a one-way analysis of variance (ANOVA) was carried out to compare the means of normally distributed data with Tukey tests. Wherever it is mentioned in the text that a result is significant indicates that a statistical test was performed to determine its significance.

3.10 Screening Tests

Screening tests were carried out in quarter 4 2021 and quarter 1 2022, before carrying out the experiments described in subsequent chapters. The purpose of the screening tests was to verify the materials and methods described in the previous sections of this chapter and ensure they were suitable for the experiments (in terms of practicality and resource requirements) that are presented in chapters 4, 5 and 6.

The screening tests also functioned as a chance to get used to working with various pieces of equipment and computer software. For example, water quality probes, GCMS equipment and associated software, HPLC equipment and associated software, rotary evaporators etc.

3.10.1 Methods

The screening test results presented below started on the 1st April 2022 and ended on the 29th April 2022. Chitosan formulations were made according to the specifications described in Section 3.2 (Chitosan Formulations). Ragworms and aquaculture wastewater were sourced as per Section 3.3 (Source of Experimental Animals and Husbandry) and 3.4 (Source of Aquaculture Wastewater), respectively. The engineered set-up is described in Section 3.5 (General Experimental Set-Up). Ragworm weights and growth rates were recorded as per Section 3.7 (Determining Ragworm Weights and Growth Rates) and summarised in Table 3.2 below. The number of mortalities was also recorded and included in Table 3.2. Biochemical analyses of the ragworms were carried out as per Section 3.8 (Biochemical Analyses of Ragworms and Feeds), however, the results were not recorded as they were not intended to be included in this thesis. Statistical analysis was not carried out.

Five groups (beakers) were set up with 10 ragworms in each. Each group were fed 1 g daily of the following feeds:

- **Group 1** – Commercial fish (**CF**) feed pellets (Tetra® Pond Pellets Mini) [no chitosan]
- **Group 2** – Cleaner fish aquaculture wastewater (**AW**) [no chitosan]
- **Group 3** – 90% Cleaner fish aquaculture wastewater + 10% chitosan (**AW + 10% C**)
- **Group 4** – 80% Cleaner fish aquaculture wastewater + 20% chitosan (**AW + 20% C**)
- **Group 5** – 70% Cleaner fish aquaculture wastewater + 30% chitosan (**AW + 30% C**)

Refer to section 4.2.4 (Making Feeds) to see how the aquaculture wastewater was processed before feeding to ragworms. In this screening test, 10% means 10% of the weight of the 1 g fed daily was chitosan (i.e., 0.1 g).

3.10.2 Results

In the screening test results (Table 3.2), Group 1 (**CF**) showed the highest final mean weight, as well as the highest specific growth rate (0.037 mg d-1) compared to the other groups. Group 2 (**AW**) and Group 3 (**AW + 10% C**) had similar initial weights, final weights, and specific growth rates, with Group 3 showing a slight increase in final weight (101.3 mg) over Group 2 (99.6 mg). Group 4 (**AW + 20% C**) had a slightly higher initial weight than Groups 2 and 3, but a similar specific growth rate (0.016 mg d-1). Group 5 (**AW + 30% C**) had the lowest final weight (84.2 mg) and the lowest specific growth rate (0.013 mg d-1). Mortality was low across all groups, with 1 or 2 deaths in each group.

Table 3.2 – Screening Test Results

	Group 1 CF	Group 2 AW	Group 3 AW + 10% C	Group 4 AW + 20% C	Group 5 AW + 30% C
Initial Mean Weight (mg)	56.9	63.1	63.5	65	58.3
Final Mean Weight (mg)	161.9	99.6	101.3	100.8	84.2
Specific Growth Rate (mg d^{-1})	0.037	0.016	0.017	0.016	0.013
Number of Mortalities	2	1	1	2	2

3.10.3 Discussion

The screening test results were positive and provided crucial confirmation of the conditions necessary for future experiments. Group 1 (**CF**) demonstrated the highest growth rate and final weight, establishing a strong baseline for comparison. The consistent performance across Groups 2 (**AW**), 3 (**AW + 10% C**), and 4 (**AW + 20% C**) indicated that the inclusion of 10% and 20% chitosan concentrations did not adversely affect growth significantly, though the specific growth rates were slightly lower than Group 1. Despite Group 5 (**AW + 30% C**) showing a lower growth rate and final weight, the overall trends confirmed that a specific range of conditions were viable for further experimentation. The low mortality rates across all groups further validated the robustness of the test conditions. These findings were instrumental in shaping the experimental designs discussed in subsequent chapters, ensuring that the foundational conditions were both appropriate and reliable for detailed investigations.

Chapter 4

The Effect of Chitosan on *Hediste diversicolor* Growth, Mortality and Nutritional Composition

4.1 Introduction

Most aquafeeds used in aquaculture systems contain ingredients derived from fish meal and fish oil processed from wild-caught fish, which is of significant sustainability concern (Tacon and Metian, 2015). Many wild fish stocks are overfished or fished at their maximal sustainable level (FAO, 2022). Therefore, sourcing high-quality, sustainable, and alternative aquafeed ingredients is vital.

Ragworms are highly nutritious for fish, making them attractive candidates for alternative aquafeeds (Fidalgo e Costa, Narciso and Cancela da Fonseca, 2000; García-Alonso and CT, 2008; A. Bischoff, Fink and Waller, 2009; Santos *et al.*, 2016; O Pajand *et al.*, 2017; Marques *et al.*, 2018; Wang *et al.*, 2019; Yousefi-Garakouei, Kamali and Soltani, 2019; Jerónimo *et al.*, 2021). Some aquafeeds already include ragworms as an ingredient (Scaps, 2002). Ragworms naturally feed on mud, sand, detritus, phytoplankton, plankton, and other macrofauna (Budd, 2020). In addition, they have a natural affinity for coprophagous feeding, meaning ragworms have great potential for intense production when raised on aquaculture wastewater (**AW)** (Riisgård, 1994; Vedel, Andersen and Riisgard, 1994; Scaps, 2002; Wang *et al.*, 2019; Jerónimo *et al.*, 2021). **AW** production is of significant sustainability concern for the aquaculture industry (Ahmad *et al.*, 2022). It has been stated by several global environmental and climate change accords that wastewater is a product that should be reused, recycled and recovered (United Nations, 2015, 2020; European Commission, 2016). Previous research has confirmed that ragworms can be cultivated on various sources of **AW** with favourable growth rates and nutritional profiles (Bradshaw *et al.*, 1990; Riisgård, 1994; Vedel, Andersen and Riisgard, 1994; Olivier *et al.*, 1997; Scaps, 2002; Batista *et al.*, 2003; Nesto *et al.*, 2012; O Pajand *et al.*, 2017; Wang *et al.*, 2019; Jerónimo *et al.*, 2021). Therefore, feeding **AW** to ragworms so they grow in terms of their biomass can provide a suitable alternative high-quality aquafeed ingredient while reducing the impact of overfishing by reducing the burden on natural fish stocks to create aquafeeds. In addition, the process can reduce the impact of wastewater treatment and subsequent pollution.

However, feeding ragworms directly with **AW** would mean that excess nutrients, especially those in the dissolved form, would be lost, as ragworms mainly consume their food in the solid form (Scaps, 2002). Previous research has, however, shown that chitosan can coagulate

and flocculate these excess nutrients (Chung, Li and Chen, 2005; Renault *et al.*, 2009). Hence chitosan coagulation of **AW** for maximum nutrient recovery and feeding to ragworms is a promising idea. However, feeding chitosan-coagulated and flocculated **AW** to ragworms has not been researched. Moreover, the impact of chitosan on ragworm growth, mortality and nutritional composition has not been investigated. An understanding of the latter is vital if the ragworms fed chitosan-processed sources of **AW** are intended for further use as an aquafeed ingredient. Ragworms with a compromised nutritional profile will not be a suitable alternative aquafeed ingredient, as the animals they are intended to feed in an aquaculture system will not develop to the same level as when fed with currently used aquafeeds, reducing the value of the aquacultured product.

Chitosan is FDA-approved and generally recognised as safe (GRAS) (Thanou, Verhoef and Junginger, 2001; USA FDA, 2019), suggesting chitosan is non-toxic to ragworms. However, chitosan safety data sheets and clinical trial data suggest that chitosan can be toxic at specific concentrations in trout, water fleas, rats and mice (Landes and Bough, 1976; Vahouny *et al.*, 1983; Fukada, Kimura and Ayaki, 1991; Deuchi *et al.*, 1995; Hirano, 1996; Tanaka *et al.*, 1997; Bullock *et al.*, 2000; National Institutes of Health and US Department of Health and Human Services, 2017). If chitosan affects those animals, it is, therefore, conceivable that chitosan can also affect ragworms. Therefore, the experiment presented here investigated whether **AW** with chitosan incorporated and fed to ragworms can create a viable alternative aquafeed ingredient without impacting their growth, mortality, and nutritional profile. Thus, to investigate the impact different concentrations of chitosan in feed have on ragworm growth, physiology, and nutritional composition, three chapter aims were formulated:

1. Investigate feeding **AW + chitosan** to ragworms and its impact on their growth and mortality.

2. Investigate feeding **AW + chitosan** to ragworms and its impact on their nutritional composition.

3. Evaluate ragworms' suitability as an alternative aquafeed ingredient after being fed with **AW + chitosan** by comparing ragworms fed with **AW + chitosan**, ragworms fed **AW** without chitosan and ragworms fed with a commercial fish feed (**CF**).

In the interest of clarity, **AW + chitosan** means **AW** with chitosan incorporated into the feed. In other words, they are not fed independently. The objective of the chapter was to investigate the three aims stated above to answer the following hypothesis:

Feeding different diets of commercial fish feed, aquaculture wastewater or aquaculture wastewater + chitosan to ragworms will not affect their growth, mortality, and nutritional composition.

Proving the above hypothesis will determine whether ragworms can utilise excess nutrients from **AW** that have been dosed with chitosan without detrimental impacts to the ragworms. In addition, it will determine whether ragworms can become a potentially valuable product in the form of an alternative aquafeed ingredient, hence, helping combat the sustainability and waste production issues associated with aquaculture.

4.2 Materials and Methods

Three groups of 30 ragworms were organised into triplicates of 10 and fed with cleaner fish **AW** containing different concentrations of chitosan (**10, 20 and 30 mg L^{-1}**) to determine their effect on ragworm growth, mortality, and nutritional composition. In addition, another group of 30 ragworms were fed **AW** without chitosan as a control, and another group of 30 were fed a commercial feed (**CF**) to compare to a nutritionally complete feed.

The experiment started on the 1st June 2022 and ended on the 30th June 2022. This section discusses experimental methodology specific to this experiment and not covered in chapter 3. For full methods used in this experiment, refer to Sections 3.2 (Chitosan Formulations), 3.3 (Source of Experimental Animals and Husbandry), 3.4 (Source of Aquaculture Wastewater), 3.5 (General Experimental Set-Up, 3.6 (Water Quality Measurements), 3.7 (Determining Ragworm Weights and Growth Rates), 3.8 (Biochemical Analyses of Ragworms and Feeds), and 3.9 (Statistical Analysis).

4.2.3 Feed Groups

The following bullet points describe the feed provided to the ragworms in their respective feed groups. The bold lettering refers to what they are referred to later in the text and are used interchangeably.

- **Group 1** – Commercial fish (**CF**) feed pellets (Tetra® Pond Pellets Mini) [no chitosan]
- **Group 2** – Cleaner fish aquaculture wastewater (**AW**) [no chitosan]
- **Group 3** – Cleaner fish aquaculture wastewater + 10 mg L^{-1} chitosan (**AW + 10 mg L^{-1} C**)
- **Group 4** – Cleaner fish aquaculture wastewater + 20 mg L^{-1} chitosan (**AW + 20 mg L^{-1} C**)
- **Group 5** – Cleaner fish aquaculture wastewater + 30 mg L^{-1} chitosan (**AW + 30 mg L^{-1} C**)

The equation to calculate the chitosan doses is included in Appendix C.

After a 5-day acclimation period (described in Section 3.5 – General Experimental Set-Up), alive and intact ragworms were selected for the experiment. They were not selected by sex, size, maturation, weight or other (they were all similar in size and at the same stage of the life cycle). Three glass beakers containing 10 random ragworms each (replicate) were set up for each food group (i.e., 30 ragworms per group and 150 ragworms in total.

4.2.4 Making Feeds

The **AW** was defrosted overnight in a fridge and then homogenised by vigorously shaking the jerry cans for approximately 5 min. Next, the **AW** was poured into four 1 L glass beakers and stirred in a paddle stirrer (VELP® Scientifica FP4 Portable Flocculation Tester) at 200 rpm for 5 min. The **AW** was then returned to the jerry can, and the process was repeated three times. Five hundred ml of a homogenous mixture of **AW** was then added to a clean 1 L beaker, and the respective chitosan dose was added and then made up to the 900 ml graduation line on

the beaker (no chitosan was added to the group 2 beaker). The beaker contents were then thoroughly mixed using the paddle stirrer to distribute the chitosan evenly.

Once the contents of the beakers were thoroughly mixed, they were sealed with cling film and parafilm, frozen overnight, and then freeze-dried for 24 hours (Modulyo® 4K Freeze-Dryer). Freeze-drying decreased the water content making it easier to handle and create feeding portions while ensuring no chitosan or **AW** components were lost. Freeze-drying can also inactivate potentially pathogenic food-borne microorganisms (Jiao *et al.*, 2017), hence removing the impact of these on ragworms. After freeze-drying, the beakers were re-sealed and stored in a freezer at -20 °C until required. The above description refers to groups 3, 4 and 5. Group 2 feed was prepared in the same way but without adding chitosan. Group 1 were fed commercial feed pellets, so no preparation was necessary.

4.2.5 Miscellaneous Experiment Notes

The dissolved oxygen (DO) probe broke during the experiment. However, similar experiments indicated that the set-up could sufficiently provide adequate constant DO to the beakers at around 7-8 mg L^{-1} (Appendices L & P).

4.2.6 Feeding Protocol

The initial plan for the experiment was to feed the ragworms using an iso-carbonic approach, by supplying the feeds at 3% of the initial ragworm group wet weight per day as the baseline, similar to other research (Wang *et al.*, 2019). However, upon review of that research, it was decided that a more suitable strategy for feeding was to feed the ragworms *ad libitum,* so there was feed available all the time. Wang *et al.*, (2019) discuss in their research of ragworms that were fed **AW** from a land-based Atlantic salmon smolt aquaculture system that using an iso-carbonic feeding approach led to food accessibility issues in the culture tanks, where the lack of food may have negatively impacted the growth and development of the ragworms.

The provision of food in this experiment was hence designed to ensure it was not a limiting factor that affected growth. Feeding *ad libitum* is a more representative feeding approach for industrial applications, especially if the goal is high-density cultures where growth rates slow

down (Scaps *et al.*, 1993). In other words, in industrial settings, there might not be the level of control of feeding that might be possible in the laboratory. Therefore, ragworms were fed 1 g of feed in the morning and 1 g at the end of the day if there was no feed visible on the sediment surface. On a Friday, 4 g were added to cover the weekend due to restricted access to the laboratory on weekends (Section 3.5 – General Experimental Set-Up).

4.2.7 A Problem with Group 1

Group 1 were directly fed with a pelleted commercial feed (**CF**) (Tetra® Pond Pellets Mini). Feeding the ragworms 1 g per day of this feed caused the culture water quality to deteriorate, as confirmed by the pH (Appendix G) and turbidity of the water (Appendix D), visually represented in Figure 4.1. Therefore, the ragworms in this group did not perform as well as expected. Consequently, a similar experiment was conducted where ragworms were fed half the amount of **CF** at feeding times under the same conditions and duration. Reducing the amount of feed added was found not to cause a deterioration in the water quality, and the ragworms performed much better in terms of growth and mortality. Therefore, the results for group 1 are split into group 1a and group 1b to differ between the two groups fed **CF**, respectively.

Group 1b were exposed to the same experimental conditions as all other groups. The ragworms were from the same batch received from Seabait Ltd (Section 3.3 – Sources of Experimental Animals and Husbandry), and the feed (**CF**) was from the same batch too.

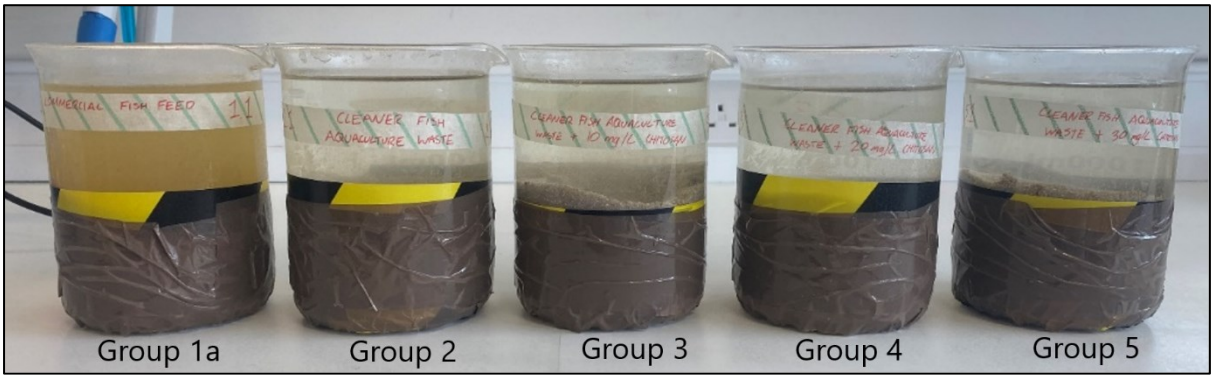

Figure 4.1 – The image shows the turbidity of the water from one beaker from each feed group
Group 1a – 5 is positioned left to right. The murky water of group 1a can be seen clearly on the left-hand side beaker. Group 1b is not included in the image.

4.3 Results

4.3.1 Feeds

To understand how different chitosan doses impact the nutritional content of **AW**, and how this affects ragworm development, total organic matter contents, carbon and nitrogen contents, protein contents, amino acid profiles, lipid contents, and fatty acid profiles were analysed. They were compared to **CF**, as this contains a high level of nutrition being a commercially manufactured feed for feeding small marine animals.

4.3.1.1 Total Organic Matter Contents

Figure 4.2 illustrates the total organic matter (TOM) content (mean ± SD) of different feeds used in the experiment, represented as a percentage. **CF** had a mean TOM percentage of 94.25 ± 0.53%, significantly higher than the **AW** feeds, which were all statistically similar (59.73 ± 1.66 – 63.06 ± 2.13%).

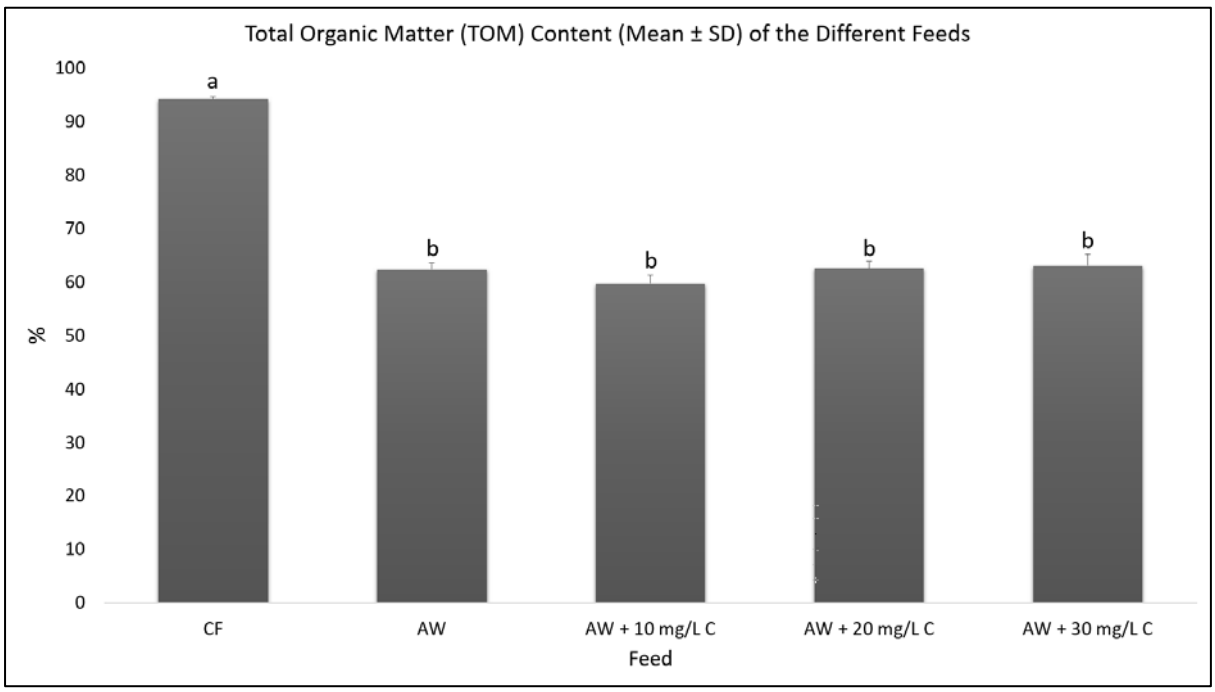

Figure 4.2 – Percentage total organic matter content of the different feeds used in the experiment (Mean ± SD)

CF = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan; **AW + 30 mg L^{-1} C** = aquaculture wastewater + 30 mg L^{-1} chitosan. Different lowercase letters indicate significant differences between groups (P > 0.05).

The TOM content results indicate that the **CF** has a significantly higher total organic matter content compared to all treatments involving **AW + Chitosan** at various concentrations. There are no significant differences in TOM content among the **AW** treatments.

4.3.1.2 Carbon and Nitrogen Contents

Figure 4.3 presents the percentage of carbon and nitrogen contents (mean ± SD) of the different feeds used in the experiment. Whilst the C content of the **CF** was higher than the **AW** feeds, the **CF** had a lower N content. In the **CF**, the C content was 43.32 ± 1.05%, and the N content was 4.62 ± 0.08%. The C contents of the **AW** feeds ranged from 32.72 ± 3.51 to 37.97 ± 0.86%, and the N contents ranged from 5.67 ± 0.11 to 5.91 ± 0.08.

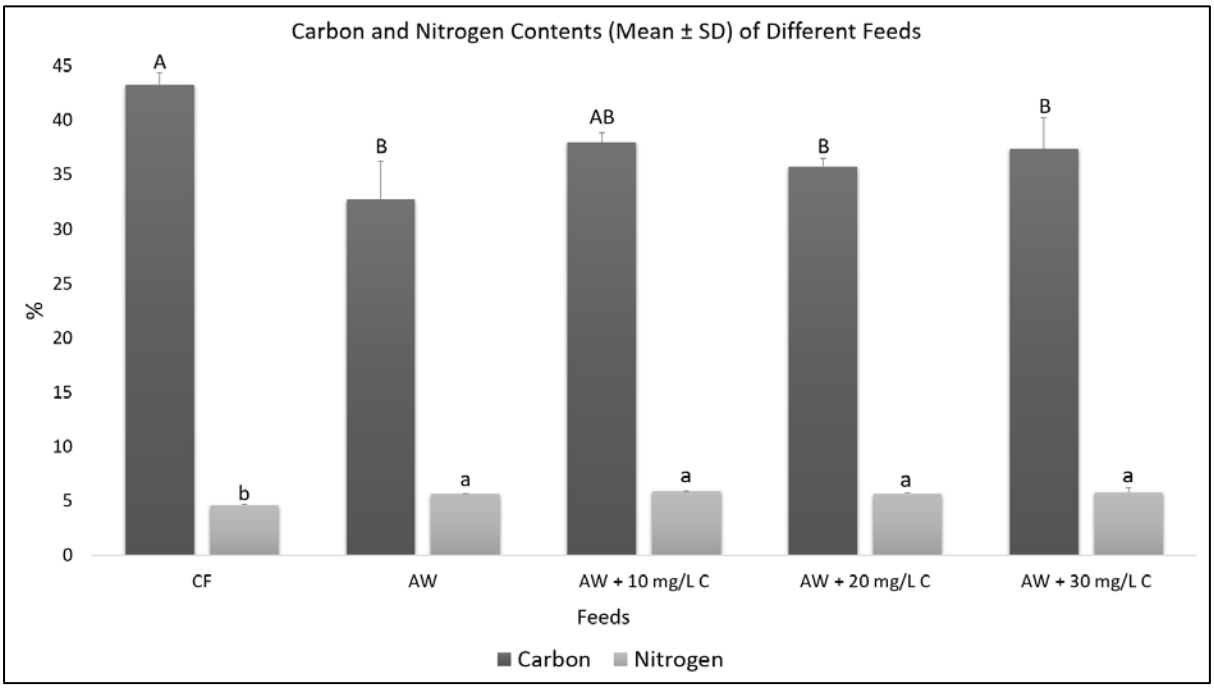

Figure 4.3 – Percentage of carbon and nitrogen contents (mean ± SD) of the different feeds used in the experiment
CF = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan; **AW + 30 mg L^{-1} C** = aquaculture wastewater + 30 mg L^{-1} chitosan. Different uppercase letters indicate significant differences in carbon percentages. Different lowercase letters indicate significant differences in nitrogen percentages ($P > 0.05$).

The carbon and nitrogen content results suggest that while **CF** has a higher carbon content, adding chitosan to **AW** maintains a significant portion of these nutrients, although at lower levels than the commercial feed. Nitrogen levels are similar but slightly higher in the **AW** feeds.

4.3.1.3 Protein Contents

Figure 4.4 presents the protein content (mean ± SD) of the different feeds used in the experiment, measured in mg g^{-1}. The **CF** protein content was significantly lower compared to the **AW** feeds. However, the **AW**-based feeds protein contents were similar and did not differ significantly. The **CF** had a mean protein content of 288.91 ± 4.95 mg g^{-1}. The **CF** protein value obtained agrees with the manufacturer's website, which states a crude protein percentage of 28%. The **AW** feeds mean protein contents ranged from 354.49 ± 7.07 to 369.47 ± 5.19.

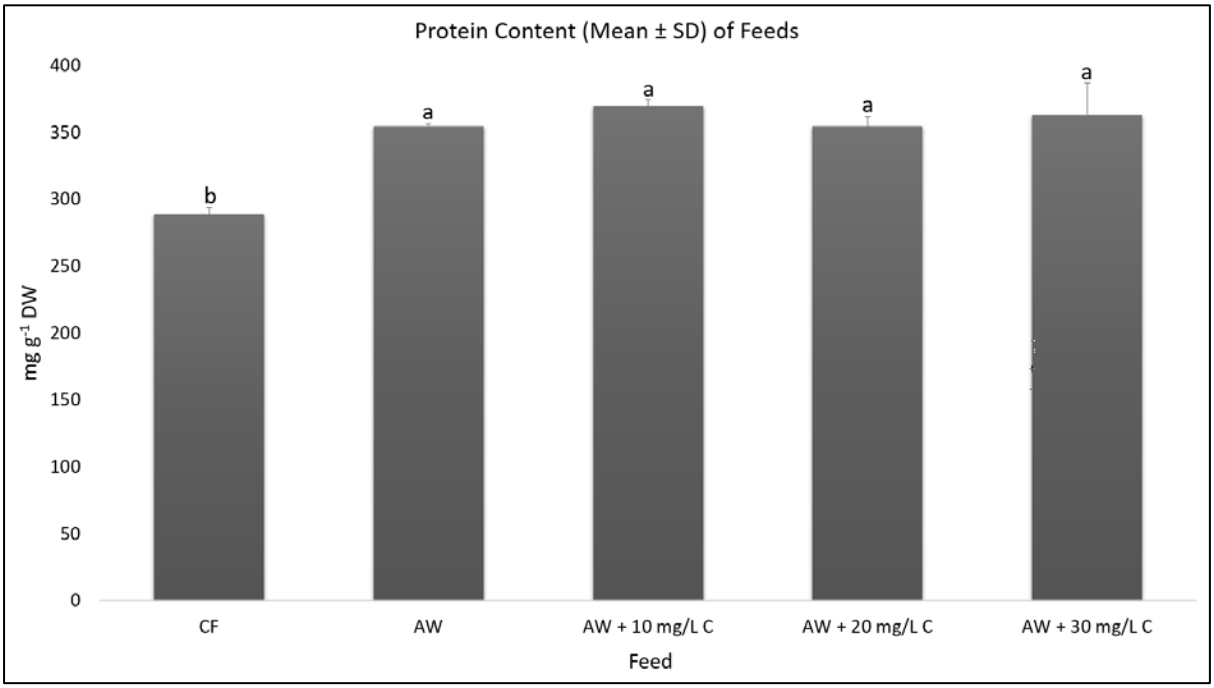

Figure 4.4 - The protein content of the different feeds used in the experiment (mean ± SD)
CF = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan; **AW + 30 mg L^{-1} C** = aquaculture wastewater + 30 mg L^{-1} chitosan. Different lowercase letters indicate significant differences between feeds (P > 0.05).

These results indicate that the **AW** used in this experiment (refer to section 3.4 – Source of Aquaculture Wastewater), both with and without chitosan treatment, provides a higher protein content compared to the **CF** used in this experiment. This suggests that using **AW** as a feed source, with or without the addition of chitosan, can enhance the protein content in the feed, potentially offering a nutritious feed for ragworms to be used as an alternative to traditional commercial feed.

4.3.1.4 Amino Acid Profiles

Figure 4.5 presents the amino acid (AA) composition (% of total amino acids) of the different feeds. The percentage compositions of the **AW** feeds were very similar, as expected. However, the **CF** had much larger percentage compositions of aspartic acid (ASP) and glutamic acid (GLU). In addition, it is important to note that protein is composed only of AA and therefore the total protein content and total AA content of the feeds and ragworms should be the same. However, using the method used to determine AA concentrations were not able to determine every AA, therefore the total AA content value is lower than the total protein content values.

Furthermore, similar to the protein contents the **CF** had a significantly lower total mean AA concentration than the **AW** feeds (154.34 ± 0.69 mg g^{-1}). The **AW** feeds ranged between 188.65 ± 1.79 and 195.22 ± 0.18 mg g^{-1}. Furthermore, there were significant differences in the concentration of essential amino acids (EAA) in **CF** compared with **AW** feeds. The **CF** had a significantly lower mean total EAA concentration of 78.33 ± 0.32 mg g^{-1} (50.75 ± 0.02%) compared to the **AW** feeds, which ranged between 109.75 ± 0.57 (58.18 ± 0.25%) and 113.73 ± 0.37 mg g^{-1} (58.26 ± 0.13%). Also, the **CF** had a significantly lower concentration of non-essential amino acids (NEAA) than the **AW** feeds. However, the **CF** had a higher percentage composition of NEAA. **CF** had a mean NEAA concentration of 76.02 ± 0.37 mg g^{-1} (49.25 ± 0.02%), and the **AW** feeds ranged between 78.90 ± 1.22 (41.82 ± 0.25%) and 82.17 ± 1.87 mg g^{-1} (42.24 ± 0.48%).

Regarding the EAA, in **CF** and **AW** feeds, lysine (LYS) and Leucine (LEU) had the highest percentage composition. For LYS and LEU, the mean percentage composition was slightly higher in the **AW** feeds compared to **CF**, ≈8.89 and ≈9.61% compared to ≈7.13 and ≈8.93%, respectively. The mean percentage composition after LYS and LEU was followed in descending order by arginine (ARG), isoleucine (ILEU), valine (VAL), phenylalanine (PHE), threonine (THR), histidine (HIS) and methionine (MET). For all EAA, each percentage composition was higher in the **AW** feeds than in the **CF**, except for THR.

Glutamic acid (GLU) was the NEAA with the highest percentage composition in the **CF** and **AW** feeds and the AA with the highest overall percentage composition. In **CF**, the mean percentage composition of GLU was ≈16.46% and in **AW** feeds, it was ≈12.34%. The second-

highest NEAA in the **CF** and **AW** feeds was aspartic acid (ASP), with mean percentage composition values of ≈13.63 and ≈9.33%, respectively. The mean percentage composition of AA in descending order after GLU and LYS in **AW** feeds was glycine (GLY), tyrosine (TYR), alanine (ALA), serine (SER), ornithine (ORN), BALA and GABA. In the **CF**, it was GLY, ALA, TYR, SER, BALA, ORN, and GABA.

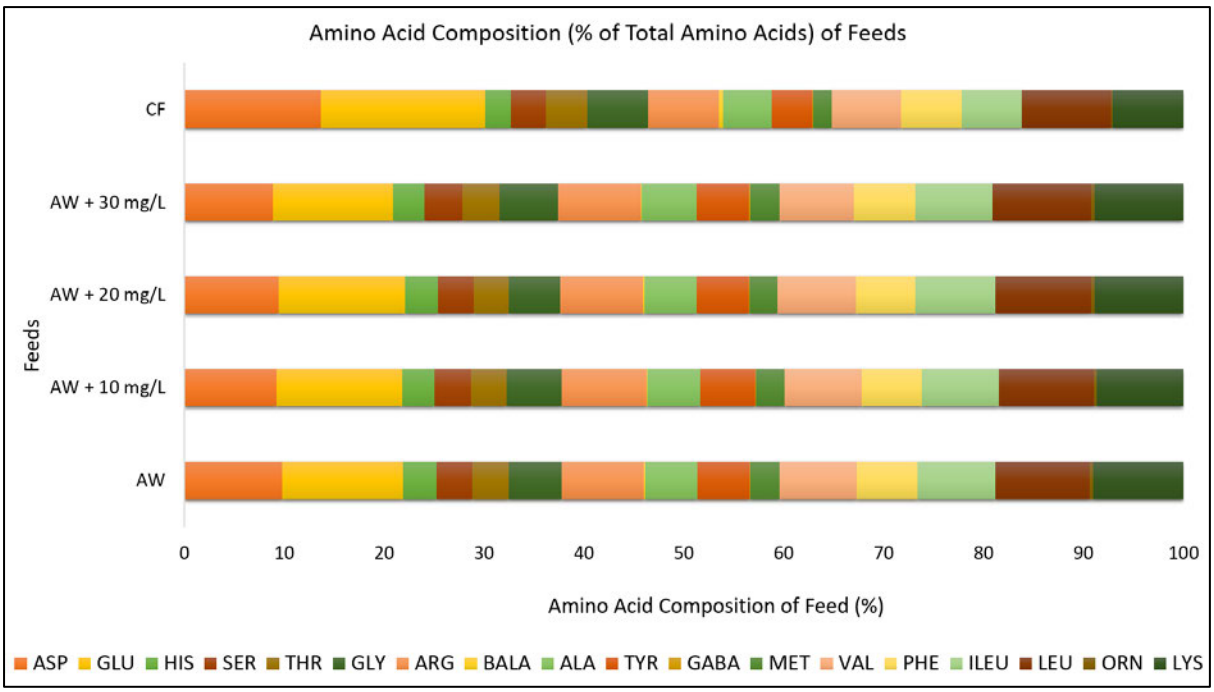

Figure 4.5 – Amino acid composition of the different feeds (% of total amino acids)
CF = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan; **AW + 30 mg L^{-1} C** = aquaculture wastewater + 30 mg L^{-1} chitosan. ASP = aspartic acid; GLU = glutamic acid; HIS = histidine; SER = serine; THR = threonine; GLY = glycine; ARG = arginine; BALA = beta-alanine; ALA = alanine; TYR = tyrosine; GABA = gamma-aminobutyric acid; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; ORN = ornithine; LYS = lysine.

The amino acid composition of the **AW** feeds shows that the different treatment types, including the addition of chitosan, do not significantly alter the amino acid profile of the feeds. This consistency in amino acid composition suggests that the feeds, regardless of the treatment, provide a similar nutritional profile in terms of amino acid content.

4.3.1.5 Lipid Contents

Figure 4.6 displays the lipid concentrations (mean ± SD) of different feeds used in the experiment, measured in mg g^{-1}. There were no statistically significant differences in mean

lipid concentrations between the feeds. However, the **CF** had a lower mean lipid concentration of 116.63 ± 13.72 mg g^{-1} compared to the **AW** feeds which ranged between 126.26 ± 4.31 and 157.04 ± 2.9 mg g^{-1}.

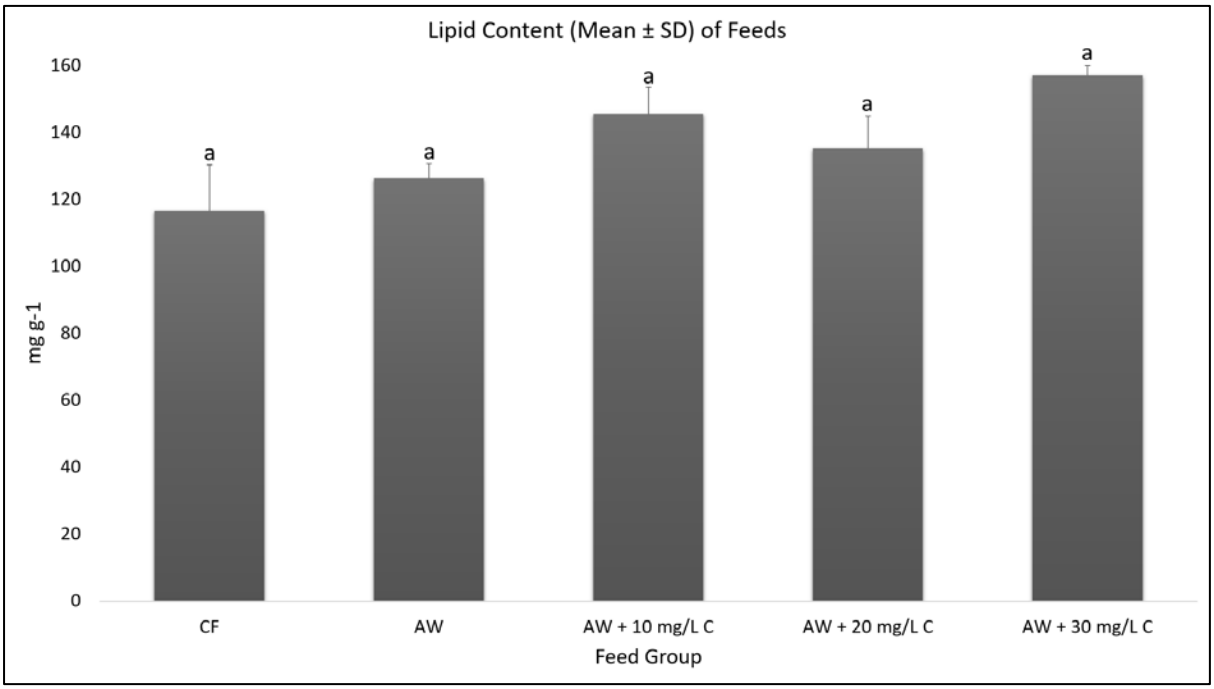

Figure 4.6 – Lipid concentrations (mean ± SD) of the different feeds used in the experiment
CF = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan; **AW + 30 mg L^{-1} C** = aquaculture wastewater + 30 mg L^{-1} chitosan. Different lowercase letters indicate significant differences between feeds (P > 0.05).

These results suggest that the addition of chitosan to **AW** does not significantly alter the lipid content of the feed compared to **AW** without chitosan. All feed types provide similar levels of lipids, indicating that chitosan treatment maintains the lipid content, and perhaps even enhances it slightly.

4.3.1.6 Fatty Acid Profiles

Table 4.1 provides the mean ± SD fatty acid (FA) composition (also expresses them as a percentage of total fatty acids) of the different feeds used in the experiment. One-way ANOVA demonstrated no significant differences between the total concentrations of FA between the feeds, even though the **CF** had a lower content than the **AW feeds**. The **CF** had a much higher concentration and percentage proportion of polyunsaturated fatty acids

(PUFA) (35.26 ± 0.53 mg g^{-1}) (≈48.47% of total FA) compared with the **AW feeds**, which ranged between 6.84 ± 1.44 (≈9.36%) and 10.35 ± 2.00 mg g^{-1} (≈10.18% of total FA). The **AW feeds** had a higher concentration and percentage proportion of saturated fatty acids (SFA) than **CF**. SFA **AW** values ranged between 43.58 ± 9.18 (≈59.64%) and 67.10 ± 11.69 mg g^{-1} (≈61.29% of total FA), and **CF** had an SFA value of 25.13 ± 1.18 mg g^{-1} (≈34.54% of total FA). The **AW feeds** also had a higher concentration and proportion of monounsaturated fatty acids (MUFA) than **CF**. MUFA **CF** values ranged between 22.66 ± 4.78 mg g^{-1} (≈31.01%) and 33.27 ± 5.80 mg g^{-1} (≈30.39% of total FA), and **CF** had a MUFA value of 12.36 ± 0.58 mg g^{-1} (≈16.99% of total FA).

The FA with the highest percentage proportion in all feeds was the SFA palmitic acid (C16:0), with values ranging between ≈20.54 and ≈33.15% of total FA. Followed by the omega-3 PUFA eicosapentaenoic acid (EPA) (C20:5) (≈15.39% of total FA) in **CF**, and the omega-9 MUFA isomers oleic acid/elaidic acid (C18:1) in the **AW**-based feeds (≈19.25 - ≈20.59% of total FA).

Regarding the essential omega-3, docosahexaenoic acid (DHA), the **CF** had a much higher concentration and proportion than the **AW**-based feeds with values of 11.00 ± 0.01 mg g^{-1} (≈15.12%) and 0.27 ± 0.06 mg g^{-1} (≈0.37%) to 0.47 ± 0.08 mg g^{-1} (≈0.43%), respectively.

Table 4.1 – Mean ± SD fatty acid composition of the different feeds used in the experiment

	Feed				
	CF	**AW**	**AW + 10 mg L^{-1} Chitosan**	**AW + 20 mg L^{-1} Chitosan**	**AW + 30 mg L^{-1} Chitosan**
Σ FA (mg g^{-1}) DW	72.25 ± 2.29[a]	73.07 ± 15.40[a]	101.70 ± 19.64[a]	90.93 ± 1.53[a]	109.49 ± 19.08[a]
FA	**FA Mean ± SD mg g^{-1} (% of Total FA)**				
C6:0	-	0.01 ± 0.00 (≈0.02%)	-	-	-
C8:0	-	0.01 ± 0.00[a] (≈0.02%)	0.01 ± 0.00[a] (≈0.01%)	0.02 ± 0.00[b] (≈0.02%)	0.01 ± 0.00[a] (≈0.01%)
C10:0	0.02 ± 0.00[b] (≈0.03%)	0.08 ± 0.02[a] (≈0.11%)	0.07 ± 0.01[a] (≈0.07%)	0.08 ± 0.00[a] (≈0.08%)	0.06 ± 0.01[ab] (≈0.06%)
C11:0	-	0.02 ± 0.00[a] (≈0.03%)	0.02 ± 0.00[a] (≈0.02%)	0.02 ± 0.00[a] (≈0.03%)	0.03 ± 0.01[a] (≈0.03%)

C12:0	0.13 ± 0.01^{b} (≈0.18%)	0.45 ± 0.09^{ab} (≈0.61%)	0.60 ± 0.12^{a} (≈0.59%)	0.54 ± 0.01^{a} (≈0.60%)	0.72 ± 0.12^{a} (≈0.65%)
C13:0	0.04 ± 0.00^{c} (≈0.05%)	0.11 ± 0.02^{bc} (≈0.15%)	0.16 ± 0.03^{ab} (≈0.15%)	0.14 ± 0.00^{b} (≈0.16%)	0.26 ± 0.04^{a} (≈0.24%)
C14:0	3.63 ± 0.17^{b} (≈4.99%)	6.99 ± 1.47^{ab} (≈9.56%)	9.89 ± 1.91^{a} (≈9.73%)	8.92 ± 0.15^{ab} (≈9.81%)	12.39 ± 2.16^{a} (≈11.32%)
C15:0	0.63 ± 0.03^{b} (≈0.86%)	1.70 ± 0.36^{ab} (≈2.32%)	2.90 ± 0.56^{a} (≈2.85%)	2.65 ± 0.04^{a} (≈2.91%)	3.24 ± 0.57^{a} (≈2.96%)
C16:0	14.94 ± 0.70^{a} (≈20.54%)	24.23 ± 5.11^{a} (≈33.15%)	32.45 ± 6.27^{a} (≈31.91%)	29.14 ± 0.49^{a} (≈32.05%)	32.16 ± 5.60^{a} (≈29.37%)
C17:0	0.48 ± 0.02^{b} (≈0.66%)	1.64 ± 0.35^{ab} (≈2.25%)	2.39 ± 0.46^{a} (≈2.35%)	2.01 ± 0.03^{a} (≈2.21%)	2.44 ± 0.43^{a} (≈2.23%)
C18:0	4.88 ± 0.23^{b} (≈6.71%)	7.97 ± 1.68^{b} (≈10.91%)	11.74 ± 2.27^{ab} (≈11.54%)	10.28 ± 0.17^{ab} (≈11.30%)	15.08 ± 2.63^{a} (≈13.77%)
C20:0	0.12 ± 0.01^{b} (≈0.17%)	0.20 ± 0.04^{b} (≈0.27%)	0.28 ± 0.05^{ab} (≈0.28%)	0.28 ± 0.00^{ab} (≈0.30%)	0.40 ± 0.07^{a} (≈0.37%)
C21:0	-	0.02 ± 0.00^{b} (≈0.02%)	0.03 ± 0.01^{ab} (≈0.03%)	0.02 ± 0.00^{ab} (≈0.02%)	0.04 ± 0.01^{a} (≈0.04%)
C22:0	0.06 ± 0.00^{b} (≈0.08%)	0.09 ± 0.02^{b} (≈0.13%)	0.14 ± 0.03^{ab} (≈0.13%)	0.13 ± 0.00^{ab} (≈0.15%)	0.18 ± 0.03^{a} (≈0.17%)
C23:0	-	0.04 ± 0.01^{a} (≈0.05%)	0.05 ± 0.01^{a} (≈0.05%)	0.05 ± 0.00^{a} (≈0.06%)	0.06 ± 0.01^{a} (≈0.05%)
C24:0	0.20 ± 0.00^{a} (≈0.27%)	0.02 ± 0.00^{b} (≈0.02%)	0.03 ± 0.00^{b} (≈0.03%)	0.02 ± 0.00^{b} (≈0.03%)	0.03 ± 0.00^{b} (≈0.02%)
Σ SFA	**25.13 ± 1.18^{b} (≈34.54%)**	**43.58 ± 9.18^{ab} (≈59.64%)**	**60.75 ± 11.73^{a} (≈59.74%)**	**54.32 ± 0.91^{ab} (≈59.74%)**	**67.10 ± 11.69^{a} (≈61.29%)**
C14:1 (ω -5)	0.01 ± 0.00^{b} (≈0.01%)	0.05 ± 0.01^{a} (≈0.07%)	0.04 ± 0.01^{a} (≈0.04%)	0.04 ± 0.00^{a} (≈0.04%)	0.03 ± 0.00^{ab} (≈0.03%)
C16:1 (ω – 7)	1.16 ± 0.05^{b} (≈1.59%)	3.89 ± 0.82^{a} (≈5.32%)	4.82 ± 0.93^{a} (≈4.74%)	4.27 ± 0.07^{a} (≈4.69%)	4.67 ± 0.81^{a} (≈4.26%)
C17:1 (ω -7)	-	0.31 ± 0.07^{a} (≈0.42%)	0.56 ± 0.11^{a} (≈0.55%)	0.47 ± 0.01^{a} (≈0.51%)	0.63 ± 0.11^{a} (≈0.58%)

C18:1 (ω – 9)	9.27 ± 0.44^{b} (≈12.75%)	14.30 ± 3.01^{ab} (≈19.57%)	19.57 ± 3.78^{ab} (≈19.25%)	18.72 ± 0.31^{ab} (≈20.59%)	21.51 ± 3.75^{a} (≈19.65%)
C20:1 (ω – 9)	1.19 ± 0.06^{c} (≈1.63%)	2.70 ± 0.57^{abc} (≈3.69%)	3.55 ± 0.69^{ab} (≈3.50%)	2.26 ± 0.04^{bc} (≈2.48%)	4.43 ± 0.77^{a} (≈4.04%)
C22:1 (ω – 9)	0.69 ± 0.03^{b} (≈0.95%)	1.27 ± 0.27^{ab} (≈1.74%)	1.84 ± 0.36^{a} (≈1.81%)	1.67 ± 0.03^{ab} (≈1.83%)	1.80 ± 0.31^{a} (≈1.64%)
C24:1 (ω -9)	0.04 ± 0.00^{b} (≈0.05%)	0.14 ± 0.03^{ab} (≈0.19%)	0.20 ± 0.04^{a} (≈0.20%)	0.18 ± 0.00^{a} (≈0.19%)	0.21 ± 0.04^{a} (≈0.19%)
Σ MUFA	**12.36 ± 0.58^{b} (≈16.99%)**	**22.66 ± 4.78^{ab} (≈31.01%)**	**30.59 ± 5.91^{a} (≈30.08%)**	**27.59 ± 0.46^{ab} (≈30.35%)**	**33.27 ± 5.80^{a} (≈30.39%)**
C18:2 (ω – 6)	10.27 ± 0.48^{a} (≈14.12%)	3.55 ± 0.75^{b} (≈4.85%)	4.45 ± 0.86^{b} (≈4.37%)	3.42 ± 0.06^{b} (≈3.76%)	3.86 ± 0.67^{b} (≈3.53%)
C20:2 (ω – 6)	0.29 ± 0.01^{b} (≈0.40%)	1.71 ± 0.36^{ab} (≈2.34%)	3.17 ± 0.61^{a} (≈3.12%)	3.03 ± 0.05^{a} (≈3.33%)	2.59 ± 0.45^{a} (≈2.37%)
C20:3 (ω – 3)	0.10 ± 0.00^{b} (≈0.14%)	0.21 ± 0.04^{ab} (≈0.28%)	0.32 ± 0.06^{a} (≈0.32%)	0.29 ± 0.00^{a} (≈0.31%)	0.32 ± 0.05^{a} (≈0.30%)
C20:4 (ω – 6)	1.20 ± 0.00^{a} (≈1.65%)	0.08 ± 0.02^{b} (≈0.11%)	0.07 ± 0.01^{b} (≈0.07%)	0.07 ± 0.00^{b} (≈0.08%)	0.08 ± 0.01^{b} (≈0.07%)
C20:5 (EPA) (ω – 3)	11.20 ± 0.02^{a} (≈15.39%)	0.51 ± 0.11^{b} (≈0.70%)	0.77 ± 0.15^{b} (≈0.75%)	0.74 ± 0.01^{b} (≈0.81%)	0.82 ± 0.14^{b} (≈0.75%)
C22:4 (ω – 6)	-	0.02 ± 0.00^{b} (≈0.03%)	0.09 ± 0.02^{b} (≈0.09%)	0.26 ± 0.00^{a} (≈0.28%)	0.08 ± 0.02^{b} (≈0.08%)
C22:5 (ω – 3)	1.20 ± 0.01^{c} (≈1.65%)	0.49 ± 0.10^{bc} (≈0.67%)	1.06 ± 0.20^{a} (≈1.04%)	0.81 ± 0.01^{ab} (≈0.89%)	0.87 ± 0.15^{ab} (≈0.80%)
C22:6 (DHA) (ω – 3)	11.00 ± 0.01^{a} (≈15.12%)	0.27 ± 0.06^{b} (≈0.37%)	0.42 ± 0.08^{b} (≈0.42%)	0.42 ± 0.01^{b} (≈0.46%)	0.47 ± 0.08^{b} (≈0.43%)
Σ PUFA	**35.26 ± 0.53^{a} (≈48.47%)**	**6.84 ± 1.44^{b} (≈9.36%)**	**10.35 ± 2.00^{b} (≈10.18%)**	**9.02 ± 0.15^{b} (≈9.92%)**	**9.12 ± 1.59^{b} (≈8.33%)**

Different superscript letters indicate significant differences in individual FA between feeds, with a to c indicating descending concentrations ($p < 0.05$). **CF** = commercial feed; **AW** = aquaculture wastewater; **AW + 10 mg L^{-1} C** = aquaculture wastewater + 10 mg L^{-1} chitosan; **AW + 20 mg L^{-1} C** = aquaculture wastewater + 20 mg L^{-1} chitosan;

AW + 30 mg L^{-1} C = aquaculture wastewater + 30 mg L^{-1} chitosan. **SFA** = saturated fatty acids; **MUFA** = monounsaturated fatty acids; **PUFA** = polyunsaturated fatty acids. **Σ** = Total. **ω - n** = omega – position of first double bond from the terminal methyl group of chemical structure. **Cn:n** = number of carbons:number of double bonds.

The **CF** has a higher content of PUFA, particularly EPA and DHA, compared to **AW** and chitosan-treated feeds. The chitosan-treated feeds (**AW + 10, 20, 30 mg L^{-1} C**) exhibit higher contents of SFAs and MUFAs but lower levels of PUFAs. These differences highlight the nutritional variations between **CF** and **AW**, emphasising the potential need for supplementation of essential fatty acids like EPA and DHA in alternative feed formulations if the ragworms are unable to match the nutritional quality of commercial feeds.

4.3.2 Ragworms

The sections below contain the results of ragworm analyses (growth, mortality, and biochemical analysis) that were carried out at the end of the experiment.

4.3.2.1 Growth

Figure 4.7 shows the mean wet weights ± standard deviation of ragworms before and after the experiment across the different feed groups. The initial mean wet weights ± SD for groups 1a, 1b, 3, 4 and 5 were 73.30 ± 7.38, 72.45 ± 5.76, 73.03 ± 9.30, 68.81 ± 6.95 and 73.99 ± 12.68 mg, respectively. The initial mean wet weight for group 2 was lower at 57.25 ± 0.90 mg but not significantly lower. As ragworms were randomly stocked to beakers, it appears, that group 2 had a skew towards smaller ragworms. The final mean wet weights were higher when compared to the initial mean wet weights for all tested groups, but they were significantly higher for groups 1b and 4.

There were differences between the groups' final mean wet weights. For example, group 1b had the highest final mean wet weight (241.32 ± 59.48 mg), which was significantly higher than all other groups indicating **CF** is a superior feed compared to **AW**.

Ragworms fed with **AW + 30 mg L^{-1} chitosan** (group 5) had the lowest increase in their final mean wet weight (76.93 ± 13.16 mg). This suggests chitosan at this concentration has negatively impacted their growth rate.

Finally, with final mean wet weights of 117.49 ± 17.20, 99.46 ± 56.35, 87.95 ± 15.11 and 85.18 ± 13.49 mg, groups 4, 2, 3 and 1a were positioned in descending order between the other two, respectively.

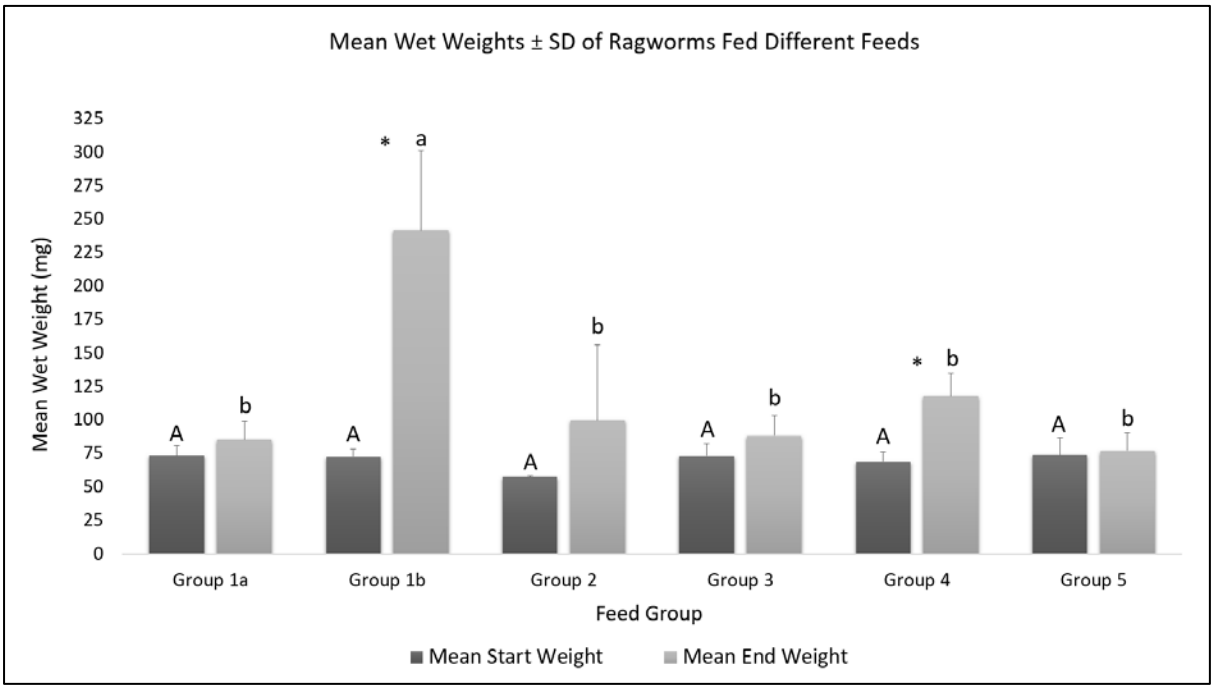

Figure 4.7 – Mean wet weights ± standard deviation of ragworms before and after the experiment fed different feeds
Group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**. Significant differences between initial and final mean wet weights within a group are marked with an asterisk (*). Significant differences in initial mean wet weights between groups are marked by different uppercase letters. Significant differences in final mean wet weights between groups are marked by different lowercase letters ($P < 0.05$).

These results suggest that while **CF** is most effective in promoting growth, the presence of chitosan in **AW** can also enhance growth significantly.

Figure 4.8 displays the specific growth rates (SGR) (mean ± standard deviation) of ragworms fed the different feeds. The SGR for ragworms fed **CF** (group 1b) was highest at 0.047 ± 0.023 d^{-1} (4.8 ± 2.4 % d^{-1}), as **CF** is a commercially manufactured fish that includes everything that growing marine animals require, so this finding was expected and logical.

The lowest SGR was recorded for group 5 (**AW + 30 mg L^{-1} C**), with an SGR of 0.005 ± 0.008 d^{-1} (0.5 ± 0.8 % d^{-1}). The other groups were placed in between groups 1b and 5 with values ranging between 0.010 ± 0.010 d^{-1} (1.0 ± 1.0 % d^{-1}) and 0.028 ± 0.002 d^{-1} (2.8 % ± 0.2 % d^{-1}). Group 1b was substantially higher than group 5. These results demonstrate that adding

chitosan to a source of **AW** does not impact ragworm growth, since the SGR results for groups 3 (**AW + 30 mg L^{-1} chitosan**) and 4 (**AW + 20 mg L^{-1} C**) are not significantly different from group 2 (**AW**).

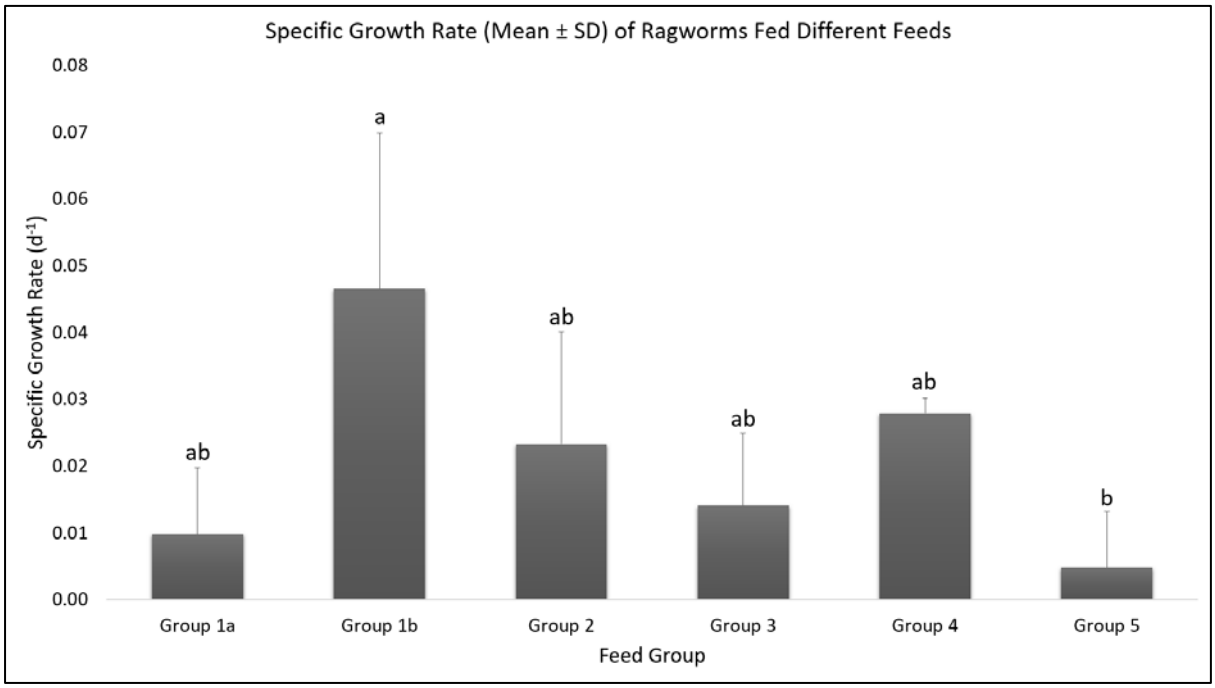

Figure 4.8 – Mean wet weights ± standard deviation of ragworms before and after the experiment fed different feeds
Group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**. Significant differences between initial and final mean wet weights within a group are marked with an asterisk (*). Significant differences in initial mean wet weights between groups are marked by different uppercase letters. Significant differences in final mean wet weights between groups are marked by different lowercase letters ($P < 0.05$).

These results highlight that **CF** is the most effective for growth, while certain concentrations of chitosan in **AW** can support moderate growth. However, a higher concentration of chitosan (30 mg L^{-1}) appears less effective.

4.3.2.2 Mortality

Group 5 had the highest average percentage of mortalities. Figure 4.9 shows that mortality throughout the experiment was low to moderate for all treatments, except group 1b (**CF**), where no mortalities were recorded. Group 5 (**AW + 30 mg L^{-1} C**) had the highest mean percentage mortality (23.33%), followed by group 1a (**CF**) (20%), groups 2 and 3 (**AW** and **AW + 10 mg L^{-1} C**) (13.33%), group 4 (**AW + 20 mg L^{-1} C**) (10%) and group 1b (**CF**) (0%).

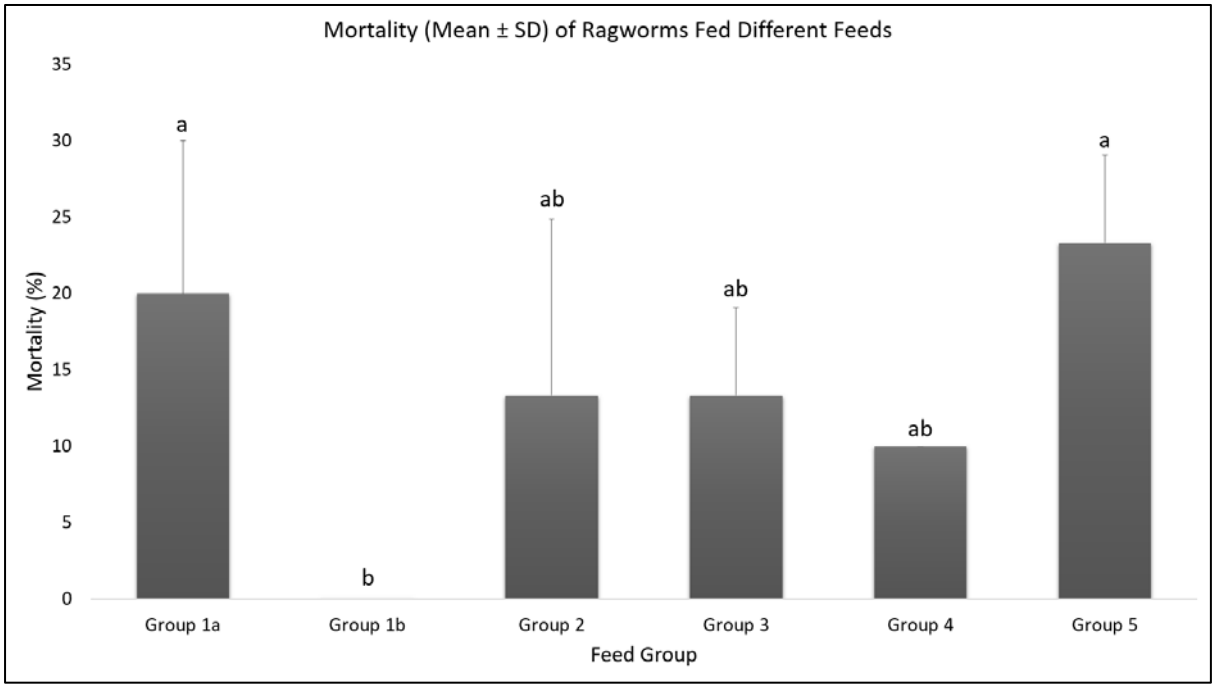

Figure 4.9 – Percentage mortality (mean ± SD) of ragworms fed different feeds
Group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**. Different letters indicate significant differences between groups (P > 0.05).

These results suggest that while **CF** can be highly effective in promoting growth and survival (as seen in Group 1b), there can be inconsistencies, as seen in Group 1a (refer to Section 4.2.7 – A Problem with Group 1). Additionally, moderate concentrations of chitosan (10 mg L^{-1} and 20 mg L^{-1}) in **AW** can support reasonable survival rates, but higher concentrations (30 mg L^{-1}) may increase mortality.

4.3.2.3 Total Organic Matter Contents

Figure 4.10 illustrates the percentage of total organic matter (TOM) content (mean ± SD) of ragworms fed the different feeds. The TOM in ragworms was high in all groups, including the initial ragworms that were not exposed to experimental conditions. The means of all groups ranged from 90.08 ± 0.35 to 91.32 ± 0.05 % and were not significantly different.

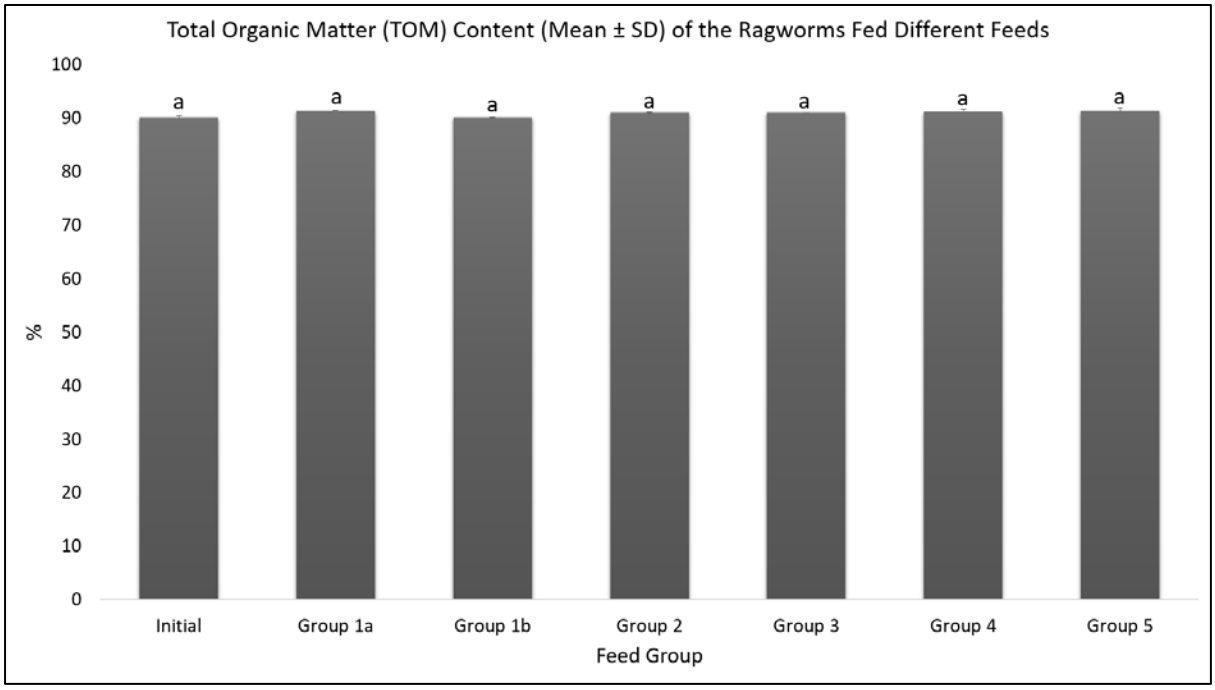

Figure 4.10 – Percentage total organic matter content of ragworms fed different diets (Mean ± SD) Initial = ragworms frozen and stored after acclimation period; group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**. Same lowercase letters indicate non-significant differences ($P > 0.05$).

These results suggest that the type of feed, including the addition of chitosan to **AW**, does not significantly affect the TOM content in ragworms. This consistency indicates that the ragworms maintain a stable organic matter composition regardless of dietary variations.

4.3.2.4 Carbon and Nitrogen Contents

Figure 4.11 illustrates the percentage of carbon and nitrogen contents (mean ± SD) of ragworms fed the different feeds. Excluding the initial group, carbon contents did not vary significantly between groups ranging from 45.28 ± 1.43 to 46.71 ± 0.23%. The initial group had a mean carbon content of 43.8 ± 0.98%.

Nitrogen contents varied, ranging between 9.31 ± 0.15 and 9.76 ± 0.41%. Group 1a (**CF**) had a mean nitrogen content of 10.25 ± 0.03%, significantly higher than group 1b (**CF**), group 2 (**AW**) and group 3 (**AW + 10 mg L^{-1} C**).

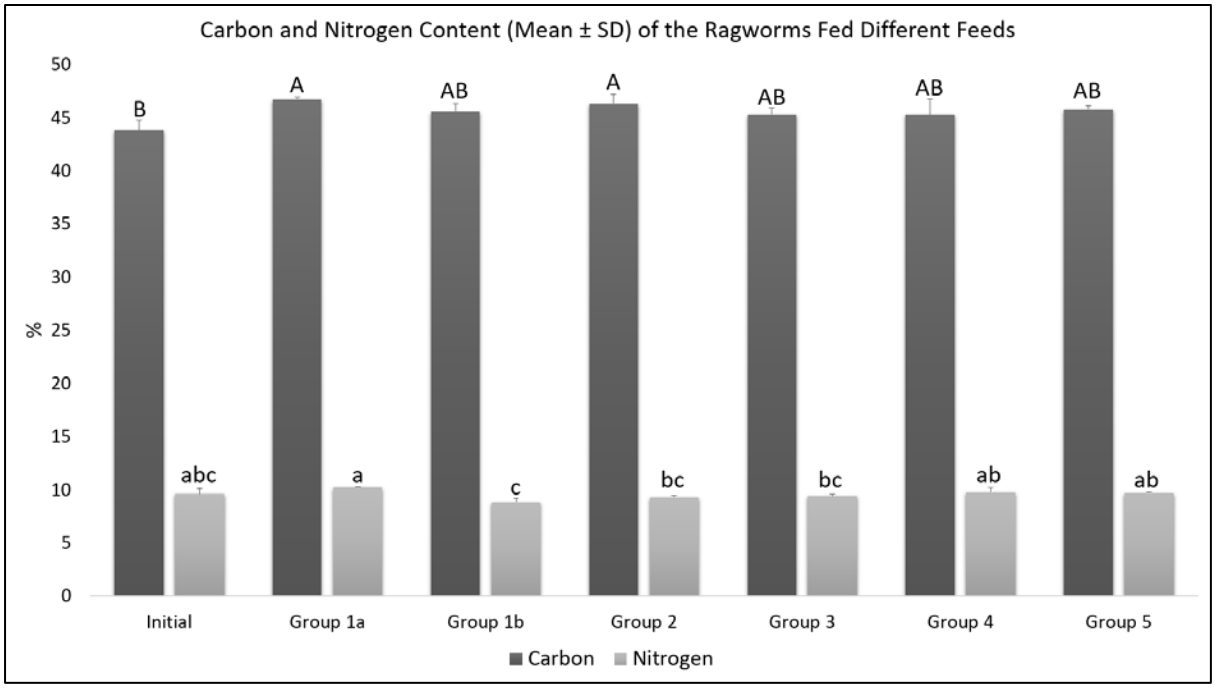

Figure 4.11 – Percentage carbon and nitrogen contents of ragworms fed different diets (Mean ± SD) Initial = ragworms frozen and stored after acclimation period; group 1a = ragworms fed **CF**; group 1b = ragworms fed CF; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed AW + **20 mg L^{-1} chitosan**; group 5 = ragworms **fed AW + 30 mg L^{-1} chitosan**. Different uppercase letters indicate significant differences in carbon percentages between groups. Different lowercase letters indicate significant differences in nitrogen percentages between groups (P > 0.05).

In ragworms fed **AW** or **AW + chitosan** (groups 2, 3, 4 and 5), the carbon and nitrogen contents were not significantly different, suggesting the presence of chitosan has not impacted these parameters.

4.3.2.5 Protein Contents

Figure 4.12 displays the protein content (mean ± SD) of ragworms fed different feeds after the experiment, measured in mg g^{-1}. All groups, except for group 1b (**CF**), had mean protein contents higher than the initial ragworms. Group 1a (**CF**) had a higher value (640.80 ± 2.09 mg g^{-1}) than all other groups (range = 552.04 ± 23.59 to 609.85 ± 25.84 mg g^{-1}), and group 1b (**CF**) had a lower value (552.04 ± 23.59 mg g^{-1}) than all other groups (range = 582.13 ± 9.21 to 609.85 ± 25.84 mg g^{-1}). Groups 2, 3, 4 and 5 protein contents were similar and did not differ significantly. The initial ragworms and group 2 (**AW**) protein contents were similar but lower than other groups (except group 1b).

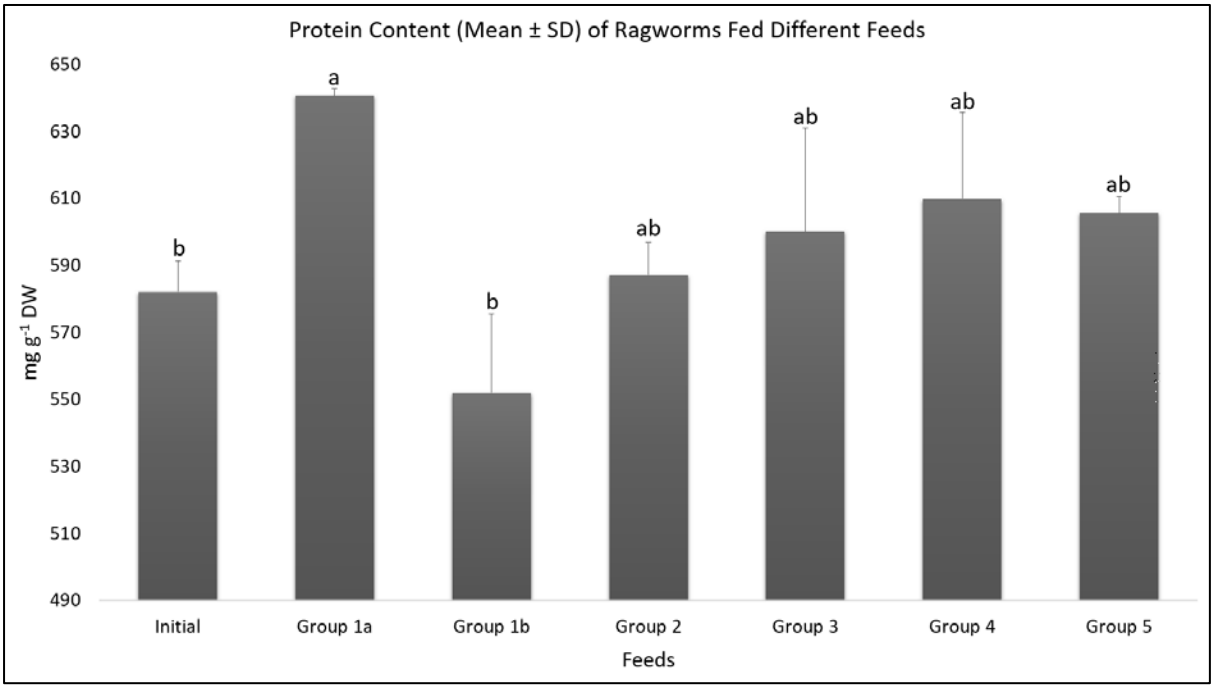

Figure 4.12 – Protein contents of the ragworms fed different feeds after the experiment (mean ± SD) Initial = ragworms frozen and stored after acclimation period; group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**. Different lowercase letters indicate significant differences between groups ($P > 0.05$).

Group 1a (ragworms fed **CF**) had the highest protein content, indicating that this feed type is highly effective in promoting protein assimilation. The initial group and Group 1b (another set of ragworms fed **CF**) had the lowest protein contents, suggesting that there may be inconsistencies in protein assimilation with the same feed type. However, this is likely related to the situation described in Section 4.2.7 – A Problem with Group 1). Groups 2, 3, 4, and 5 (ragworms fed **AW** with or without chitosan) had moderate protein contents, indicating that **AW** with or without chitosan supports reasonable protein assimilation.

These results suggest that while **CF** (Group 1a) is highly effective for protein assimilation, there can be variability even with the same feed (Group 1b). **AW**, with or without chitosan, supports moderate protein assimilation, making it a viable alternative feed for ragworms before their inclusion as an ingredient in aquafeeds, though not reaching the protein levels achieved with the **CF** in Group 1a.

4.3.2.6 Amino Acid Profiles

Figure 4.13 illustrates the amino acid composition (% of total amino acids) of ragworms fed the different feeds. The initial group had a significantly lower total mean AA concentration than all other groups (429.75 ± 1.79 mg g^{-1}). Moreover, group 1b (**CF**) and group 4 (**AW + 20 mg L^{-1} C**) total mean AA concentrations of 494.56 ± 1.90 and 495.66 ± 1.02 mg g^{-1} were significantly lower than groups 1a, 2, 3 and 5, which ranged between 511.45 ± 2.03 and 524.10 ± 2.95 mg g^{-1}.

Regarding mean total EAA concentrations, group 5 (**AW + 30 mg L^{-1} C**) had a statistically significantly higher mean concentration than all other groups (277.70 ± 0.12 mg g^{-1}). Group 2 (**AW**), group 3 (**AW + 10 mg L^{-1} C**) and group 4 (**AW + 20 mg L^{-1} C**) were not significantly different, ranging between 262.47 ± 1.69 and 271.53 ± 0.71 mg g^{-1}. However, it is important to note the standard deviations for the amino acid results are small. Group 1a (**CF**), group 1b (**CF**), and group 4 (**AW + 20 mg L^{-1} C**) were not significantly different, with values ranging between 253.07 ± 0.36 and 262.47 ± 1.69 mg g^{-1}. Initial ragworms were significantly lower than all other groups (243.95 ± 0.16 mg g^{-1}).

Ragworms in all groups contained all of the dietary EAA for fish and shrimp (THR, LEU, MET, LYS, ARG, VAL, ILEU, HIS, PHE) (154), except for tryptophan (TRY), which was not able to be identified using the chosen method. TRY is damaged by hydrolysis and becomes undetectable using HPLC. The EAA with the highest percentage proportion varied between groups. In the initial group, it was ARG (≈9.76%). In group 1a (**CF**), group 3 (**AW + 10 mg L^{-1} C**) and group 4 (**AW + 20 mg L^{-1} C**), it was LEU (≈8.72, ≈9.75 and ≈9.39%, respectively). In group 1b (**CF**), group 2 (**AW**) and group 5 (**AW + 30 mg L^{-1} C**), it was LYS (≈9.49, ≈9.10 and ≈9.89%, respectively). In all groups, the most prominent EAAs were ARG (6.76 – 9.76%), VAL (5.98 – 7.25%), ILEU (≈5.77 – ≈6.98%), LEU (≈8.72 – ≈9.75%) and LYS (≈7.59 – ≈9.89%). Less prominent EAA were THR (≈2.81 – ≈5.22 %), HIS (≈2.33 – ≈2.63%), MET (≈2.38 – ≈2.76%) and PHE (≈4.48 – ≈5.14%).

For the mean total NEAA concentrations, initial ragworms were significantly lower than all other groups (185.80 ± 1.63 mg g^{-1}). Group 1a (**CF**) and group 2 (**AW**) had the highest mean concentrations and were significantly higher than all other groups (255.32 ± 1.89 and 260.61 ± 2.86 mg g^{-1}, respectively). Group 1b (**CF**), group 3 (**AW + 10 mg L^{-1} C**), group 4 (**AW + 20 mg L^{-1} C**), and group 5 (**AW + 30 mg L^{-1} C**) concentrations were not significantly different, lower

than groups 1a and 2, and higher than the initial group with values between 233.18 ± 0.67 and 241.49 ± 1.53 mg g^{-1}.

The NEAA detected in the ragworm samples were ASP, GLU, SER, GLY, ALA, TYR and ORN. The most prominent of all groups was GLU ranging between ≈13.03 and ≈16.48%. ALA was also prominent in all groups (≈8.42 – ≈12.04%), except in the initial group (≈6.23%). Followed by ASP (≈5.27 – ≈9.73%), GLY (≈5.69 – ≈8.90%), TYR (≈4.17 – ≈5.03%), SER (≈3.15 – ≈4.38%) and ORN (≈0.05 – ≈0.84%).

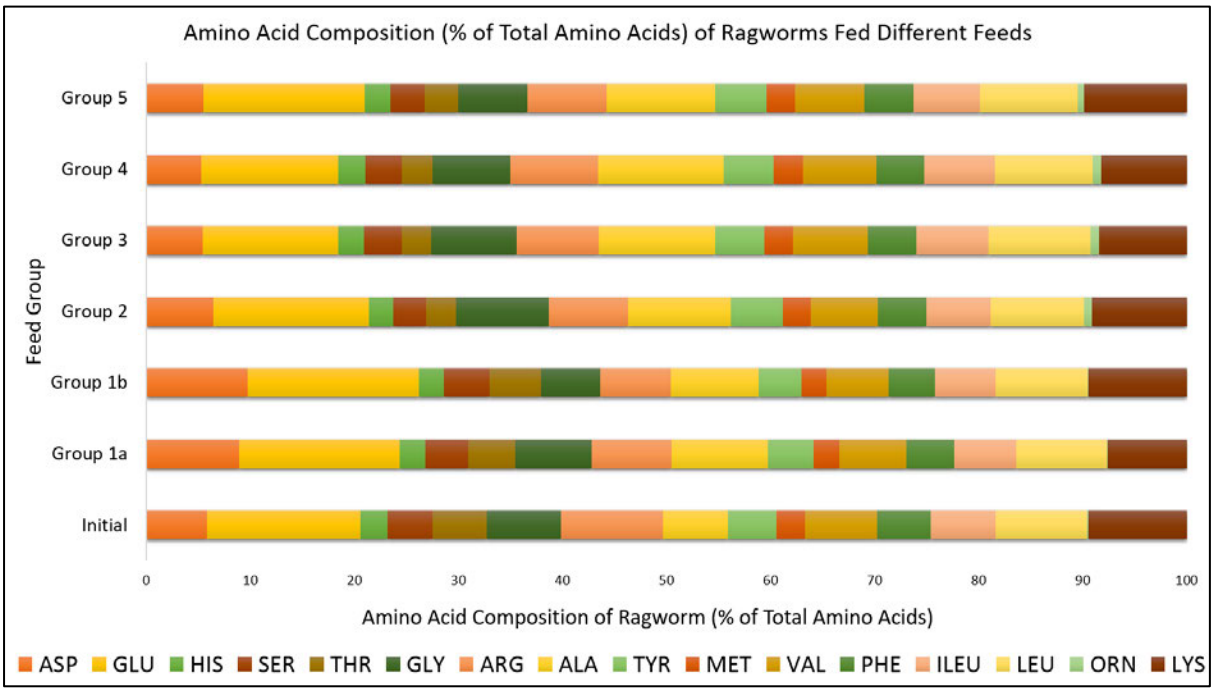

Figure 4.13 – Amino acid composition of the ragworms fed different feeds (% of total amino acids) **Initial** = ragworms frozen and stored after acclimation period; **group 1a** = ragworms fed CF; **group 1b** = ragworms fed CF; **group 2** = ragworms fed AW; **group 3** = ragworms fed AW + 10 mg L^{-1} chitosan; **group 4** = ragworms fed AW + 20 mg L^{-1} chitosan; **group 5** = ragworms fed AW + 30 mg L^{-1} chitosan. ASP = aspartic acid; GLU = glutamic acid; HIS = histidine; SER = serine; THR = threonine; GLY = glycine; ARG = arginine; ALA = alanine; TYR = tyrosine; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; ORN = ornithine; LYS = lysine.

These results suggest that ragworms can maintain a stable amino acid profile regardless of dietary variations, making them versatile in terms of their nutritional intake from different feed sources before their utilisation as an ingredient in aquafeeds.

4.3.2.7 Lipid Contents

Figure 4.14 displays the lipid concentrations (mean ± SD) of ragworms fed different feeds, measured in mg g^{-1}. all groups had higher lipid concentrations than the initial ragworms. Perhaps, 5 days of acclimation before being put into frozen storage and fed with CF was not enough time for the initial ragworms to build up fat reserves. Furthermore, only group 1b (**CF**) was significantly higher. There were no significant differences between ragworms fed **CF**, **AW,** or **AW + chitosan**. Group 1b (**CF**) had a mean lipid concentration of 100.9 ± 0.57 mg g^{-1}, which was higher than ragworms fed with **AW** or **AW + chitosan** which ranged between 54.1 ± 5.8 and 81.04 ± 6.08 mg g^{-1}.

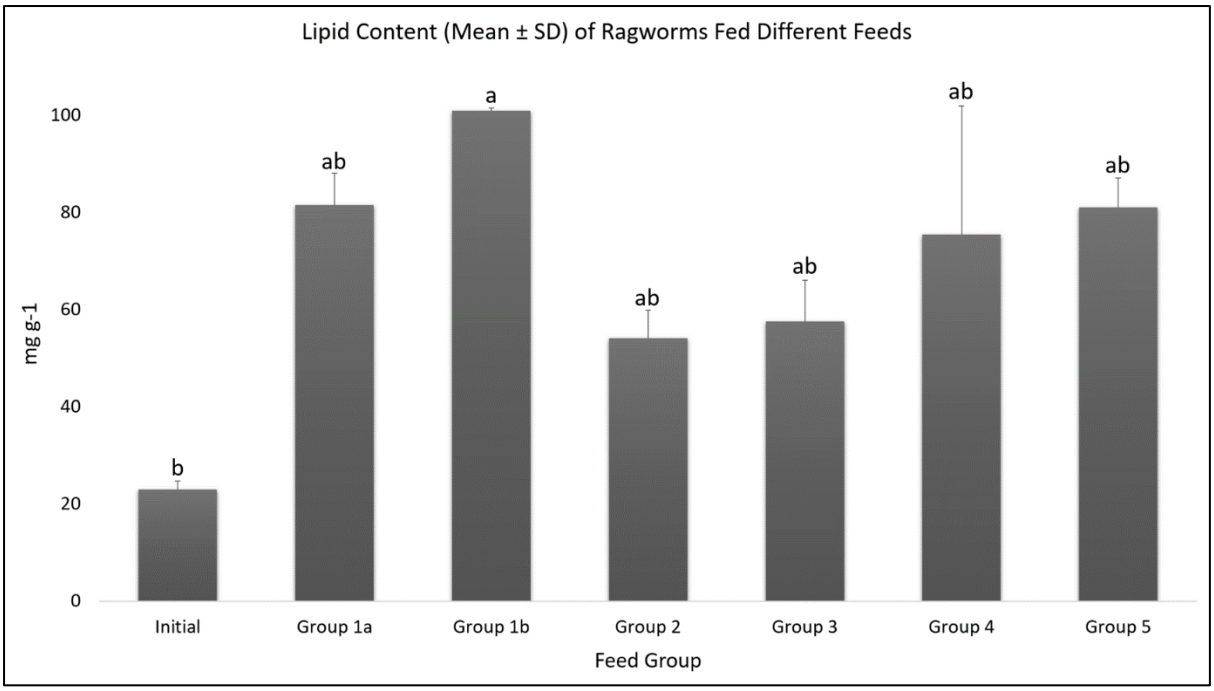

Figure 4.14 – Lipid concentrations (mean ± SD) of the ragworms fed different feeds
Initial = ragworms frozen and stored after acclimation period; **group 1a** = ragworms fed CF; **group 1b** = ragworms fed CF; **group 2** = ragworms fed AW; **group 3** = ragworms fed AW + 10 mg L^{-1} chitosan; **group 4** = ragworms fed AW + 20 mg L^{-1} chitosan; **group 5** = ragworms fed AW + 30 mg L^{-1} chitosan. Different lowercase letters indicate significant differences between groups ($P > 0.05$).

These results suggest that **CF** promotes higher lipid accumulation in ragworms, as seen in Group 1b. The initial group has the lowest lipid levels, showing the starting point before the feeding trials. **AW**, with or without chitosan, supports moderate lipid levels, indicating its viability as a feeding source for ragworms before their inclusion in aquafeeds. The variations

in lipid content among the groups highlight the impact of different feed types on lipid accumulation in ragworms.

4.3.2.8 Fatty Acid Profiles

Table 4.2 provides the mean ± SD fatty acid composition (also expresses them as a percentage of total fatty acids) of the ragworms. The total FA content increased in all groups compared to the initial group, but the percentage of those FA that was PUFA decreased. Group 1b (**CF**) had a significantly higher total FA concentration (74.49 ± 5.34 mg g^{-1}) compared to the initial and **AW** feed groups (10.02 ± 4.59 – 31.48 ± 15.66 mg g^{-1}), except for group 5 (**AW + 30 mg L^{-1} C**) (44.75 ± 10.75 mg g^{-1}). Group 1b (**CF**) also had a significantly higher PUFA concentration than all other groups (26.84 ± 7.61 mg g^{-1} compared to 4.32 ± 1.98 – 12.85 ± 0.91 mg g^{-1}) and a higher percentage proportion (≈36.03% compared to ≈18.23 – ≈31.14%), except for the initial group (43.12%). Similarly, group 1b (**CF**) also had a significantly higher concentration of MUFA compared to all other groups (27.35 ± 8.17 mg g^{-1} compared to 2.87 ± 1.31 – 15.65 ± 1.39 mg g^{-1}); however, group 2 (**AW**) had a slightly higher percentage proportion of MUFA than other groups (≈38.01% compared to ≈28.64 – ≈36.71%). All groups had a significantly higher concentration of SFA (9.54 ± 4.75 – 23.31 ± 2.06 mg g^{-1}) compared to the initial group (2.83 ± 1.30 mg g^{-1}). Group 5 (**AW + 30 mg L^{-1} C**) had the highest percentage proportion of SFA at ≈51.62% compared to between ≈27.26 and ≈44.99% in the other groups.

Moreover, the FA with the highest percentage proportion varied between groups. In group 1a (**CF**), group 3 (**AW + 10 mg L^{-1} C**), group 4 (**AW + 20 mg L^{-1} C**) and group 5 (**AW + 30 mg L^{-1} C**), the FA with the highest percentage proportion was C16:0, with values of ≈28.04, ≈ 23.35, 25.14 and 34.06%, respectively. In group 1b (**CF**) and group 2 (**AW**), the FA with the highest percentage was C18:1, with values of ≈26.70 and ≈25.25%, respectively. In the initial group, the FA with the highest percentage was C18:2 (≈25.08%).

Regarding the essential omega-3 FA for aquafeeds EPA and DHA, all groups displayed a higher percentage proportion than the initial group. In the initial group, EPA had an average percentage proportion of ≈4.03%; in the other groups, it ranged between ≈4.39 and ≈9.29%. DHA had an average percentage proportion of ≈0.61% in the initial group and between ≈0.64 and 5.33% in the other groups.

Table 4.2 – Means ± SD (% of total fatty acids) fatty acid composition of ragworms fed different feeds

	Feed Group						
	Initial	**1a**	**1b**	**2**	**3**	**4**	**5**
	CF	**CF**	**CF**	**AW**	**AW + 10 mg L^{-1} C**	**AW + 20 mg L^{-1} C**	**AW + 30 mg L^{-1} C**
Σ FA (mg g^{-1}) DW	10.02 ± 4.59^{c}	51.82 ± 4.36^{ab}	74.49 ± 5.34^{a}	31.48 ± 15.66^{bc}	27.66 ± 2.40^{bc}	25.74 ± 12.53^{bc}	44.75 ± 10.78^{abc}
FA	**FA Mean ± SD mg g^{-1} (% of Total FA)**						
C8:0	0.001 ± 0.00^{a} (≈0.01%)	0.01 ± 0.00^{a} (≈0.02%)	0.01 ± 0.02^{a} (≈0.02%)	0.004 ± 0.00^{a} (≈0.01%)	-	-	-
C10:0	0.003 ± 0.00^{a} (≈0.03%)	0.02 ± 0.00^{a} (≈0.04%)	0.04 ± 0.04^{a} (≈0.06%)	0.07 ± 0.04^{a} (≈0.23%)	0.04 ± 0.00^{a} (≈0.14%)	0.05 ± 0.03^{a} (≈0.21%)	-
C11:0	-	-	0.03 ± 0.03^{a} (≈0.04%)	0.08 ± 0.04^{a} (≈0.25%)	0.02 ± 0.00^{a} (≈0.07%)	0.02 ± 0.01^{a} (≈0.07%)	0.01 ± 0.00^{a} (≈0.03%)
C12:0	0.01 ± 0.00^{b} (≈0.08%)	0.07 ± 0.01^{b} (≈0.13%)	0.09 ± 0.04^{ab} (≈0.13%)	0.39 ± 0.20^{a} (≈1.25%)	0.16 ± 0.01^{ab} (≈0.56%)	0.13 ± 0.06^{ab} (≈0.50%)	0.09 ± 0.02^{ab} (≈0.20%)
C13:0	-	0.01 ± 0.00^{a} (≈0.02%)	0.04 ± 0.03^{a} (≈0.06%)	0.06 ± 0.03^{a} (≈0.19%)	0.03 ± 0.00^{a} (≈0.11%)	0.03 ± 0.02^{a} (≈0.13%)	0.02 ± 0.01^{a} (≈0.05%)
C14:0	0.11 ± 0.05^{b} (≈1.15%)	1.79 ± 0.16^{a} (≈3.45%)	1.34 ± 0.38^{a} (≈1.80%)	0.63 ± 0.31^{ab} (≈1.99%)	1.38 ± 0.12^{a} (≈4.98%)	1.06 ± 0.52^{ab} (≈4.11%)	1.35 ± 0.33^{a} (≈3.02%)
C15:0	0.13 ± 0.06^{b} (≈1.27%)	0.39 ± 0.03^{b} (≈0.75%)	0.95 ± 0.12^{a} (≈1.27%)	0.28 ± 0.14^{b} (≈0.89%)	0.45 ± 0.04^{b} (≈1.62%)	0.31 ± 0.15^{b} (≈1.20%)	0.38 ± 0.09^{b} (≈0.84%)
C16:0	1.80 ± 0.82^{c} (≈17.94%)	14.53 ± 1.29^{a} (≈28.04%)	12.33 ± 2.38^{ab} (≈16.55%)	4.93 ± 2.45^{bc} (≈15.67%)	6.46 ± 0.56^{abc} (≈23.35%)	6.47 ± 3.15^{abc} (≈25.14%)	15.24 ± 3.67^{a} (≈34.06%)
C17:0	0.16 ± 0.08^{c} (≈1.65%)	0.82 ± 0.07^{ab} (≈1.58%)	1.00 ± 0.13^{a} (≈1.34%)	0.35 ± 0.17^{bc} (≈1.11%)	0.41 ± 0.04^{bc} (≈1.47%)	0.33 ± 0.16^{bc} (≈1.29%)	0.63 ± 0.15^{abc} (≈1.41%)
C18:0	0.60 ± 0.27^{b} (≈5.97%)	5.59 ± 0.49^{a} (≈10.79%)	4.26 ± 0.33^{a} (≈5.72%)	2.66 ± 1.32^{ab} (≈8.45%)	2.24 ± 0.19^{ab} (≈8.10%)	2.38 ± 1.16^{ab} (≈9.26%)	5.28 ± 1.27^{a} (≈11.81%)

C20:0	0.01 ± 0.01^{b} (≈0.14%)	0.09 ± 0.01^{ab} (≈0.18%)	0.15 ± 0.01^{a} (≈0.20%)	0.08 ± 0.04^{ab} (≈0.27%)	0.06 ± 0.00^{b} (≈0.20%)	0.07 ± 0.03^{ab} (≈0.25%)	0.09 ± 0.02^{ab} (≈0.20%)
C22:0	-	-	0.06 ± 0.02 (≈0.08%)	-	-	-	-
Σ SFA	**2.83 ± 1.30^{b} (≈28.24%)**	**23.31 ± 2.06^{a} (≈44.99%)**	**20.31 ± 3.53^{a} (≈27.26%)**	**9.54 ± 4.75^{ab} (≈30.30%)**	**11.23 ± 0.97^{ab} (≈40.61%)**	**10.85 ± 5.28^{ab} (≈42.16%)**	**23.10 ± 5.57^{a} (≈51.63%)**
C14:1 (ω -5)	-	0.01 ± 0.00^{a} (≈0.02%)	0.07 ± 0.06^{a} (≈0.10%)	0.02 ± 0.01^{a} (≈0.08%)	0.04 ± 0.00^{a} (≈0.13%)	0.02 ± 0.01^{a} (≈0.09%)	0.01 ± 0.00^{a} (≈0.01%)
C16:1 (ω – 7)	0.23 ± 0.11^{b} (≈2.30%)	2.45 ± 0.22^{a} (≈4.72%)	2.74 ± 0.25^{a} (≈3.68%)	1.57 ± 0.78^{ab} (≈4.98%)	1.87 ± 0.16^{ab} (≈6.77%)	1.54 ± 0.75^{ab} (≈5.98%)	1.48 ± 0.36^{ab} (≈3.31%)
C17:1 (ω -7)	0.05 ± 0.02^{a} (≈0.52%)	0.11 ± 0.01^{a} (≈0.22%)	0.29 ± 0.34^{a} (≈0.39%)	0.22 ± 0.11^{a} (≈0.68%)	0.06 ± 0.01^{a} (≈0.22%)	0.05 ± 0.02^{a} (≈0.20%)	0.06 ± 0.01^{a} (≈0.13%)
C18:1 (ω – 9)	2.18 ± 1.00^{a} (≈21.73%)	8.87 ± 0.79^{a} (≈17.12%)	19.89 ± 7.26^{a} (≈26.70%)	7.63 ± 3.80^{a} (≈25.25%)	4.45 ± 0.39^{a} (≈16.07%)	4.09 ± 1.99^{a} (≈15.89%)	7.88 ± 1.90^{a} (≈17.62%)
C20:1 (ω – 9)	0.35 ± 0.16^{b} (≈3.47%)	3.75 ± 0.33^{a} (≈7.24%)	3.84 ± 0.16^{a} (≈5.16%)	1.95 ± 0.97^{ab} (≈6.18%)	1.81 ± 0.16^{ab} (≈6.53%)	1.96 ± 0.96^{ab} (≈7.62%)	3.54 ± 0.85^{a} (≈7.92%)
C22:1 (ω – 9)	0.06 ± 0.03^{a} (≈0.62%)	0.45 ± 0.04^{a} (≈0.87%)	0.50 ± 0.10^{a} (≈0.68%)	0.58 ± 0.29^{a} (≈1.83%)	0.41 ± 0.04^{a} (≈1.49%)	0.58 ± 0.28^{a} (≈2.23%)	0.50 ± 0.12^{a} (≈1.13%)
C24:1 (ω -9)	-	0.01 ± 0.00^{a} (≈0.02%)	-	-	0.01 ± 0.00^{a} (≈0.05%)	0.02 ± 0.01^{a} (≈0.07%)	0.02 ± 0.00^{a} (≈0.04%)
Σ MUFA	**2.87 ± 1.31^{b} (≈28.64%)**	**15.65 ± 1.39^{ab} (≈30.21%)**	**27.35 ± 8.17^{a} (≈36.71%)**	**11.96 ± 5.95^{b} (≈38.01%)**	**8.65 ± 0.75^{b} (≈31.26%)**	**8.26 ± 4.02^{b} (≈32.09%)**	**13.49 ± 3.25^{ab} (≈30.14%)**
C18:2 (ω – 6)	2.51 ± 1.15^{a} (≈25.08%)	2.66 ± 0.24^{a} (≈5.14%)	9.72 ± 6.36^{a} (≈13.05%)	4.19 ± 2.09^{a} (≈13.32%)	1.90 ± 0.16^{a} (≈6.88%)	1.34 ± 0.65^{a} (≈5.20%)	1.86 ± 0.45^{a} (≈4.15%)
C20:2 (ω – 6)	0.53 ± 0.24^{b} (≈5.32%)	1.54 ± 0.14^{b} (≈2.97%)	5.43 ± 0.08^{a} (≈7.29%)	1.32 ± 0.66^{b} (≈4.20%)	0.71 ± 0.06^{b} (≈2.58%)	0.60 ± 0.29^{b} (≈2.31%)	1.07 ± 0.26^{b} (≈2.39%)
C20:3 (ω – 3)	0.37 ± 0.17^{b} (≈3.68%)	1.18 ± 0.10^{b} (≈2.28%)	2.73 ± 0.20^{a} (≈3.66%)	0.91 ± 0.46^{b} (≈2.91%)	0.61 ± 0.05^{b} (≈2.20%)	0.57 ± 0.28^{b} (≈2.21%)	0.88 ± 0.21^{b} (≈1.97%)

C20:4 (ω – 6)	0.24 ± 0.11[b] (≈2.36%)	0.71 ± 0.06[b] (≈1.38%)	2.08 ± 0.19[a] (≈2.79%)	0.54 ± 0.27[b] (≈1.71%)	0.40 ± 0.03[b] (≈1.46%)	0.40 ± 0.19[b] (≈1.54%)	0.52 ± 0.12[b] (≈1.15%)
C20:5 (EPA) (ω – 3)	0.40 ± 0.19[b] (≈4.03%)	2.80 ± 0.25[ab] (≈5.41%)	3.27 ± 0.51[a] (≈4.39%)	1.69 ± 0.84[ab] (≈5.48%)	2.74 ± 0.24[ab] (≈9.90%)	2.39 ± 1.16[ab] (≈9.29%)	2.21 ± 0.53[ab] (≈4.95%)
C22:2 (ω – 6)	0.13 ± 0.06[c] (≈1.26%)	0.68 ± 0.06[ab] (≈1.32%)	0.97 ± 0.05[a] (≈1.30%)	0.48 ± 0.24[abc] (≈1.51%)	0.28 ± 0.02[bc] (≈1.02%)	0.41 ± 0.20[bc] (≈1.57%)	0.62 ± 0.15[abc] (≈1.38%)
C22:4 (ω – 6)	0.04 ± 0.02[b] (≈0.45%)	0.14 ± 0.01[b] (≈0.28%)	0.48 ± 0.05[a] (≈0.65%)	0.13 ± 0.06[b] (≈0.41%)	0.10 ± 0.01[b] (≈0.37%)	0.10 ± 0.05[b] (≈0.39%)	0.13 ± 0.03[b] (≈0.30%)
C22:5 (ω – 3)	0.03 ± 0.02[c] (≈0.33%)	0.36 ± 0.03[ab] (≈0.70%)	0.23 ± 0.04[bc] (≈0.31%)	0.18 ± 0.09[bc] (≈0.56%)	0.57 ± 0.05[a] (≈2.06%)	0.44 ± 0.21[a] (≈1.70%)	0.58 ± 0.14[a] (≈1.29%)
C22:6 (DHA) (ω – 3)	0.06 ± 0.03[a] (≈0.61%)	2.76 ± 0.02[a] (≈5.33%)	1.91 ± 0.09[a] (≈2.56%)	0.54 ± 0.27[a] (≈1.71%)	0.46 ± 0.04[a] (≈1.67%)	0.39 ± 0.19[a] (≈1.52%)	0.29 ± 0.07[a] (≈0.64%)
Σ PUFA	**4.32 ± 1.98[b] (≈43.12%)**	**12.85 ± 0.91[b] (≈24.80%)**	**26.84 ± 7.61[a] (≈36.03%)**	**9.80 ± 4.88[b] (≈31.14%)**	**7.78 ± 0.67[b] (≈28.13%)**	**6.63 ± 3.23[b] (≈25.74%)**	**8.16 ± 1.97[b] (≈18.23%)**

Different superscript letters indicate significant differences in individual FA between feeds, with a to c indicating descending concentration ($p < 0.05$). Initial = ragworms frozen and stored after acclimation period; group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1}** chitosan. **SFA** = saturated fatty acids; **MUFA** = monounsaturated fatty acids; **PUFA** = polyunsaturated fatty acids. **Σ** = Total. **ω - n** = omega – position of first double bond from the terminal methyl group of chemical structure. **Cn:n** = number of carbons:number of double bonds.

CF tends to increase total fatty acids, especially saturated and monounsaturated fats while maintaining a high proportion of PUFAs. On the other hand, **AW,** and **AW + chitosan** shows varying effects on the fatty acid composition. The presence of chitosan in **AW** affects the distribution of fatty acids, with specific concentrations showing different impacts The ragworms fed **AW**-derived feeds had much lower PUFA contents, which reflects what was observed in their feed (Section 4.3.1.5 – Lipid Contents).

4.4 Discussion

4.4.1 Growth

The growth results (Section 4.3.2.1 - Growth) show that ragworms grew in all tested scenarios, responding well to the **CF** and **AW** feeds with different chitosan concentrations, as reflected by the positive SGRs. The best results were obtained for **CF**, which as previously explained is due to this feed being optimal for small marine organisms. The poorest results were obtained for chitosan concentrations of **30 mg L^{-1}** (group 5) showing a detrimental influence on ragworm growth.

Except for group 5 (**AW + 30 mg L^{-1} C**), the SGR of ragworms fed **AW** or **AW + chitosan** in the experiment varied from 0.014 to 0.028 d^{-1}. In comparison with similar research, ragworms fed Atlantic salmon smolt (*Salmo* salar) **AW** had an average SGR of 0.012 d^{-1} (Wang *et al.*, 2019), ragworms fed Beluga sturgeon (*Huso huso*) **AW** had an average SGR of 0.03 d^{-1} (O Pajand *et al.*, 2017). Ragworms fed European bass (*Dicentrarchus labrax*) and gilt-head sea bream (*Sparus aurata*) **AW** had SGR up to 0.02 d^{-1} (Bischoff, 2007). Therefore, the results of this experiment were hence similar to, or higher than the results from the research referenced.

To determine whether chitosan was the sole reason for reduced growth rates in group 5 (**AW + 30 mg L^{-1} C**), it is necessary to consider other factors which impact growth rates.

One factor is food quality. When comparing group 1b (**CF**) to those fed **AW** feeds (groups 2, 3, 4 and 5), the growth was higher (0.047 d^{-1} compared to 0.005 – 0.028 d^{-1}). Therefore, this shows that **CF** is a superior feed to **AW** and confirms that food quality plays a significant role in growth rates (Nielsen *et al.*, 1995). In addition, due to the **CF** being in pellet form and specifically formulated, the ragworms could eat less and obtain the same quantity of nutrients as ragworms fed **AW** or **AW + chitosan** feeds, therefore using less energy in the process. The **CF** being of better quality to the **AW** is confirmed by far higher total organic matter content, higher carbon percentage and better fatty acid profile, discussed in greater detail in subsequent sections.

Moreover, food quantity can also impact the growth rates of ragworms (Nielsen *et al.*, 1995). For example, food quantity can indirectly affect growth rates by the unintended deterioration of water quality, as seen with group 1a (**CF**), which had an SGR of 0.010 d^{-1}. In addition,

logically thinking, a quantity of feed that is too low to sustain a ragworm will lower growth rates too. However, except for group 5 (**AW + 30 mg L^{-1} C**), the other groups did not have affected growth, pH, or turbidity values. Group 5 had the lowest growth rate; this was likely due to the presence of chitosan and not a lack of feed. In addition, group 5 did not have affected pH or turbidity values like group 1a. Therefore, except for group 1a (**CF**), food quantity was not a factor that directly or indirectly affected ragworm growth.

Furthermore, food availability is another parameter that can affect the growth rates of ragworms (Scaps, 2002). During the experiment, the ragworms would protrude their heads out of their burrows, move around near them, and immediately ingest feeds after they had been added. This shows the ragworms were hungry at feeding times. This is evidenced by video recordings, photographs (Figure 4.15) and personal observations. The feeds were also dispersed evenly throughout the culture beakers. Very rarely (on one or two occasions in 28 days) a ragworm was observed entirely out of its burrow and actively scavenging on top of the sediment surface.

The favoured feeding behaviour that is frequently seen in ragworms is surface-deposit feeding (Aberson, Bolam and Hughes, 2016), which in turn determines territory size. Ragworms are naturally territorial, with a territorial area of around 4 cm^2 (Reise, 1979; Scaps, 2002). Ragworm size determines the territory size because ragworms often do not exit their burrow to look for food but instead maintain a good amount of their posterior body in their burrow to be ready for fast retraction when disturbed (Wang *et al.*, 2019). This was observed (not during the experimental period) by waving a hand over a culture beaker when ragworms were partly out of their burrow to mimic a predatory situation. The ragworms would instantly retract into their burrow. In this experiment, the stocking density of the culture beakers (0.12 ind cm^2) was low compared with previous research (Nesto *et al.*, 2012; Jerónimo *et al.*, 2021). Therefore, food availability and accessibility likely did not affect growth. Research has shown that high stocking densities can obtain a higher biomass production (Rasines, Martín and Aguado-Giménez, 2023), however, this research aimed to observe chitosan impact upon growth, mortality and the nutritional composition, not optimal biomass production for commercial reasons.

Figure 4.15 – Ragworms from group 5 protruded from their burrows to consume aquaculture waste feed

Also, it is necessary to consider internal and external environmental factors potentially affecting ragworm growth. The ragworms for this study were obtained in summer, before autumn, when maturation processes typically begin (Kristensen, 1984). Due to seasonal variations in day length, food availability, temperature and internal resource allocation, maturation co-occurs when growth rates start to decrease (Wang *et al.*, 2019). Ragworms have been seen to alternate between biomass growth and the formation of gametes in response to artificially manipulated day durations (Last and Olive, 1999). Higher feeding rates and ragworm growth correlate with higher culture temperatures and a more extended photoperiod (Arias and Drake, 1995; Olivier *et al.*, 1997; Last and Olive, 1999). As this experiment was carried out in June, and photoperiod, salinity and temperature conditions were kept constant to replicate summertime settings, sexual maturation was not likely to have been a factor that affected the growth of the ragworms. Also, the ragworms did not display signs of sexual maturation, such as colour change (Budd, 2020).

Additionally, SGR of around 0.06 – 0.07 d^{-1} has only been documented in juvenile ragworms fed an optimal diet with very low starting weights, and as animals get larger, growth rates typically decline (Heip and Herman, 1979; Nesto *et al.*, 2012).

In addition, in group 1a, where feeding 1 g of **CF** caused the quality of the culture water to deteriorate (Section 4.2.7 – A Problem with Group 1), mucus was attached and stretched across the glass walls of the culture beaker. Mucus production is an example of the ragworms

switching from surface-deposit feeding to a filter-feeding mechanism. Harley (1950) was the first to describe the existence of ragworms filter-feeding, and a study by Riisgard (1994) confirmed a similar attachment of mucus threads to the walls of glass culture tubes used in their study to create a mucus net (Harley, 1950; Riisgård, 1994). The ragworms then force water through the net by manipulating their bodies. The mucus net catches any suspended particles in the water, which are subsequently ingested along with the mucus net (Riisgård, 1994). However, when ragworms switch to filter feeding, there is an energy cost due to the body's movements to pump water through the net (Riisgård, 1994; Bischoff, 2007). Therefore, the increased energy cost of the filter-feeding mechanism may be why lower growth rates were observed in group 1a compared with groups 1b, 2, 3 and 4.

Perhaps due to the high concentration of dissolved CF in the culture water, the group 1a ragworms thought there were suspended particles in the water and switched to the filter-feeding mechanism. In the wild, filter-feeding in ragworms is relatively common and initiated when a high phytoplankton concentration is present in the water (Riisgård *et al.*, 1992; Vedel and Riisgard, 1993; Riisgård, 1994; Vedel, Andersen and Riisgard, 1994).

Moreover, as previously stated, growth rates in group 5 (**AW + 30 mg L^{-1} C**) were lower than in all other groups. This group had the highest concentration of chitosan incorporated into the feed at **30 mg L^{-1}**, suggesting chitosan may be a significant factor as to why the growth rates were lower than the other groups fed **AW + chitosan**. All other parameters were the same as other groups. For instance, in auxiliary experiments, the dose of chitosan that is sufficient to flocculate **AW** with high percentage turbidity removal was routinely seen around 10 mg L^{-1}. Therefore, at 30 mg L^{-1}, there can potentially be around 20 mg L^{-1} of 'free' or 'part-free' chitosan. The free chitosan – only partly associated with the feed – could be released from the feed into the water when the feed is placed in water. The free dissolved chitosan may interfere with (i.e., stick to) the mucous-secreting membranes of the ragworm, preventing this function. In the same way that 'free chitosan' will interfere with the membranes of fish gills (Valenzuela *et al.*, 2003). This hypothesis would fit with group 5 having the highest number of mortalities.

4.4.2 Mortality

When comparing the overall mean percentage values displayed in Figure 4.9 (Section 4.3.2.2 – Mortality), it becomes apparent that in groups 1a (**CF**) and 5 (**AW + 30 mg L^{-1} C**), factors not present in other groups resulted in more mortalities (i.e., unknown variables or conditions not included or accounted for in this research). However, it is also important to note that groups 1a and 5 had lower growth rates than other groups, even when mortalities were accounted for with the adapted SGR equation (Section 3.7.2.1 – Adaptation to the Specific Growth Rate Equation).

For group 1a (**CF**), the higher percentage of mortality observed can be attributed to the decrease in water quality as a direct result of adding too much feed, as seen in the pH value (Appendix G) and turbidity of the water (Figure 4.1) (Appendix D). The pH of group 1a culture water had a mean value of ≈7.3. Past research has found that ragworms at a pH range between 7.1 and 7.5 had reduced mean wet weights by 5.5 to 8.1% (Sokołowski, Brulińska and Sokołowska, 2020). The addition of too much feed as the indirect cause of higher mortality in group 1a is confirmed by group 1b (**CF**) results. In group 1b, the rationing of feed was halved and did not affect the culture water pH (Appendix G), turbidity (Appendix D) or mortalities, as zero deaths were recorded in this group.

For group 5 (**AW + 30 mg L^{-1} C**), the feed's only factor different from other groups was the chitosan concentration. Therefore, it may be a reasonable assumption that chitosan in feed at this concentration may be detrimental to ragworms regarding growth rates and mortality rates. For example, in a clinical trial with rats, they displayed no detectable chitosan toxicity effects at doses of 5% in their food. However, at 10%, growth rates decreased, and clinical pathology changes were detected (Landes and Bough, 1976). Chitosan toxicity thresholds in rats may help to explain why lower growth rates and higher mortality values were seen in group 5 ragworms compared to other feed groups with chitosan incorporated into their feed (groups 3 and 4). Groups 3 and 4 (**AW + 10 mg L^{-1} C** and **AW + 20 mg L^{-1} C**) chitosan concentrations could be below the threshold at which growth rates and mortality start to be affected in ragworms. There was no significant difference in the values of growth and mortality from group 2 (**AW**), so chitosan does not seem to have an impact at these concentrations.

In research investigating ragworms fed with Beluga sturgeon (*Huso huso*) **AW** for eight weeks, the mean percentage mortality was 39.6% (O Pajand *et al.*, 2017). In research investigating ragworms fed with Atlantic salmon (*Salmo salar*) smolt **AW** for 30 days, the mean percentage mortality was 53.3% (Seekamp, Reitan and Handå, 2017). Both are much higher than observed in any of the groups in this 28-day study (0 – 23.33%). The lower mortality suggests the experimental set-up and the source of cleaner fish **AW** is a good choice for utilising an **AW** stream to feed to ragworms to create an alternative aquafeed ingredient. Subsequent discussion regarding the cleaner fish **AW**'s nutritional composition confirms whether this is the case. However, in group 5 (**AW + 30 mg L^{-1} C**), the SGR (0.5 % d^{-1}) was lower than those mentioned in the abovementioned studies, which were 3.4% and 1.1 % d^{-1}, respectively. Therefore, despite mortalities being lower in the current study, the lower growth rates in group 5 indicate another factor causing the ragworms not to perform well, most likely chitosan toxicity.

The ragworms were kept in culture beakers at low stocking densities. Pertinent because in the studies mentioned above, the stocking densities were higher, and higher stocking densities are associated with higher polychaete mortality (Nesto *et al.*, 2012).

The build-up of toxins such as ammonia, nitrate, and phosphate as a cause of mortalities can be ruled out as the culture water was changed three times weekly. Furthermore, as part of the process, the culture beakers and sand were rinsed with deionised water and artificial seawater. Therefore, potential contaminants will have been washed out along with ragworm excretions and uneaten feed. In previous research, the build-up of toxins such as ammonia has been determined as a potential reason for high mortalities (Bischoff, 2007; Seekamp, Reitan and Handå, 2017).

Cannibalism has been observed in ragworms (Costa, Oliveira and Da Fonseca, 2006). However, the feed was abundant, and stocking densities and mortalities were lower than in previous studies and the wild (Riisgård, 1994; Batista *et al.*, 2003; Bischoff, 2007; Nesto *et al.*, 2012; Seekamp, Reitan and Handå, 2017). Therefore, cannibalism as a cause of mortality can be ruled out.

It was observed that there were no mortalities in any group in the first 7 days of the experiment. Therefore, stress-related deaths during the transition to the experimental

system may be ruled out. Besides, all ragworms were acclimated to experimental conditions for 5 days before the start of the experiment.

In summary, regarding mortalities, ragworms fed **AW** did not perform as well as ragworms fed **CF**. Ragworms fed with **AW + chitosan** at concentrations of **10 or 20 mg L^{-1}** performed similarly to ragworms fed **AW without chitosan**. However, in ragworms fed with **AW + chitosan** at a concentration of **30 mg L^{-1}**, higher mortalities were recorded. Therefore, it can be suggested that high concentrations of chitosan are detrimental to ragworms in terms of growth and mortality.

4.4.3 Total Organic Matter, Carbon and Nitrogen Contents of Feeds and Ragworms

To better understand the growth and mortality results and partly assess the nutritional composition of the feeds and the ragworms, total organic matter (TOM), carbon, and nitrogen contents were analysed. Briefly, organic matter consists of carbon-containing compounds. Inorganic matter or 'ash' is the proportion that contains inorganic compounds which animals cannot utilise. Therefore, feeds with a higher TOM content are superior as they have a higher carbon content which is required as energy for living organisms to survive. Nitrogen is also crucially important for living organisms as it is a component of amino acids, proteins, and DNA.

The higher TOM content of the **CF** compared with **AW** could be a factor as to why the ragworms in group 1b (**CF**) had the highest growth rate (Section 4.3.2.1 - Growth). For instance, TOM is a significant component of sediment in the wild that serves as a primary food source for the benthic fauna of water bodies (Arfiati *et al.*, 2019). Therefore, feed with a higher TOM content is more beneficial to ragworms.

The TOM content of ragworms is not impacted by the TOM content in the feeds. Furthermore, the presence of chitosan in the feed did not impact the TOM contents. Chitosan is an organic polymer (Teixeira, Santini and Souto, 2017). Therefore, presumably, it would contribute to the TOM content, but its presence in the feeds did not impact the TOM contents of the feeds or the ragworms.

TOM content results for the ragworms are comparable to a study that also found similar TOM contents in ragworms fed different diets (**CF**, Atlantic salmon (*Salmo salar*) smolt **AW** or a shellfish diet), resulting in TOM percentage values between 86.2 ± 2.3 and 89.1 ± 5.7% (Seekamp, Reitan and Handå, 2017). In that same research, the **CF,** and the salmon smolt **AW** values were 89.4 ± 0.3% and 57.3 ± 2.2%, respectively, which are not too dissimilar to what was found in this study. Therefore, the TOM results in both studies suggest that the ragworms regulate their internal inorganic matter.

However, **AW** containing around 40% inorganic matter seems very high. Therefore, to get an idea of the inorganic compounds present in the feeds, an inductively coupled plasma atomic emission spectroscopy (ICP-OES) elemental analysis was carried out for **AW** (Appendix H).

As a result, the following elements were identified in significant quantities: calcium (149.39 ± 10.61 mg kg^{-1}), iron (4.72 ± 0.33 mg kg^{-1}), potassium (5.93 ± 1.27 mg kg^{-1}), magnesium (16.67 ± 3.88 mg kg^{-1}), sodium (124.29 ± 30.14 mg kg^{-1}, phosphorus (67.21 ± 7.72 mg kg^{-1}) and sulphur (46.54 ± 7.58 mg kg^{-1}). In addition, according to the **CF** manufacturer's website (not ICP-OES analysed), trace elements of inorganic manganese (manganese (II) sulphate, monohydrate) (74 mg kg^{-1}), zinc (zinc sulphate, monohydrate) (44 mg kg^{-1}) and iron (iron (II) sulphate, monohydrate) (29 mg kg^{-1}) are present in **CF**.

The inorganic matter content is potentially very important if aquaculture wastewater is required as a feedstock for ragworms. For example, if one tonne of aquaculture waste costs £1000 and has an inorganic matter content of 10%, then almost 100 kg (i.e., £100) is wasted. The economics of such a system is out of the scope of this project, but it is a key area which must be considered in future research.

In ragworms fed **AW** or **AW + chitosan** (groups 2, 3, 4 and 5), the carbon and nitrogen contents were not significantly different, suggesting the presence of chitosan in their feed has not impacted them.

4.4.4 Protein Contents and Amino Acid Profiles of Feeds and Ragworms

Aquafeeds must have a protein content that can satisfy the requirements of the species they are intended to feed. Furthermore, they must have a well-balanced amino acid (AA) profile, as deficiencies can lead to detrimental impacts on fish health (Tacon, 1987).

Finding alternatives to aquafeeds from capture fisheries with a high protein content, which is often the most expensive component of fish meal, has been a focus of study for many years now (Ayadi, Rosentrater and Muthukumarappan, 2012). As the mean protein contents of the **AW** feeds were higher than that of the **CF** feeds, the cleaner fish **AW** used is potentially a good source of protein. Therefore, this waste stream could help to reduce reliance on capture fisheries to produce aquafeed ingredients and help to address the waste management issues facing the aquaculture sector. Additionally, chitosan did not affect the protein contents of the **AW** feeds, as they were statistically similar. Furthermore, the protein contents in ragworms fed **AW**, or **AW + chitosan**, did not differ significantly; therefore, chitosan also did not affect the protein contents of the ragworms.

The protein content agrees with the lower nitrogen content seen in **CF** (group 1b) compared to the **AW** feeds (Section 4.3.1.2 – Carbon and Nitrogen Contents). The lower **CF** protein content makes sense as protein contains nitrogen, in contrast to carbohydrates and fats, which only have carbon, hydrogen, and oxygen, so the lower observed protein content in the **CF** compared to **AW** feeds is logical.

Regarding the protein contents in the different groups of ragworms, all groups, except for group 1b (**CF**), had higher protein contents than the initial ragworms. Therefore, despite all groups of ragworms having a sufficient food supply, ragworms in groups other than 1b (**CF**) perhaps had to use more energy when feeding. As a result, the increased use of muscle to feed builds more muscle, mainly consisting of proteins (Young and Schmidt-Nielsen, 1985). Protein development is most clearly seen in group 1a (**CF**) ragworms that utilised the more energy-intensive feeding mechanism of filter-feeding discussed in Section 4.4.1 – Growth. Research investigating growth rates in ragworms when utilising filter feeding has been lower compared to other feeding modes (Vedel and Riisgard, 1993).

Moreover, regardless of the feed group, the ragworms displayed a high protein content (55 – 64%). The protein contents were similar to 54 – 58% reported in a study using Atlantic salmon (*Salmo salar*) smolt **AW** to feed ragworms (Wang *et al.*, 2019). Furthermore, this study's protein contents were higher than a study feeding Beluga sturgeon (*Huso huso*) **AW** to ragworms, which were around 49% (O Pajand *et al.*, 2017) and another study of ragworms found in the wild, which had protein contents of around 50.3% (Luis and Passos, 1995). Again, this suggests that the cleaner fish **AW** used in this study was a good source of protein, and the presence of chitosan did not negatively impact the protein contents in the ragworms.

Over 30 fish and shrimp species have defined protein requirements, and the findings point to a consistently high dietary protein demand in the range of 24 to 57% (Tacon, 1987). Therefore, the protein contents determined in the ragworms used in this experiment suggest that ragworms fed cleaner fish **AW** can contribute to the protein requirements for many fish species. Furthermore, the inclusion of chitosan into a feed does not negatively impact the protein contents of the ragworms when fed a source of **AW**. Therefore, ragworms fed **AW + chitosan** up to and including concentrations of **30 mg L^{-1}** can satisfy aquafeed protein requirements.

However, the protein quality is not just determined by content. Proteins are made up of smaller units called amino acids (AA), and a favourable AA profile is crucial concerning the needs of the marine species the ragworms are intended to feed (Wang *et al.*, 2019). It is, therefore, also necessary to discuss chitosan's impact on the AA profiles of ragworms.

The AA composition concerning the consumer's needs determines protein quality (Wang *et al.*, 2019). In addition, the protein quality is correlated with the bioavailability and digestibility of AA (Gaudichon and Calvez, 2021). Many studies have demonstrated that plant-derived protein is of lower quality than animal-derived protein in terms of digestibility and bioavailability (Mambrini *et al.*, 1999; Kaushik *et al.*, 2004; Parisi *et al.*, 2004; Espe *et al.*, 2006; Collins *et al.*, 2013; Egerton *et al.*, 2020). Therefore, ragworms (marine animals) are an appropriate alternative aquafeed ingredient as they are a bioavailable protein source.

AA are crucial in fish as significant building blocks of proteins and polypeptides and as regulators of the metabolic pathways determining growth, reproduction and immunity (Li *et al.*, 2009). EAA are AA that must be provided in a ready-made form in the diet since they

cannot be produced by the animal body or produced at a rate fast enough to suit their physiological demands (Tacon, 1987). On the other hand, NEAA are those that animals can produce *de novo* in sufficient proportions to fulfil requirements for maintenance, growth, development and health and, as a result, are not required to be provided in the diet (Hou, Yin and Wu, 2015).

Moreover, the necessity for a well-balanced AA profile in aquafeeds can be demonstrated by deficiency indicators found in experiments with fish-fed feeds lacking in one or more EAA. The following is in addition to the fact that all fish exhibit slower development when fed EAA-deficient diets (Tacon, 1987). In juvenile rainbow trout (*Salmo gairdneri*), LYS deficiency has been reported to cause fin erosion and increased mortality (Ketola, 1983; Walton, Cowey and Adron, 1984). In juvenile rainbow trout and Atlantic salmon (*Salmo salar*), MET deficiency has been reported to cause cataracts (Poston *et al.*, 1977; Barash, Poston and Rumsey, 1982; Walton, Cowey and Adron, 1982). In juvenile rainbow trout, TRY deficiency has been reported to cause scoliosis, lordosis, renal calcinosis, cataracts, caudal fin erosion, decreased lipid content and elevated calcium, magnesium, sodium and potassium concentrations (Shanks, Gahimer and Halver, 1962; Kloppel and Post, 1975; Poston and Rumsey, 1983; Walton *et al.*, 1984). Furthermore, in juvenile sockeye salmon (*Oncorhynchus nerka*), TRY deficiency has been reported to cause scoliosis (Halver and Shanks, 1960).

Well-proportioned AA profiles in aquafeeds is critical (Nakagawa, Sato and Gatlin, 2007; Wu, 2009). Poorly balanced AA profiles can lead to chemical antagonisms, such as LEU/ILEU and ARG/LYS antagonisms (Tacon, 1987). Blood meal, for example, is high in VAL, LEU and HIS but low in MET and ILEU. Therefore, because excess LEU antagonises ILEU, animals fed with high levels of blood meal suffer from ILEU deficiency induced by an overabundance of dietary LEU (Taylor, Cole and Lewis, 1977).

The AA profiles of ragworms in this study demonstrated that ragworms, regardless of their feed group, including those fed **AW + chitosan**, had well-balanced AA profiles.

By comparison of AA profiles of ragworms fed **CF** with ragworms fed **AW** or **AW + chitosan**, the suitability of ragworms fed with **AW + chitosan** as an alternative aquafeed ingredient can be demonstrated. There was a high variation in the means of individual AA in each group. For example, the means of one or more groups were significantly different for each EAA (except

for PHE) and each NEAA (except for TYR). However, specific trends between similar groups could be observed. For example, for MET, ragworms fed **AW** or **AW + chitosan** (groups 2, 3, 4 and 5) were significantly higher in ragworms fed **CF** (Groups initial, 1a and 1b). Therefore, indicating ragworm AA profiles are impacted by the feed.

Figure 4.16 depicts a direct comparison of EAA in ragworms fed with **AW** or **AW + chitosan** with ragworms fed **CF** to illustrate their usefulness as a feed resource. All EAA, except for THR and LYS, were in higher concentrations in ragworms fed with **AW** or **AW + chitosan** than in ragworms fed **CF**.

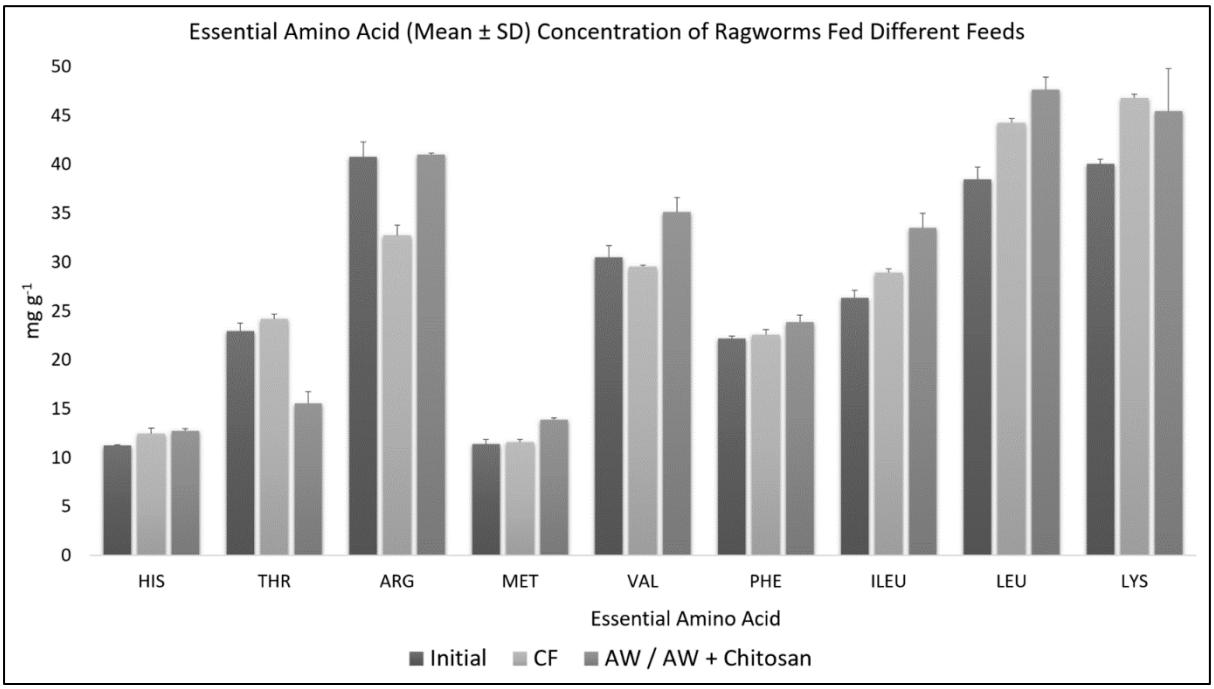

Figure 4.16 – EAA concentrations (mean ± SD) of ragworms fed different feeds
Initial = ragworms frozen and stored after acclimation period; **CF** = group 1b; **AW / AW + chitosan** = groups 2, 3, 4 and 5. HIS = histidine; THR = threonine; ARG = arginine; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; LYS = lysine.

To further demonstrate ragworms fed with **AW + chitosan** suitability as an aquafeed ingredient, a comparison of limiting essential amino acids (LEAA) with ragworms fed **CF** can be made. LEAA are considered the most critical EAA as they improve the utilisation of other AA and reduce their oxidation rates (Nunes *et al.*, 2014; Wang *et al.*, 2019). LEAA are AA that are in shortest supply in relation to need. The idea of LEAA is crucial when choosing the types, amounts and combinations of feeds to provide to fish. For example, even if total protein and all other AA requirements are fulfilled, if one of the LEAA is not, then the feed is still seen as

having a nutritional limit (Lopez and Mohiuddin, 2020). MET, LYS, ARG and THR are the most critical LEAA in fish, especially if fishmeal is substituted with plant-based alternatives because plant-based sources typically have lower bioavailability (Li *et al.*, 2009; Wang *et al.*, 2019). The LEAA requirements (% of dry protein) for 7 fish species are displayed in Table 4.3 and the LEAA contents (% of dry protein) of the ragworms fed the different diets from the experiment are displayed in Table 4.4.

Table 4.3 – LEAA percentage requirements of various fish species

LEAA Requirement (% of Protein)	**Rainbow Trout**	**Common Carp**	**Japanese Eel**	**Chinook Salmon**	**Channel Catfish**	**Chum Salmon**	**Mozambique Tilapia**
MET	≈1.8	≈2.1	≈2.1	≈1.5	≈1.3	N/A	≈1.3
LYS	≈5.3	≈5.7	≈4.8	≈5.0	≈5.1	≈4.8	≈4.1
ARG	≈3.5	≈3.3	≈3.9	≈6.0	≈4.3	N/A	≈4.0
THR	≈3.4	≈3.9	≈3.6	≈2.2	≈2.2	≈3.0	N/A
References	(Kaushik, 1979; Walton, Cowey and Adron, 1982, 1984; Ketola, 1983; Kim, Kayes and Amundson, 1983; Rumsey, Page and Scott, 1983)	(Nose, 1978; Ogino, 1980)	(Tacon, 1987)	(Nutrition and Council, 1983)	(Nutrition and Council, 1983)	(Akiyama *et al.*, 1985)	(Jackson and Capper, 1982)

Values are displayed as a percentage of dietary protein. LEAA = limiting essential amino acid; MET = methionine; LYS = lysine; ARG = arginine; THR = threonine.

Table 4.4 – LEAA percentage composition of ragworms fed different feeds

LEAA Requirement (% of protein)	Initial	Group 1a	Group 1b	Group 2	Group 3	Group 4	Group 5
MET	≈1.95	≈2.10	≈1.94	≈2.31	≈2.33	≈2.28	≈2.32
LYS	≈6.88	≈8.48	≈6.21	≈7.75	≈7.20	≈6.71	≈8.50
ARG	≈7.00	≈5.93	≈6.03	≈6.98	≈6.82	≈6.75	≈6.75
THR	≈3.94	≈4.39	≈3.73	≈2.71	≈2.47	≈2.37	≈2.82

Values are displayed as a percentage of dietary protein. LEAA = limiting essential amino acid; MET = methionine; LYS = lysine; ARG = arginine; THR = threonine. Initial = ragworms frozen and stored after acclimation period; group 1a = ragworms fed **CF**; group 1b = ragworms fed **CF**; group 2 = ragworms fed **AW**; group 3 = ragworms fed **AW + 10 mg L^{-1} chitosan**; group 4 = ragworms fed **AW + 20 mg L^{-1} chitosan**; group 5 = ragworms fed **AW + 30 mg L^{-1} chitosan**.

In rainbow trout (*Salmo gairdneri*), the requirement (as a percentage of total dietary protein) for MET, LYS, ARG, and THR is approximately 1.8, 5.3, 3.5 and 3.4%, respectively. In the ragworm groups fed with **AW** or **AW + chitosan** (groups 2, 3, 4 and 5), the mean ± SD percentage composition of total protein for MET, LYS and ARG were above the requirements in rainbow trout (2.31 ± 0.02, 7.57 ± 0.73 and 6.83 ± 0.11%, respectively). However, the percentage composition of THR was only close to what is required (2.59 ± 0.21%). The same findings were observed for common carp (*Cyprinus carpio carpio*) and Japanese eel (*Anguilla japonica*).

In Chinook salmon (*Oncorhynchus tshawytscha*), the requirements of MET, LYS, ARG and THR are estimated to be 1.5, 5.0, 6.0 and 2.2%, respectively (Nutrition and Council, 1983; Tacon, 1987). The ragworms fed **AW**, or **AW + chitosan** had higher percentage compositions than required (2.31 ± 0.02, 7.57 ± 0.73 and 6.83 ± 0.11%). The results were also higher for channel catfish (*Ictalurus punctatus*) (Nutrition and Council, 1983; Tacon, 1987). The same can be supposed for chum salmon (*Oncorhynchus keta*); however, MET and ARG data is unavailable, and the same can be supposed for Mozambique tilapia (*Oreochromis mossambicus*); however, THR data is unavailable.

The LEAA percentage composition of total protein in the other groups (initial, 1a and 1b) was lower than the requirements mentioned for all fish species above for MET (1.95, 2.10 and

1.94%, respectively) (except common carp and Japanese eel for group 1a), but higher for LYS (6.88, 8.48 and 6.21%, respectively), ARG (7.00, 5.93 and 6.03%, respectively) and THR (3.94, 4.39 and 3.73%, respectively). Suitable LEAA compositions were expected in these groups as they were fed commercial feeds developed for the growth of small fish and, therefore, included everything growing fish require, including LEAA. However, as it turns out, the MET percentage compositions were lower than what was required in most instances, again highlighting the high protein quality of the **AW** used in the experiment.

Overall, ragworms fed **AW** or **AW + chitosan** can satisfy the LEAA nutritional requirement of several fish species. Supplementation of one AA may be necessary for some species, but the same was observed in ragworms fed **CF**. Secondly, incorporating chitosan up to and including concentrations of **30 mg L^{-1}** does not negatively impact the AA profiles of ragworms fed **AW**. Finally, the ability of ragworms to synthesise AA from waste sources highlights why ragworms are an ideal candidate for use as an alternative aquafeed ingredient.

Finally, there are 10 EAA vital for aquafeeds (HIS, THR, ARG, MET, VAL, PHE, ILEU, LEU, LYS, and TRY) (Tacon, 1987; Kaushik and Seiliez, 2010). However, it has been demonstrated that NEAA such as GLY, ALA and GLU enhance feeding behaviour in several fish species (Carr *et al.*, 1996; Wang *et al.*, 2019). GLU is also significant for metabolic activity. ALA is crucial for DNA synthesis and an essential nitrogen carrier for AA metabolism (Li *et al.*, 2009). Moreover, it has also been demonstrated with Senegalese sole (*Solea senegalensis*) that they are attracted to odours released by ragworms in the form of AA, which encourages feeding (Li *et al.*, 2009). In this study, GLU, GLY and ALA made up 30.24 ± 1.95% in groups fed with a commercial feed (initial, 1a and 1b) and 32.90 ± 0.57% in groups fed **AW** or **AW + chitosan** (groups 2, 3, 4 and 5).

4.4.5 Lipid Contents and Fatty Acid Profiles of Feeds and Ragworms

Like protein, aquafeeds must have a lipid content that can satisfy the requirements of the species they are intended to feed. Deficiencies in FA can lead to detrimental impacts such as disease and stunted growth (Tacon, 1987).

Fish and shrimp use lipids to oxidise AA and generally have a dietary protein requirement of 24 – 57% DW (Tacon, 1987), which requires a lipid content of around 10 to 20% to be oxidised

without causing excessive lipid deposits in their tissues (Rust, 2003). Ragworms fed with **CF** (groups initial, 1a and 1b) had percentage lipid contents of 2.29 ± 0.09, 8.16 ± 0.35 and 10.09 ± 0.02%, respectively. The ragworms fed **AW**, or **AW + chitosan** (groups 2, 3, 4 and 5), had values between 5.41 ± 2.54 and 8.16 ± 0.35%. This demonstrates that only group 1b fed **CF** had a lipid content suitable for fish and shrimp requirements. Therefore, although cleaner fish **AW** was demonstrated as a suitable source of protein and AA, the lipid content may not be sufficient for the ragworms to meet (complete) aquafeed requirements. However, it could be achieved with supplementation or a combination of the cleaner fish **AW** with another high-fat source of **AW** for ragworms. But it is intended that ragworms are utilised as an ingredient in aquafeeds.

In all groups, the lipid concentrations were lower than the results in other research. For example, research investigating feeding Atlantic salmon smolt **AW** to ragworms recorded mean lipid concentrations of 123.6 ± 12.8 mg g^{-1} (Wang *et al.*, 2019). In addition, the lipid contents of ragworms fed with Atlantic salmon smolt **AW** closely resembled those found in ragworms fed Beluga sturgeon **AW** (O Pajand *et al.*, 2017). In the current research with cleaner fish **AW**, lipid concentrations ranged between 54.10 ± 5.80 and 81.04 ± 6.08 mg g^{-1}. Therefore, it may be the case that cleaner fish **AW** is not suitable for ragworm development in terms of lipid content.

It is also important to note that the lipid contents of ragworms can vary depending on what stage of their life cycle and the season. For example, it has been shown that ragworms store lipids before winter and sexual maturation, even in times of high food supply (Luis and Passos, 1995; García-Alonso and CT, 2008). However, this study was conducted with juvenile ragworms in spring. So, the ragworms were not at the sexual maturation stage or preparing for colder winter conditions, which may have contributed to lower lipid contents.

Also, the ragworms fed **AW** (groups 2, 3, 4 and 5) had slightly higher lipid concentrations increasing with higher chitosan concentrations. As discussed previously, chitosan has a positive charge and attaches to negatively charged molecules like lipids, cholesterol and fatty acids when solubilised in acidic environments, such as the digestive tract. Therefore, a potential reason for increased lipid contents in ragworms when fed **AW + chitosan** could be that the chitosan was binding to lipids in the digestive tract, increasing their capacity for

consumption. On the other hand, the opposite could be the case. In other words, chitosan can bind lipids in the digestive tract preventing their utilisation.

In the digestive tract of ragworms, lipids are emulsified by bile salts, broken down by lipases into fatty acids and monoglycerides, and then absorbed through the intestinal walls. These smaller molecules are utilised for energy, stored as fat, or incorporated into cell membranes. It is unclear whether chitosan enhances or limits the utilisation of lipids.

The ragworms, regardless of feed, did not vary significantly in terms of their protein and lipid contents. Excluding the initial group, protein contents were between ≈ 58.73 and ≈ 64.08%, and fat contents were between ≈ 5.41 and 10.09%. It has been shown that ragworms control their internal energy storage by aiming for a protein content of 55 - 60% and a lipid content of 10 - 15% (Wang *et al.*, 2019). Group 1b (**CF**) had optimal protein and lipid contents. However, those fed **AW** or **AW + chitosan** had protein contents within the typical range, but the lipid contents were lower than group 1b. Therefore, it can be concluded that the source of cleaner fish **AW** was not a good source of total lipid. However, the inclusion of chitosan potentially can make lipids more accessible to ragworms, as demonstrated by higher lipid contents with ragworms fed **AW + chitosan** (groups 3, 4 and 5) compared to those fed **AW** without chitosan (group 2). A reason for this could be that the chitosan binds more lipids present in the **AW** than **AW** without chitosan, which is seen in Figure 4.15. The **AW** without chitosan can leave more lipids in the solution than would be in **AW + chitosan**.

Furthermore, the fact that the **AW** used in this study was poor in terms of lipid content can be further demonstrated. Ragworms are effective at assimilating lipids even when fed a food source with relatively low amounts (Bradshaw *et al.*, 1990). Therefore, although the lipid contents of the **AW feeds** were like **CF**, the lipids in the **AW feeds** were not of the same bioavailability and quality as **CF**.

Fatty Acid Profiles

Regarding the essential omega-3 FA for aquafeeds EPA and DHA, all groups displayed a higher percentage proportion than the initial group. In the initial group, EPA had an average percentage proportion of ≈4.03%; in the other groups, it ranged between ≈4.39 and ≈9.29%.

DHA had an average percentage proportion of ≈0.61% in the initial group and between ≈0.64 and 5.33% in the other groups.

Similar to past research, compared to the initial group, the ragworms in other groups increased their total FA content throughout the experiment (Bischoff, 2007; A. Bischoff, Fink and Waller, 2009; Wang *et al.*, 2019). Thus, the increase in FA content is comparable to the increase in lipid contents in Figure 4.15. However, the increase in total FA in ragworms fed with **CF** was higher, and individual FA concentrations differed significantly from those fed **AW** or **AW + chitosan**. Therefore, this implies that **CF** is an excellent source of FA for ragworms compared to the cleaner fish **AW** used in this experiment.

Group 1b (**CF**) had a total FA concentration of 74.49 ± 5.34 mg g^{-1}, similar to other research with ragworms fed **CF** (73.7 ± 6.2 mg g^{-1}) (Wang *et al.*, 2019). However, the total FA concentration in this research with ragworms fed cleaner fish **AW** (25.74 ± 12.53 – 44.75 ± 10.78 mg g^{-1}) was lower than research which fed ragworms Atlantic salmon smolt **AW** (56.9 ± 7.8 mg g^{-1}) (Wang *et al.*, 2019). Therefore, this source of cleaner fish **AW** may not be of the same quality, in terms of FA, as Atlantic salmon smolt **AW** and **CF**. Despite this, the ragworms in groups fed **AW** or **AW + chitosan** maintained a good proportion of PUFA relative to the ragworms fed **CF**, highlighting why ragworms are an appropriate candidate for inclusion as an ingredient in aquafeeds.

The primary FA present in the ragworms included EPA, palmitic acid, elaidic/oleic acid and linoleic acid, which is the same found in similar studies (Luis and Passos, 1995; Fidalgo e Costa, Narciso and Cancela da Fonseca, 2000; Bischoff, 2007; García-Alonso and CT, 2008; A. Bischoff, Fink and Waller, 2009; Brown, Eddy and Plaud, 2011; Santos *et al.*, 2016; O Pajand *et al.*, 2017; Wang *et al.*, 2019). Some FA are essential because they cannot be synthesised or synthesised in minimal amounts and, therefore, must be supplied in a ready-made form within the diet (Tacon, 1987). EPA, DHA and arachidonic acid (ARA) (C20:4) are considered essential in marine fish (Sargent *et al.*, 1999). EPA, DHA and ARA are long-chain PUFA (LC-PUFA) and come from two closely related families of FA, the omega-3 and omega-6 families and have numerous biological functions (Mejri *et al.*, 2021). In aquafeeds, the most valuable FA are the omega-3s, EPA and DHA, as they are the predominant PUFA in freshwater fish, marine fish, and shrimp. (Tacon, 1987; Wang *et al.*, 2019). DHA helps fish larvae better withstand stress and is necessary for gonad development (García-Alonso and CT, 2008). EPA,

like DHA, is a significant component of fish oil and plays a central role in regulating several biological processes and the immune system (Horn *et al.*, 2019). In addition to playing a role in growth maturation and immunological responses (Friesen and Innis, 2009), ARA serves as the primary precursor for eicosanoids (FA with 20 carbons) (Glencross, 2009).

While not essential, other FA have important roles in marine animals. Palmitic acid is the initial metabolite in FA production and is produced from excess carbohydrates, and serves as a precursor to several longer FA (Carta *et al.*, 2017). Oleic and linoleic acids are precursors of LC-PUFA, such as EPA, DHA and ARA (Dalsgaard *et al.*, 2003). Except for DHA in group 5, EPA and ARA made up significant percentages of the total FA in the ragworms. The **AW feeds** were significantly lower than **CF** for DHA, EPA, ARA, and linoleic acid but higher in oleic acid/elaidic acid and palmitic acid, which is reflected in the contents of the ragworms.

Past has demonstrated that ragworms' FA profile reflects their diets' FA profile (Cowey and Sargent, 1972). FA profile reflection is seen when comparing group 1b (**CF**) to groups fed **AW** or **AW + chitosan**. For example, the **CF** feed had higher concentrations of EPA, DHA and ARA, which is reflected in the ragworm FA profiles where group 1b ragworms (**CF**) also had higher concentrations of EPA, DHA and ARA

On the other hand, it has also been demonstrated that ragworms can synthesise EPA and DHA when fed with diets low or absent in EPA and DHA (Fidalgo e Costa, Narciso and Cancela da Fonseca, 2000). To demonstrate, in the groups fed **AW** or **AW + chitosan** (groups 2, 3, 4 and 5), DHA made up less than 0.5% of total FA. However, in the ragworms, the DHA percentage proportion increased to about 1.6% (except in group 5). An increase was also observed with EPA. Again, this highlights why ragworms are an attractive candidate for alternative aquafeed ingredients.

Group 5 (**AW + 30 mg L^{-1} C**) had the lowest proportion of PUFA and the highest proportion of SFA. Group 5 also had the highest concentration of lipids (Figure 4.15) and FA among the ragworm groups fed with **AW** or **AW + chitosan** (groups 2, 3, 4 and 5). A reason for this could be that the 30 mg L^{-1} chitosan concentration binds the highest concentration of lipid in the **AW** (i.e., fewer escapes) and therefore has a higher lipid concentration available than the other **AW** feeds. A higher lipid content in the ragworms fed **AW + 30 mg L^{-1} C**, primarily SFA may be evidence of chitosan disruption in ragworm development and higher mortalities and

lower growth rates than other groups. Group 5 also had a significantly lower percentage proportion of DHA. Alternatively, it may be the case that the **AW** lipids are of lower quality in terms of EFA compared to the **CF**.

Furthermore, once dissolved in an acidic environment like the gastrointestinal tract, chitosan acts as an emulsifier of fat. The chitosan in the emulsion transforms into an insoluble gel-like form of trapped fat that cannot be broken down by intestinal enzymes (Ahn, Cho and Choi, 2021). Therefore, group 5 (**AW + 30 mg L^{-1} C**) ragworms may have been ingesting fat in the presence of chitosan. There is no other source of lipids other than what is contained in the **AW** for these ragworms. So, any lipids present and bound by chitosan come from the same **AW** with the same composition for the preparation of all **AW** feeds. Yet, observations in lipid concentrations containing chitosan have a higher concentration of lipid than the **AW** not containing chitosan. A conversation with the industrial supervisor of this project stated that in his experience, chitosan flocculation gathers more lipids than separation without chitosan (J Hughes, 2023, personal communication).

Additionally, because the intestinal enzymes could not break down the FA, the SFA, such as palmitic acid, are not as readily synthesised into longer chain FA, thus giving the group 5 ragworms a higher proportion of SFA. Moreover, a decreasing trend in the percentage proportion of PUFA is seen with increasing chitosan concentration (groups 3, 4 and 5). Furthermore, an increasing trend in the percentage proportion of SFA is seen with increasing chitosan concentration (groups 3, 4 and 5). Therefore, it may be suggested that if chitosan is to be used to coagulate and flocculate **AW** before feeding to ragworms intended for inclusion in aquafeeds, the concentration of chitosan must be carefully considered not to impact the FA profiles of the ragworms negatively.

Overall, similar to other research, ragworms successfully integrated FA from sources of **AW** into their tissues (Luis and Passos, 1995; Bischoff, 2007; A. Bischoff, Fink and Waller, 2009; Wang *et al.*, 2019). However, in this research, the same can be said when chitosan is incorporated into the **AW**. However, at higher concentrations of chitosan, while concentrations of PUFA and SFA were moderate, lower percentage proportions of PUFA and higher percentage proportions of SFA are observed, which can impact the LC-PUFA content in ragworms and, therefore, their value as an aquafeed ingredient.

To determine ragworms fed with **AW + chitosan** suitability as an aquafeed ingredient, Figure 4.17 displays the ragworms fed with **AW + chitosan** average FA profile against the **CF** (the feed itself, not the ragworms fed **CF**). The primary point of the project is to demonstrate that ragworms fed **AW** can be a more sustainable approach for producing aquafeed ingredients. Therefore, the ragworms fed **AW**-derived feeds are compared with the commercially available and nutritionally suitable **CF**. The ragworms contained 23 of the 24 FA that could be identified in the **CF**, missing only the SFA lignoceric acid (C24:0). Three more FA were identified in ragworms (26 in total) than the **CF** with undecanoic acid (C11:0), heptadecenoic acid (C17:1), and docosatetraenoic acid (C22:4) identified in low quantities. Of the 26 identified FA across all samples, the ragworms contained 21 in equal or more significant percentage proportions. One-way ANOVA concluded no significant difference between SFA contents in **CF** or ragworms. However, the percentage composition of MUFA and PUFA was significantly higher in the **CF**.

Regarding differences in individual FA between the **CF** and ragworms fed **AW + chitosan**, the most important to note is the difference in EPA and DHA percentage compositions. The **CF** had significantly higher percentage compositions than the ragworms fed **AW + chitosan**. The **CF** was also significantly higher in linoleic acid (C18:2). However, the ragworms were significantly higher in palmitic acid (C16:0), oleic/elaidic acid (C18:1) and stearic acid (C18:0).

Overall, the ragworms fed cleaner fish **AW + chitosan** included the necessary FA, EPA, DHA and ARA but were not in the same percentage composition as the **CF**. However, this says more about the **AW** than it does about the performance of the ragworms or the presence of chitosan. As previously stated, ragworms reflect the FA profile of their feed, and the batch of cleaner fish **AW** used was not a great source of PUFA. However, other studies have successfully shown that different sources of **AW** have proved to be an excellent supply of FA in ragworms (Bischoff, 2007; O Pajand *et al.*, 2017; Wang *et al.*, 2019). Therefore, in conjunction with the results of this experiment, it can be said that chitosan at low to moderate concentrations will not impact the FA profile of ragworms when fed with a source of **AW**.

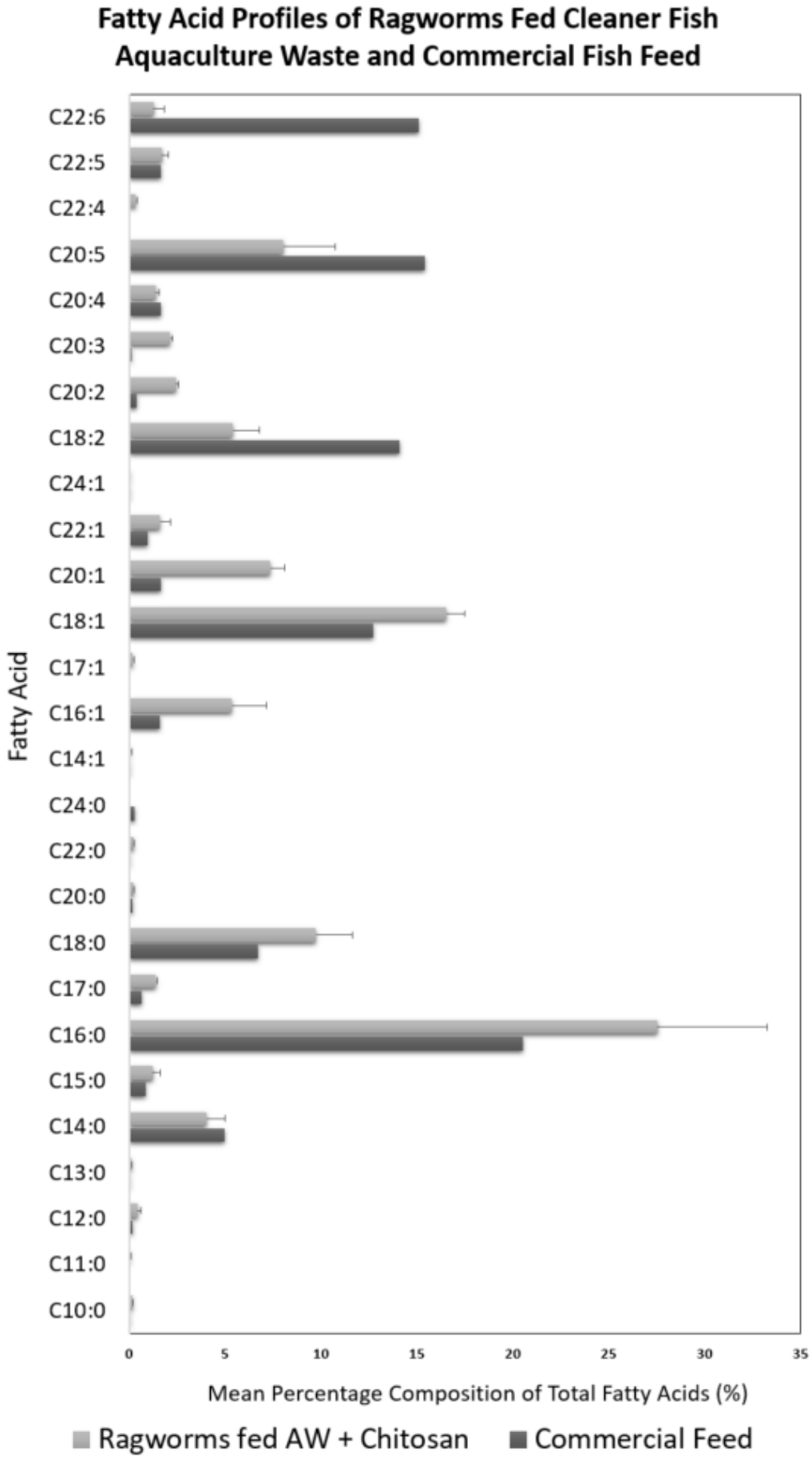

Figure 4.17 – Fatty acid profiles of ragworms fed AW + Chitosan versus a commercial fish feed

4.5 Conclusion

The findings of this study show that ragworms may be effectively grown using **AW + chitosan**-derived feeds from a cleaner fish aquaculture system as a sole food source. Furthermore, it has been demonstrated that ragworms utilise and integrate nutrients found in the **AW** that are often discarded. Therefore, potentially making the aquaculture industry more sustainable by using resources more efficiently. However, at high concentrations of chitosan (**30 mg L^{-1}**) in the **AW**, detrimental effects in ragworms are observed. Namely, lower growth rates, higher mortalities, and negatively impacted FA profiles.

The growth rates and mortalities of ragworms in the experiment varied across different feeds. **AW** is less energy-dense than **CF**, which was included as a control, and therefore, growth rates were lower in ragworms fed **AW**. Furthermore, ragworms fed **AW** with the highest dose of chitosan (**30 mg L^{-1}**) had higher mortalities and lower growth rates than other feed groups, signifying that chitosan at higher concentrations has adverse physiological effects on ragworms.

Similar TOM, C and N contents were found in ragworms fed **CF**, **AW,** and **AW + chitosan**, suggesting chitosan, even at high concentrations, does not impact these parameters.

Protein contents, while statistically similar, were higher in ragworms fed **AW** or **AW + chitosan** than in ragworms fed **CF**, suggesting that the batch of cleaner fish **AW** was a good source of protein. The presence of chitosan did not impact the protein contents in the ragworms either.

It was discussed that fish require a balanced amino acid (AA) profile in their diet. Regardless of the feed group, the ragworms in this experiment were found to meet the requirements of various fish species. For almost all measured essential AA, the ragworms fed **AW** or **AW + chitosan** was found in higher concentrations than those fed with the **CF**. The AA profiles demonstrated two things pertinent to the aims of this study: 1) the cleaner fish **AW** used is a high-quality source of AA, and 2) chitosan does not negatively influence the AA profiles of the feeds or the ragworms.

Regarding 1), differences in fatty acids between different RAS housing different fish species is expected because each species has unique dietary needs, metabolic rates, and ways of

processing and storing lipids, leading to variations in the composition of fatty acids in their bodies and the wastewater produced.

Lipid contents, like protein contents, were statistically similar, but ragworms fed **CF** had a higher content than those fed **AW** or **AW + chitosan**. Chitosan did not impact the protein contents, but ragworms fed **AW** with increasing chitosan concentrations had higher lipid contents.

Finally, the ragworms fed on **AW + chitosan** was a good source of fatty acids (FA) and contained essential FA (EPA, DHA and ARA). However, they were not present in the same concentrations or at the same percentage composition as ragworms fed with **CF**. Therefore, suggests that the **AW** used in this study was not a high-quality source of FA. Lower FA concentrations and percentage compositions of EFA were also confirmed for ragworms fed **AW** without chitosan. Furthermore, the concentration and percentage composition of EPA and other PUFA decreased with increasing chitosan concentration. As a result, the ragworms fed with **AW + chitosan** also had higher concentrations and percentage compositions of saturated fatty acids.

The differences in percentage compositions of FA were significant in group 5 (**AW + 30 mg L^{-1} C**). As discussed, differences in FA profiles are likely due to the mechanism by which chitosan binds to lipids, limiting their ability to be metabolised effectively by the ragworms. When the ragworms fed **AW + chitosan** was compared to the **CF**, the ragworms had lower percentage compositions of EFA but were equal or higher in many other FA. The FA profiles demonstrated 3 things: 1) ragworms can assimilate FA from **AW** with chitosan incorporated; 2) high concentrations of chitosan can negatively affect the concentrations of essential polyunsaturated FA; 3) the source of **AW** used in this experiment is not a great source of FA.

To conclude, ragworms fed **AW + chitosan** can utilise nutrients from the **AW**, potentially making ragworms valuable components in aquafeeds. However, at high concentrations of chitosan, specific detrimental effects on growth, mortality and nutritional composition are observed. Therefore, it can be said that coagulating and flocculating sources of **AW** with chitosan, then feeding to ragworms, and then feeding ragworms to fish in aquaculture systems can help to lessen the strain on wild fish supplies that are used to make aquafeeds.

As the share of aquaculture facilities, such as those breeding cleaner fish, continues to grow, so does waste production. However, the waste can be converted into a valuable product by developing integrated systems with cleaner fish **AW** processed with chitosan and ragworms.

Chapter 5

Feeding Chitosan Coagulated Aquaculture Wastewater to *Hediste diversicolor* creates a Viable Alternative Aquafeed Ingredient

5.1 Introduction

Aquaculture wastewater (**AW**) is aqueous and must be processed in some way before feeding to ragworms. For example, previous research has centrifuged Atlantic salmon smolt **AW** before feeding ragworms (Wang *et al.*, 2019). Culturing ragworms directly in the **AW** is inadvisable as the water quality is not suitable unless they are placed in sand filters (Marques *et al.*, 2018), or by other means to co-treat the water. One way to process **AW** before applying it as a feed for ragworms is to coagulate and flocculate the **AW** with chitosan. **AW** can be coagulated and flocculated with chitosan very efficiently (over 90% turbidity removal) (Chung, Li and Chen, 2005; Renault *et al.*, 2009). One of the benefits of using chitosan is that it can gather components in the **AW** that are solid and in the dissolved form into a particulate form which ragworms can then consume. Another benefit of using chitosan is that it is more cost-effective than other methods, such as centrifugation, which is the fastest and most efficient way of concentrating a sample with solids recovery normally better than 95% (Brandt *et al.*, 2016). Other, less effective methods include freeze-drying and settling.

Therefore, to compare feeding **AW** that has been coagulated and flocculated to ragworms against other methods, three chapter aims were formulated:

1. Investigate feeding **AW** that was coagulated and flocculated with chitosan to ragworms and its impact on their growth and mortality.

2. Investigate feeding **AW** that was coagulated and flocculated with chitosan to ragworms and its impact on their nutritional composition.

3. Evaluate ragworms' suitability as an alternative aquafeed ingredient after being fed **AW** that has been coagulated and flocculated with chitosan by comparing to ragworms fed **AW** that has either been centrifuged, freeze-dried, or settled and ragworms fed with a commercial fish feed.

The objective of the chapter was to investigate the three aims stated above to answer the following hypothesis:

Feeding ragworms with aquaculture wastewater coagulated and flocculated by chitosan creates a more valuable alternative aquafeed ingredient than feeding ragworms with centrifuged, freeze-dried or settled aquaculture wastewater.

Proving the hypothesis will determine whether ragworms fed **AW** coagulated and flocculated with chitosan provide a suitable alternative aquafeed ingredient and, potentially, a more valuable one compared to ragworms that have been fed **AW** that was processed in different ways. Therefore, it also potentially helps combat sustainability issues surrounding aquafeed production and waste production from aquaculture while concomitantly producing a high-quality alternative aquafeed ingredient.

5.2 Materials and Methods

The experiment started on the 1st July 2022 and ended on the 1st August 2022. This section discusses experimental methodology specific to this experiment and not covered in chapter 3. For full methods used in this experiment, refer to Sections 3.2 (Chitosan Formulations), 3.3 (Source of Experimental Animals and Husbandry), 3.4 (Source of Aquaculture Wastewater), 3.5 (General Experimental Set-Up, 3.6 (Water Quality Measurements), 3.7 (Determining Ragworm Weights and Growth Rates), 3.8 (Biochemical Analyses of Ragworms and Feeds), and 3.9 (Statistical Analysis).

One group was fed **AW** that was coagulated and flocculated with chitosan (**AWCH)**, one group was fed **AW** that was centrifuged (**AWCE**), one group was fed **AW** that was freeze-dried (**AWFD**), and one group was fed **AW** that was settled (**AWSE**). In addition, one group was fed a commercial feed (**CF**) to compare the before-mentioned groups to ragworms fed with a nutritionally complete feed. Each group consisted of 3 replicates containing 13 ragworms per replicate.

5.2.1 Feed Groups

The following bullet points describe the feed provided to the ragworms in their respective groups. The bold lettering denotes what the feed groups are referred to in the text and are used interchangeably.

- **Group 1** – Commercial fish feed pellets (**CF**) (Tetra® Pond Pellets Mini) [no chitosan]

- **Group 2** – Centrifuged cleaner fish aquaculture wastewater (**AWCE**) [no chitosan] [supernatant discarded]

- **Group 3** – Freeze-dried cleaner fish aquaculture wastewater (**AWFD**) [no chitosan]

- **Group 4** – Chitosan coagulated and flocculated cleaner fish aquaculture wastewater (**AWCH**) [contains chitosan] [unsettled liquid layer removed]

- **Group 5** – Settled cleaner fish aquaculture wastewater (**AWSE**) [no chitosan] [unsettled liquid layer removed]

Three glass culture beakers (replicates) containing 13 juvenile ragworms each were set up for each feed group (39 ragworms per feed group) (195 ragworms in total). This was a stocking density of 0.15 ind cm^2. After a 5-day acclimation period, alive and intact ragworms were selected for the experiment and randomly stocked in the culture beakers. They were not selected for size, maturation, weight, or any other classification. However, all ragworms used were of a similar weight and at the same stage of maturation.

5.2.2 Making Feeds

The **AW** used in this experiment was from a cleaner fish facility (refer to Section 3.4 – Source of Aquaculture Wastewater). However, it was of a different batch to the one used in the experiment described in chapter 4.

- **Group 1** ragworms were fed pelleted **CF**, so no preparation was necessary before the experiment.

- **Group 2** ragworms were fed centrifuged **AW** (**AWCE**). First, homogenised **AW** was added to multiple 50 ml centrifuge tubes (Fisherbrand™ Polypropylene) and centrifuged at 5000 rpm for 5 min (Eppendorf® Centrifuge 5810 R). After, the supernatant was discarded, and the tubes

containing the concentrated **AW** pellet were left upside down on paper towels in a fume hood for 24 hrs to drain excess water. Finally, the tubes were then re-sealed and frozen until required.

- **Group 3** ragworms were fed freeze-dried **AW** (**AWFD**), and the feed was prepared as described in chapter 4 (Section 4.2.4 – Making Feeds).

- **Group 4** ragworms were fed **AW** that was coagulated and flocculated with chitosan (**AWCH**). The chitosan solution used to prepare the **AW** was prepared according to confidential instructions from Pennosan®. To effectively coagulate and flocculate with chitosan, the pH of the **AW** was dropped from ≈7.9 to ≈6.5 using 2 M hydrochloric acid. As explained in chapter 2, the pH must be slightly acidic, or the chitosan will not function effectively. The dose that returned the highest percentage of turbidity removal was chosen as the optimum dose. The optimum dose, determined before the experiment, and specific to this source of **AW**, was 8 mg L^{-1} which removed 92.25 ± 1.43% of turbidity (n=3). Therefore, a chitosan dose of 8 mg L^{-1} was added to approximately 500 ml of homogenised and pH-adjusted **AW**. Next, the chitosan was added using a pipette (Thermo Scientific™ Finnpipette™ F2 Variable Volume Pipette) with a paddle stirrer (VELP® Scientifica FP4 Portable Flocculation Tester) to stir the **AW** at 200 rpm with the chitosan added directly into the vortex. Next, the **AW** and chitosan were stirred at 200 rpm for a further 30 sec to allow the chitosan to be distributed evenly and the coagulation process to take place. Then, the rpm was reduced to 40 rpm for 10 min allowing the flocculation process to take place before removing the beaker from the paddle stirrer, where it was allowed to settle for 15 min. After 15 min, the unsettled liquid layer was removed using a pipette. Finally, the beaker was sealed with cling film and frozen until required.

- **Group 5** ragworms were fed settled **AW** (**AWSE**), which was made by adding 900 ml of homogenised **AW** to a glass 1 L beaker. Then the beaker was left undisturbed for 24 hours in a fume hood. Next, the unsettled liquid layer was removed using a pipette. Finally, the beaker was sealed with cling film and frozen until required.

5.2.3 Feeding Protocol

The provision of food was designed to ensure that it was not a factor limiting the growth of the ragworms. From Monday to Friday, 1 g of respective feed was added to each culture beaker in the morning. If no feed was present on the sediment surface at the end of the day,

another 1 g was added. On a Friday, 4 g were added to cover the weekend due to restricted access to the laboratory on weekends (refer to Section 3.5 – General Experimental Set-Up).

5.3 Results

5.3.1 Feeds

To better understand how different treatment processes (centrifugation, coagulation and flocculation, freeze-drying, and settling) impact the nutritional content of **AW**, total organic matter contents, carbon and nitrogen contents, protein contents, amino acid profiles, lipid contents, and fatty acid profiles were analysed. They were compared to **CF**, as this contains a high level of nutrition being a commercially manufactured feed for feeding small marine animals.

5.3.1.1 Total Organic Matter Contents

Figure 5.1 shows the percentage of total organic matter content (TOM) (mean ± SD) of the different feeds used in the experiment. All feeds were significantly different from each other. The **CF** had the highest mean TOM content with a value of 94.25 ± 0.53%, followed by **AWCE** (81.06 ± 1.52%), then **AWCH** (61.28 ± 0.68%), then **AWSE** (51.56 ± 1.41%) and finally **AWFD** (38.77 ± 2.40%).

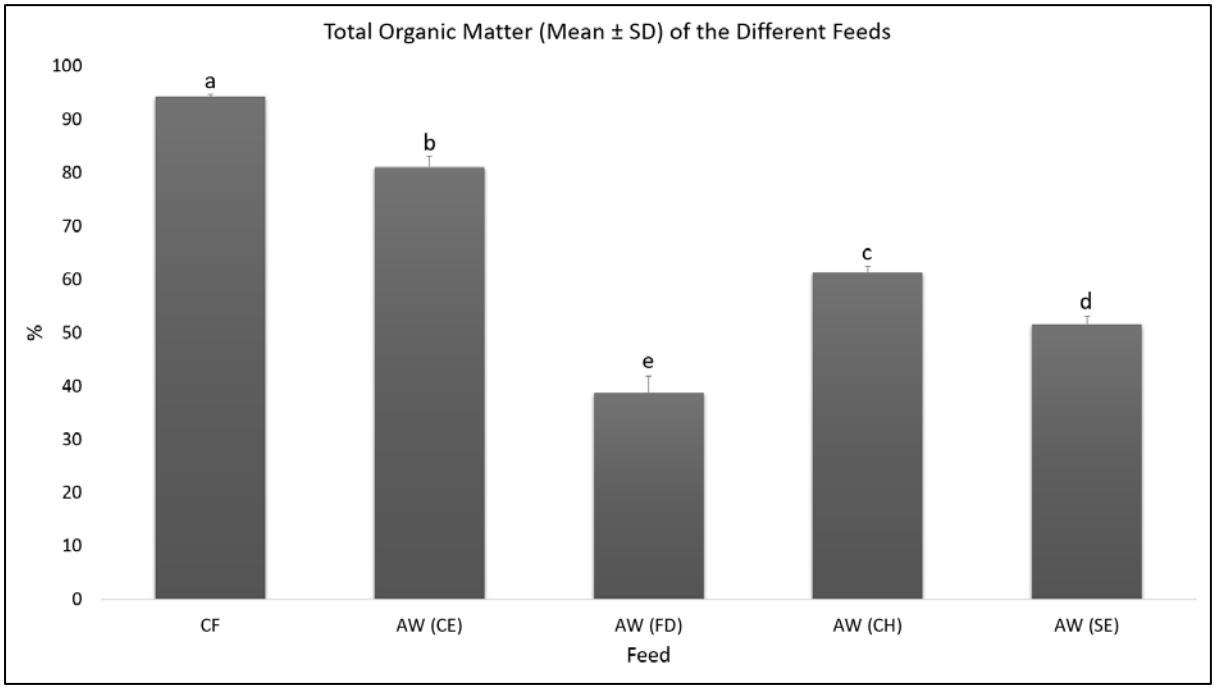

Figure 5.1 – Percentage total organic matter content (mean ± SD) of the different feeds used in the experiment

CF = commercial feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated and flocculated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. Different letters indicate significant differences between feeds ($P < 0.05$).

In summary, **CF** has the highest TOM content, indicating its rich organic composition compared to the **AW-**derived feeds. The differences highlight the impact of different treatment processes on the organic matter content in **AW**.

5.3.1.2 Carbon and Nitrogen Contents

Figure 5.2 shows the carbon and nitrogen contents of the different feeds used in the experiment. **CF** had the highest mean percentage carbon and nitrogen content with values of 43.32 ± 1.05 % and 4.62 ± 0.08%, respectively, followed by **AWCE** (22.99 ± 0.35 & 2.86 ± 0.35%), then **AWCH** (19.01 ± 0.58 & 2.63 ± 0.08), then **AWSE** (12.49 ± 0.22 & 1.8 ± 0.03%) and finally **AWFD** (5.98 ± 0.19 & 1.18 ± 0.01%).

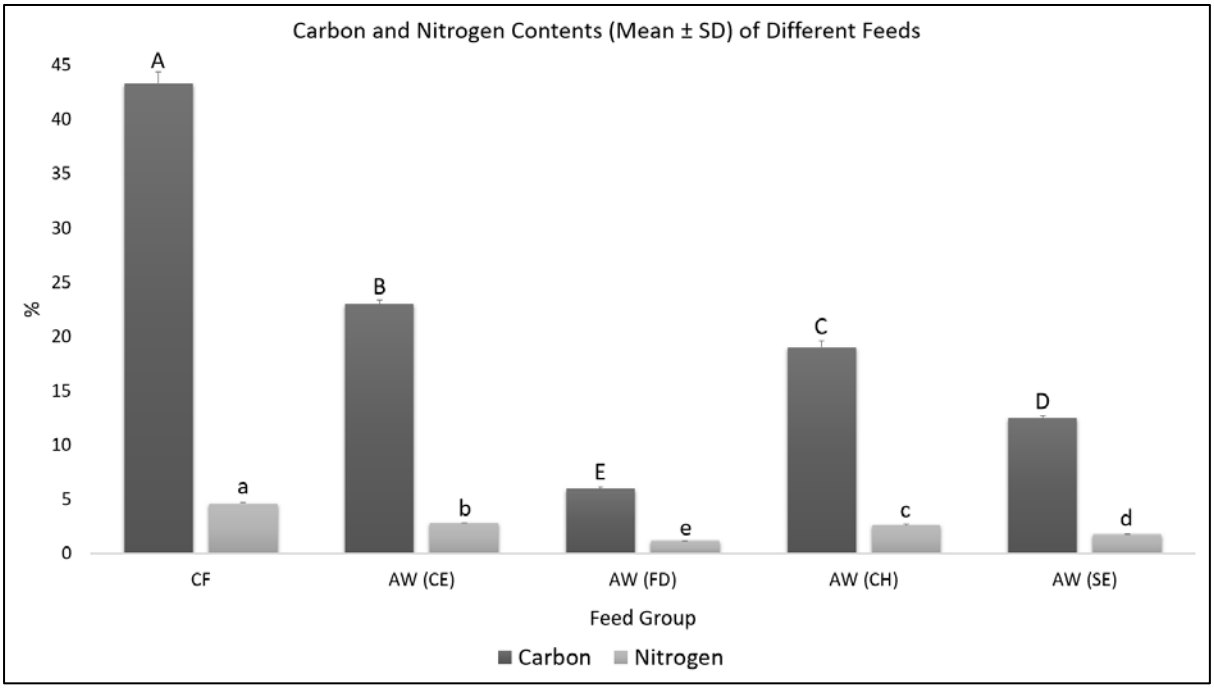

Figure 5.2 – Percentage carbon and nitrogen contents (mean ± SD) of the different feeds used in the experiment

CF = commercial feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated and flocculated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. Different uppercase letters indicate significant differences between carbon contents. Different lowercase letters indicate significant differences between nitrogen contents ($P < 0.05$).

Like the TOM contents (Section 5.3.1.1 – Total Organic Matter Contents), **CF** had the highest content of carbon and nitrogen, and the other feeds followed the same order (**AWCE**, **AWCH**, **AWSE**, and then **AWFD**). The differences further highlight the impact of different treatment processes on the nutritional content in **AW**.

5.3.1.3 Protein Contents

Figure 5.3 shows the protein content of the different feeds used in the experiment. The trend observed with protein contents was like that with TOM, C and N contents. In other words, the protein contents are higher as they become more concentrated with more efficient processing methods. Also, similar to what was observed in the TOM contents, they differed significantly from each other. **CF** had the highest mean protein content of 288.91 ± 4.95 mg g^{-1}, followed by **AWCE** (178.61 ± 1.85 mg g^{-1}), **AWCH** (164.14 ± 5.31 mg g^{-1}), **AWSE** (112.44 ± 1.68 mg g^{-1}) and **AWFD** (73.66 ± 0.58 mg g^{-1}).

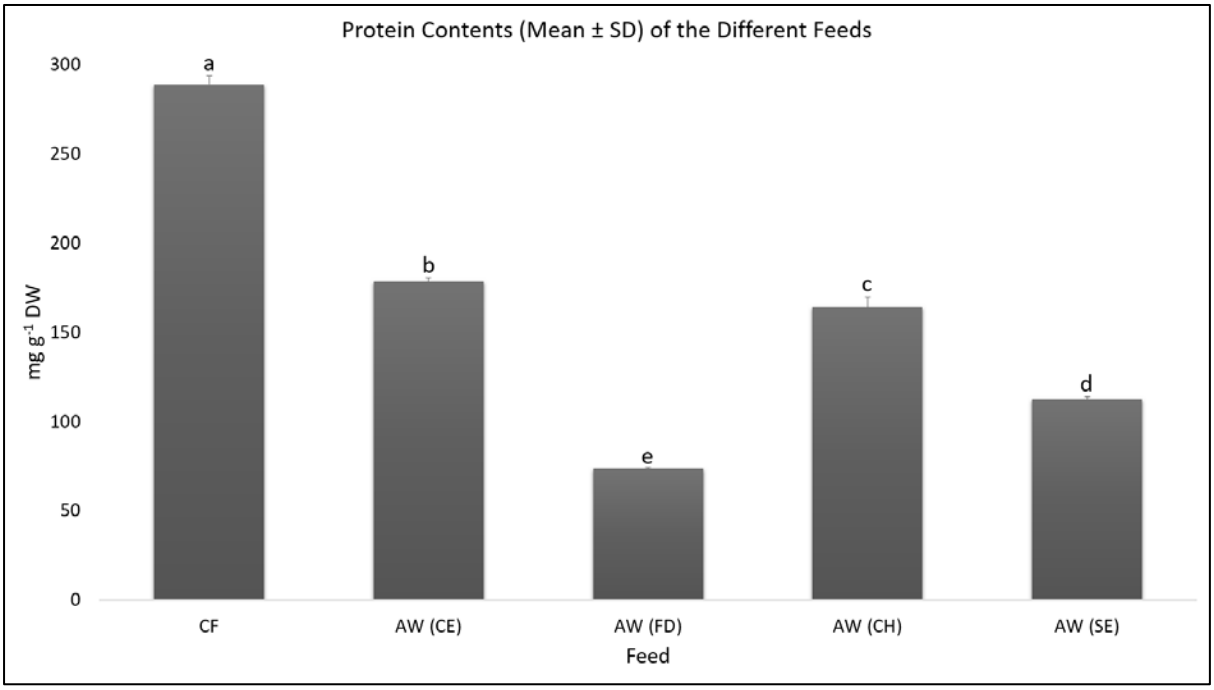

Figure 5.3 – The protein content (mean ± SD) of the CF and processed AW
CF = commercial feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated and flocculated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. Different letters indicate significant differences between feeds ($P < 0.05$).

Like the TOM contents (Section 5.3.1.1 – Total Organic Matter Contents) and carbon and nitrogen contents (Section 5.3.1.2 – Carbon and Nitrogen Contents), **CF** had the highest content of carbon and nitrogen, and the other feeds followed the same order (**AWCE**, **AWCH**, **AWFD**, and then **AWSE**), all differing significantly. The differences further highlight the impact of different treatment processes on the nutritional content in **AW**, which may have implications if the intention is to feed to ragworms before they are processed into aquafeeds as a high-quality ingredient.

5.3.1.4 Amino Acid Profiles

Figure 5.4 illustrates the amino acid composition (% of total amino acids) of the different feeds used in the experiment. Regarding percentage composition, all feeds were similar. However, the total AA concentration differed with different treatment methods. Like the TOM and protein contents of the feeds, the total AA concentrations differed significantly for each feed. Again, as with the other analyses discussed previously, the **AW** feeds followed the same trend where the concentration increases with better processing methods. The **CF** had the highest total AA concentration (154.34 ± 0.69 mg g^{-1}), followed by **AWCE** (137.59 ± 0.12 mg g^{-1}), then

AWCH (100.38 ± 0.38 mg g^{-1}), then **AWSE** (73.37 ± 0.02 mg g^{-1}) and finally **AWFD** (30.20 ± 0.05 mg g^{-1}).

Similarly, the total essential amino acids (EAA) concentrations differed significantly between feeds and followed the same trend as the total AA concentrations. The **CF** had the highest EAA concentration (78.33 ± 0.32 mg g^{-1}), followed by **AWCE** (71.80 ± 0.53 mg g^{-1}), then **AWCH** (50.93 ± 0.35 mg g^{-1}), then **AWSE** (37.14 ± 0.07 mg g^{-1}) and finally **AWFD** (14.50 ± 0.19 mg g^{-1}).

Resembling the total AA percentage compositions, the percentage compositions of EAA in the **AW** feeds were very similar. The EAA with the highest mean percentage composition in the **AW** feeds was arginine (ARG) (≈10.09%). However, in the **CF**, the EAA with the highest mean percentage composition was leucine (LEU) (8.93%). In the **AW** feeds, ARG was followed by valine (VAL) (≈8.61%), isoleucine (ILEU) (≈7.01%), leucine (LEU) (≈6.74%), phenylalanine (PHE) (≈5.84%), lysine (LYS) (≈5.25%), methionine (MET) (≈2.50%), threonine (THR) (≈2.42%) and histidine (HIS) (≈1.93%). In the **CF**, the order differed. LEU was followed by LYS (≈7.17%), ARG (≈7.15%), VAL (≈6.97%), PHE (≈6.09%), ILEU (≈6.04%), THR (≈4.18%), HIS (≈2.58%) and MET (≈1.88%).

Likewise, the total non-essential amino acids (NEAA) concentrations differed significantly between feeds and followed the same trend as the total AA and EAA concentrations. The **CF** had the highest NEAA concentration (75.16 ± 0.45 mg g^{-1}), followed by **AWCE** (65.80 ± 0.65 mg g^{-1}), then **AWCH** (49.44 ± 0.03 mg g^{-1}), then **AWSE** (36.22 ± 0.05 mg g^{-1}) and finally **AWFD** (15.70 ± 0.14 mg g^{-1}).

Also, the percentage compositions of NEAA in the **AW** feeds were similar. Again, the total concentration of NEAA differed with different treatment methods, but their percentage composition did not. The NEAA with the highest mean percentage composition in the **AW** feeds was aspartic (ASP) (≈16.22%). However, in the **CF**, it was glutamic (GLU) (≈16.56%). In the **AW** feeds, ASP was followed by GLU (≈14.27%), alanine (ALA) (≈6.36%), tyrosine (TYR) (≈5.04%), glycine (GLY) (≈3.82%), serine (SER) (≈3.58%) and ornithine (ORN) (≈0.32). In the CF, GLU was followed by ASP (≈13.70%), GLY (≈6.09%), ALA (≈4.85%), TYR (≈4.10%), SER (≈3.54%) and ORN (≈0.13%).

Moreover, the percentage compositions of total EAA and total NEAA were similar. The total EAA in all feeds ranged from ≈48 to ≈52%, and the total NEAA in all feeds ranged from ≈48 to ≈52%.

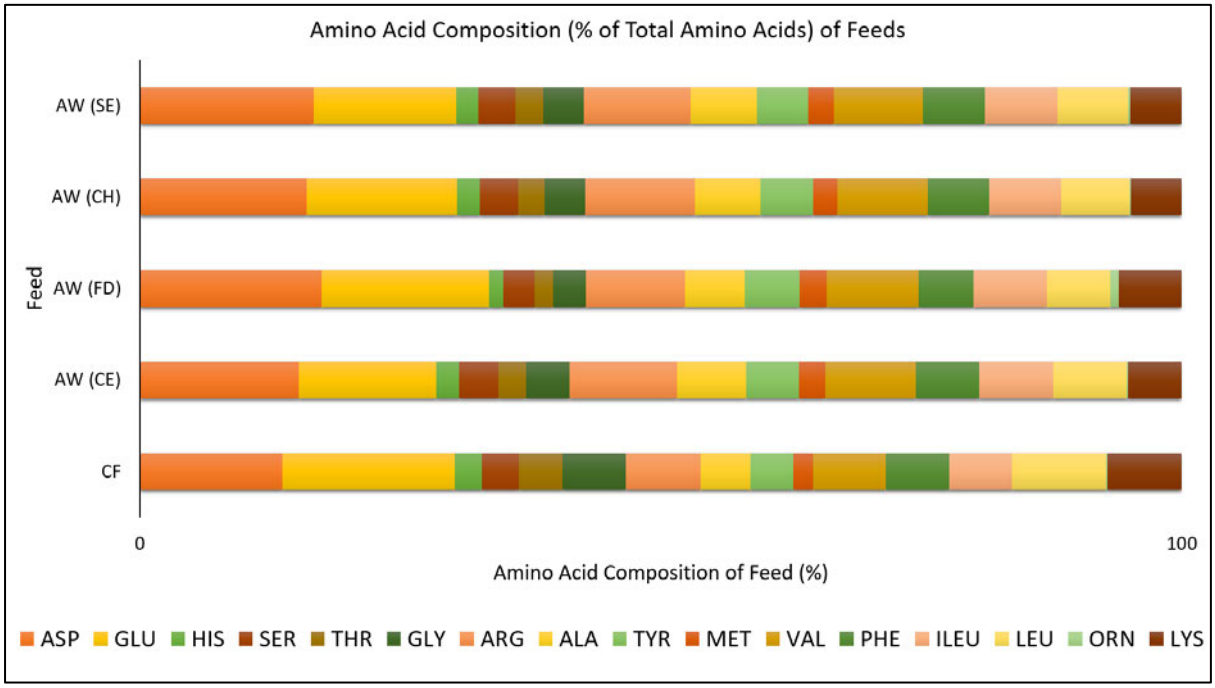

Figure 5.4 – Amino acid composition of CF and processed AW (% of total amino acids)
CF = commercial feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated and flocculated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. ASP = aspartic acid; GLU = glutamic acid; HIS = histidine; SER = serine; THR = threonine; GLY = glycine; ARG = arginine; ALA = alanine; TYR = tyrosine; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; ORN = ornithine; LYS = lysine.

The results suggest that processed **AW** (regardless of how it was processed) can potentially provide a comparable amino acid profile to **CF** as an indicator of quality for feeding to ragworms before their inclusion in aquafeeds.

5.3.1.5 Lipid Contents

Figure 5.5 shows the lipid contents of the feeds. Like what was observed in previous sections, the **AW** lipid content increases with better processing methods. **CF** and **AWCE** had the highest lipid contents (116.63 ± 13.72 mg g^{-1} and 112.15 ± 2.76 mg g^{-1}), which were statistically similar. **AWCH** followed (97.66 ± 13.72 mg g^{-1}), which was not significantly different from **CF** and **AWCE** or **AWSE** and **AWFD**. Finally, **AWSE** and **AWFD** followed (70.73 ± 2.90 mg g^{-1} and 60.41

± 7.71 mg g^{-1}), which were statistically similar. Again, the order was the same as was observed in protein, C, N and TOM contents.

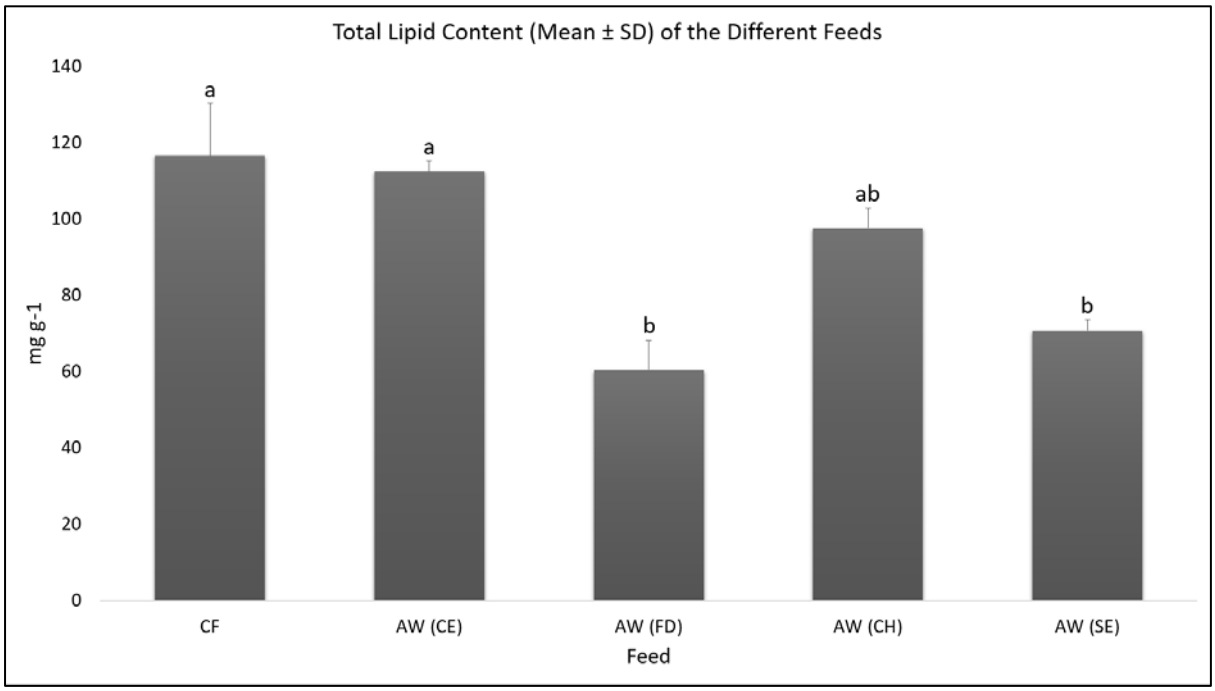

Figure 5.5 – Total lipid content (mean ± SD) of the CF and processed AW
CF = commercial fish feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. Different letters indicate significant differences between feeds ($P < 0.05$).

The differences again highlight the impact of various treatment processes on the nutritional content in **AW**, which could be significant if the intention is to feed ragworms before processing them into high-quality aquafeed ingredients.

5.3.1.6 Fatty Acid Profiles

Table 5.1 provides the mean ± SD of the fatty acid composition (also expressed as percentages of total fatty acids) for the different feeds used in the experiment. The concentration of FA in the feeds did not follow the same order as was seen in previously described analyses. **AWCE** had the highest concentration of total FA with a value of 74.29 ± 0.69 mg g^{-1}. Closely following and statistically similar were **CF** and **AWCH** with 72.25 ± 2.29 and 68.03 ± 7.76 mg g^{-1}, respectively. Then, significantly lower was **AWSE** (49.15 ± 4.39 mg g^{-1}), and finally, significantly lower again was **AWFD** (28.62 ± 3.65 mg g^{-1}).

The concentration of SFA did not follow the same order as the total concentrations of FA. **AWCH** and **AWCE** had the highest concentrations of SFA with values of 49.67 ± 5.66 and 41.44 ± 0.38 mg g^{-1}, respectively. **AWCH** was significantly higher than all other feeds except for **AWCE**. **AWSE**, **CF** and **AWFD** followed with values of 35.07 ± 3.13, 25.13 ± 1.18 and 20.41 ± 2.69 mg g^{-1}, respectively. **AWSE** was statistically similar to **AWCE** and **CF** but not **AWFD**. **AWFD** was significantly lower than all other groups except for **CF**. The percentage composition of SFA in the **AW** feeds was very similar (≈71.33 - ≈73.00%), except for **AWCE** (≈55.84%). The percentage composition of SFA in the **CF** was ≈34.54%.

The concentrations of monounsaturated fatty acids (MUFA) had a different order than SFA and total FA concentrations. **AWCE** had a significantly higher concentration than all other groups, with a value of 21.68 ± 0.20 mg g^{-1}. **AWCH**, **CF** and **AWSE** followed with statistically similar values of 13.96 ± 1.59, 12.36 ± 0.58 and 12.35 ± 1.10 mg g^{-1}, respectively. Significantly lower than all other groups was **AWFD**, with a value of 6.02 ± 0.77 mg g^{-1}. The percentage composition of MUFA in the **AW** feeds ranged between ≈20.52 and ≈29.22%. The **CF** was lowest at ≈16.99%.

The concentrations of polyunsaturated fatty acids (PUFA) again had a different order than MUFA, SFA and total FA. The **CF** had a significantly higher concentration of PUFA than all other groups at 35.26 ± 0.53 mg g^{-1}. **AWCE** followed with a value of 11.09 ± 0.10 mg g^{-1}. Then, significantly lower, **AWCH** followed with a value of 4.41 ± 0.50 mg g^{-1}. Finally, significantly lower again but statistically similar to each other, **AWFD** and **AWSE** followed with values of 2.19 ± 0.28 and 1.72 ± 0.15 mg g^{-1}, respectively. The percentage composition of PUFA in all feeds varied. **CF** had a much higher percentage composition than all other groups (≈48.47%). The **CF** was followed by **AWCE** (≈14.95%), **AWFD** (≈7.64%), **AWCH** (≈6.48%) and **AWSE** (≈3.50%).

The FA with the highest concentration in all feeds was the SFA palmitic acid (C16:0), with values ranging between 11.03 ± 1.41 and 25.40 ± 2.90 mg g^{-1} making up ≈20.54% of total FA in the **CF** and between ≈31.37 and ≈38.53% in the **AW** feeds. The next FA with the highest concentration in the **CF** was the omega-3 PUFA eicosapentaenoic acid (EPA), with a value of 11.20 ± 0.02 mg g^{-1} (≈15.39%). In the **AW** feeds, except for **AWCE**, the next FA with the highest concentration was stearic acid (octadecanoic acid) (C18:0), ranging between 4.33 ± 0.55 and 11.82 ± 1.35 mg g^{-1} (≈15.14 – ≈20.10%). In the **AWCE**, the FA with the next highest

concentration was oleic acid/elaidic acid (C18:1), with a value of 17.22 ± 0.16 mg g^{-1} (≈23.20%).

In addition to EPA, the other essential omega-3 FA, docosahexaenoic acid (DHA), was far higher in terms of concentration and percentage composition than the **AW** feeds. For example, in **CF**, the concentration was 11.00 ± 0.01 mg g-1 (≈15.12%); in the **AW** feeds, it ranged between 0.11 ± 0.01 and 0.57 ± 0.01 mg g^{-1} (≈0.22 and ≈0.76%).

Table 5.1 – Means ± SD (% of total fatty acids) fatty acid profiles of the different feeds

	Feed				
	CF	**AW (CE)**	**AW (FD)**	**AW (CH)**	**AW (SE)**
Σ FA (mg g^{-1})	72.25 ± 2.29^{a}	74.29 ± 0.69^{a}	28.62 ± 3.65^{c}	68.03 ± 7.76^{a}	49.15 ± 4.39^{b}
FA	**FA Mean ± SD mg g^{-1} (% of Total FA)**				
C10:0	0.02 ± 0.00^{b} (≈0.03%)	0.06 ± 0.00^{a} (≈0.08%)	0.02 ± 0.00^{b} (≈0.08%)	0.01 ± 0.00^{b} (≈0.02%)	0.01 ± 0.00^{b} (≈0.02%)
C11:0	-	0.02 ± 0.00 (≈0.03%)	-	-	-
C12:0	0.13 ± 0.01^{b} (≈0.18%)	0.47 ± 0.00^{a} (≈0.64%)	0.13 ± 0.02^{b} (≈0.46%)	0.07 ± 0.01^{c} (≈0.10%)	0.04 ± 0.00^{c} (≈0.08%)
C13:0	0.04 ± 0.00^{b} (≈0.05%)	0.10 ± 0.00^{a} (≈0.14%)	0.03 ± 0.00^{b} (≈0.09%)	0.12 ± 0.01^{a} (≈0.18%)	0.05 ± 0.00^{b} (≈0.10%)
C14:0	3.63 ± 0.17^{b} (≈4.99%)	7.00 ± 0.06^{a} (≈9.43%)	3.10 ± 0.40^{b} (≈10.83%)	6.44 ± 0.73^{a} (≈9.47%)	3.82 ± 0.34^{b} (≈7.77%)
C15:0	0.63 ± 0.03^{d} (≈0.86%)	2.11 ± 0.02^{b} (≈2.84%)	1.18 ± 0.15^{cd} (≈4.11%)	3.39 ± 0.39^{a} (≈4.99%)	1.55 ± 0.14^{bc} (≈3.15%)
C16:0	14.94 ± 0.70^{c} (≈20.54%)	23.38 ± 0.22^{ab} (≈31.37%)	11.03 ± 1.41^{c} (≈38.53%)	25.40 ± 2.90^{a} (≈37.33%)	17.58 ± 1.57^{bc} (≈35.76%)
C17:0	0.48 ± 0.02^{c} (≈0.66%)	1.09 ± 0.01^{b} (≈1.47%)	0.50 ± 0.06^{c} (≈1.74%)	1.70 ± 0.19^{a} (≈2.51%)	1.52 ± 0.14^{ab} (≈3.09%)
C18:0	4.88 ± 0.23^{c} (≈6.71%)	7.09 ± 0.07^{bc} (≈9.55%)	4.33 ± 0.55^{c} (≈15.14%)	11.82 ± 1.35^{a} (≈17.37%)	9.88 ± 0.88^{ab} (≈20.10%)
C20:0	0.12 ± 0.01^{bc} (≈0.17%)	0.22 ± 0.00^{b} (≈0.29 %)	0.10 ± 0.01^{c} (≈0.35%)	0.42 ± 0.05^{a} (≈0.62%)	0.38 ± 0.03^{a} (≈0.77%)

C21:0	-	-	-	0.04 ± 0.01[a] (≈0.06%)	0.04 ± 0.00[a] (≈0.08%)
C22:0	0.06 ± 0.00[b] (≈0.08%)	-	-	0.19 ± 0.02[a] (≈0.28%)	0.17 ± 0.02[a] (≈0.35%)
C24:0	0.20 ± 0.00[a] (≈0.27%)	-	-	0.05 ± 0.01[b] (≈0.07%)	0.04 ± 0.00[b] (≈0.09%)
Σ SFA	**25.13 ± 1.18[cd] (≈34.54%)**	**41.44 ± 0.38[ab] (≈55.84%)**	**20.41 ± 2.60[d] (≈71.33%)**	**49.67 ± 5.66[a] (≈73.00%)**	**35.07 ± 3.13[bc] (≈71.36%)**
C14:1 (ω -5)	0.01 ± 0.00[c] (≈0.01%)	0.06 ± 0.00[a] (≈0.08%)	0.05 ± 0.01[a] (≈0.16%)	0.06 ± 0.01[a] (≈0.09%)	0.03 ± 0.00[b] (≈0.06%)
C16:1 (ω – 7)	1.16 ± 0.05[c] (≈1.59%)	1.86 ± 0.02[b] (≈2.50%)	1.29 ± 0.16[bc] (≈4.52%)	2.54 ± 0.29[a] (≈3.73%)	1.40 ± 0.12[bc] (≈2.84%)
C17:1 (ω -7)	-	0.54 ± 0.00[a] (≈0.72%)	0.02 ± 0.00[d] (≈0.08%)	0.10 ± 0.01[c] (≈0.15%)	0.23 ± 0.02[b] (≈0.46%)
C18:1 (ω – 9)	9.27 ± 0.44[b] (≈12.75%)	17.22 ± 0.16[a] (≈23.20%)	3.50 ± 0.45[c] (≈12.23%)	7.71 ± 0.88[b] (≈11.33%)	8.24 ± 0.74[b] (≈16.78%)
C20:1 (ω – 9)	1.19 ± 0.06[a] (≈1.63%)	0.29 ± 0.00[c] (≈0.40%)	0.62 ± 0.08[bc] (≈2.17%)	1.57 ± 0.18[a] (≈2.31%)	0.71 ± 0.06[b] (≈1.45%)
C22:1 (ω – 9)	0.69 ± 0.03[b] (≈0.95%)	1.59 ± 0.01[a] (≈2.14%)	0.50 ± 0.06[b] (≈1.74%)	1.77 ± 0.20[a] (≈2.60%)	1.53 ± 0.14[a] (≈3.11%)
C24:1 (ω -9)	0.04 ± 0.00[c] (≈0.05%)	0.13 ± 0.00[b] (≈0.18%)	0.04 ± 0.00[c] (≈0.13%)	0.21 ± 0.02[a] (≈0.31%)	0.21 ± 0.02[a] (≈0.43%)
Σ MUFA	**12.36 ± 0.58[b] (≈16.99%)**	**21.68 ± 0.20[a] (≈29.22%)**	**6.02 ± 0.77[c] (≈21.03%)**	**13.96 ± 1.59[b] (≈20.52%)**	**12.35 ± 1.10[b] (≈25.13%)**
C18:2 (ω – 6)	10.27 ± 0.48[a] (≈14.12%)	5.26 ± 0.05[b] (≈7.09%)	1.28 ± 0.16[d] (≈4.46%)	2.53 ± 0.29[c] (≈3.72%)	0.59 ± 0.05[d] (≈1.20%)
C20:2 (ω – 6)	0.29 ± 0.01[d] (≈0.40%)	3.06 ± 0.03[a] (≈4.12%)	0.41 ± 0.05[d] (≈1.43%)	1.20 ± 0.14[b] (≈1.77%)	0.75 ± 0.07[c] (≈1.53%)
C20:3 (ω – 3)	0.10 ± 0.00[b] (≈0.14%)	0.26 ± 0.00[a] (≈0.35%)	0.05 ± 0.01[c] (≈0.18%)	0.05 ± 0.01[c] (≈0.07%)	0.03 ± 0.00[d] (≈0.06%)
C20:4 (ω – 6)	1.20 ± 0.00[a] (≈1.65%)	0.09 ± 0.00[b] (≈0.12%)	0.02 ± 0.00[d] (≈0.08%)	0.07 ± 0.01[c] (≈0.11%)	0.03 ± 0.00[d] (≈0.05%)
C20:5 (EPA) (ω – 3)	11.20 ± 0.02[a] (≈15.39%)	0.96 ± 0.01[b] (≈1.30%)	0.21 ± 0.03[d] (≈0.74%)	0.31 ± 0.04[c] (≈0.46%)	0.19 ± 0.02[d] (≈0.38%)

C22:4 (ω – 6)	-	0.04 ± 0.00 (≈0.05%)	-	-	-
C22:5 (ω – 3)	1.20 ± 0.01[a] (≈1.65%)	-	0.11 ± 0.01[b] (≈0.38%)	0.06 ± 0.01[c] (≈0.09%)	0.03 ± 0.00[c] (≈0.06%)
C22:6 (DHA) (ω – 3)	11.00 ± 0.01[a] (≈15.12%)	0.57 ± 0.01[b] (≈0.76%)	0.11 ± 0.01[d] (≈0.37%)	0.18 ± 0.02[c] (≈0.27%)	0.11 ± 0.01[d] (≈0.22%)
Σ PUFA	**35.26 ± 0.53[a] (≈48.47%)**	**11.09 ± 0.10[b] (≈14.95%)**	**2.19 ± 0.28[d] (≈7.64%)**	**4.41 ± 0.50[c] (≈6.48%)**	**1.72 ± 0.15[d] (≈3.50%)**

Different superscript letters indicate significant differences in individual FA between feeds; a - d indicate descending concentrations ($p > 0.05$). **CF** = commercial fish feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated aquaculture wastewater; **AWSE** = settled aquaculture wastewater. **SFA** = saturated fatty acids; **MUFA** = monounsaturated fatty acids; **PUFA** = polyunsaturated fatty acids. **Σ** = Total; **ω - n** = omega – position of first double bond from the terminal methyl group of chemical structure; **Cn:n** = number of carbons:number of double bonds.

Overall, the **CF** exhibits the highest PUFA content and a well-balanced fatty acid profile, whereas the **AW**-derived feeds, notably **AWCH**, show higher SFA proportions and lower PUFA proportions, again, highlighting the impact of different wastewater processing methods on the nutritional quality of the **AW**.

5.3.2 Ragworms

The sections below contain the results of ragworm analyses (growth, mortality, and biochemical analysis) that were carried out at the end of the experiment.

5.3.2.1 Growth

Figure 5.6 shows the mean wet weights ± standard deviation of ragworms before and after the experiment across the different feed groups. All feed group's final mean wet weights were significantly higher than their initial mean wet weights, indicating growth regardless of how the **AW** was processed. The initial mean wet weights ± SD ranged between 62.84 ± 31.53 and 74.72 ± 37.83 mg and did not differ significantly from each other. Group 1 (**CF**) and group 2 (**AWCE**) had the highest final mean wet weights of 241.32 ± 113.19 and 211.82 ± 89.62 mg, respectively. Group 1 final mean wet weights were significantly higher than all other groups except for group 2. Group 4 (**AWCH**) had the next highest final mean wet weight with a value

of 167.43 ± 109.24, which was statistically similar to group 2 and group 5 (**AWSE**). Group 5 had a value of 124.96 ± 62.60, followed by group 3 (**AWFD**), which was 105.62 ± 55.41, and both were statistically similar.

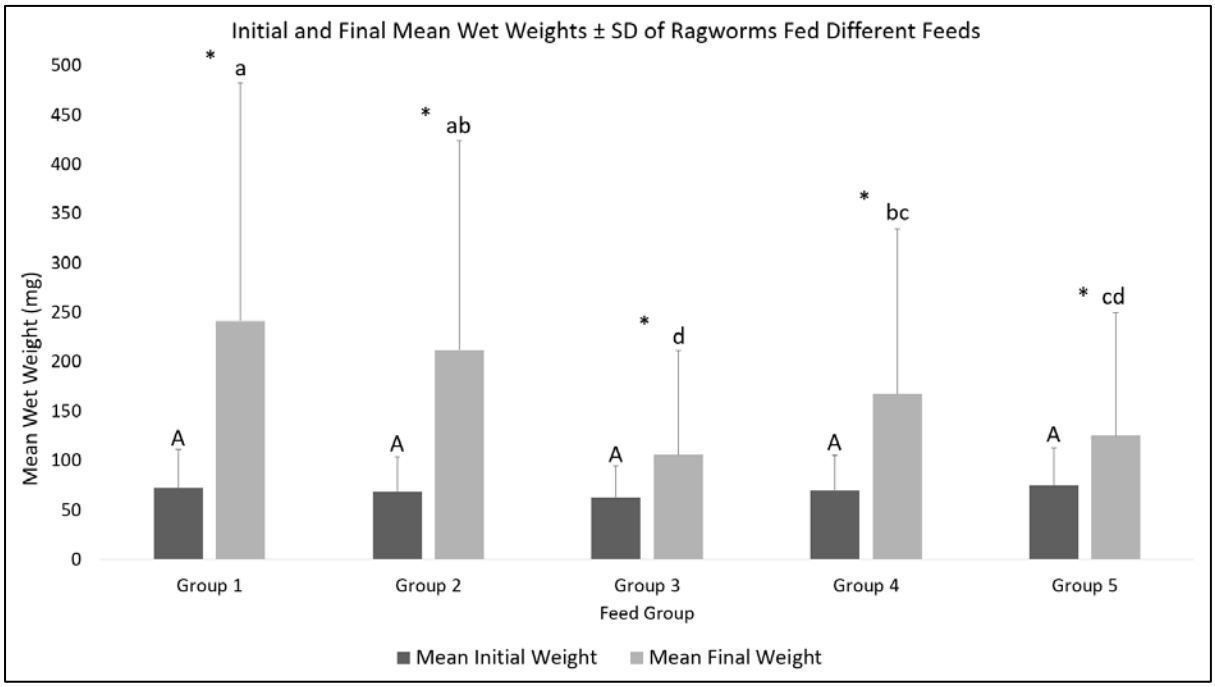

Figure 5.6 – Initial and final mean wet weights ± SD of ragworms fed CF or processed AW
Group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Significant differences between initial and final mean wet weights within groups are marked with an asterisk (*). Significant differences in initial mean wet weights between groups are marked by different uppercase letters. Significant differences in final mean wet weights between groups are marked by different lowercase letters ($P < 0.05$).

Group 1 (ragworms fed **CF**) exhibited the most significant growth, ending with the highest mean wet weight, indicating the effectiveness of this feed. Group 4 (ragworms fed **AWCH**) also showed significant growth, though not as high as Group 1. Initial mean weights were consistent across all groups, indicating similar starting conditions. Final mean weights show that while commercial feed leads to the highest growth, the action of using chitosan to coagulate and flocculate **AW** also supports significant growth in ragworms.

Figure 5.7 shows the specific growth rates (SGR) (mean ± standard deviation) of ragworms fed the different feeds. The SGR was highest in group 1 (**CF**) and group 2 (**AWCE**), respectively, with values of 0.042 ± 0.010 d^{-1} (4.3 ± 1.1 % d^{-1}) and 0.041 ± 0.003 d^{-1} (4.2 ± 0.3 % d^{-1}), which, like Figure 5.1 above, were also statistically similar. Group 4 (**AWCH**) followed with a value of 0.029 ± 0.007 d^{-1} (2.9 ± 0.7 % d^{-1}) which was not significantly higher or lower than any other

groups. The lowest SGR was recorded in group 3 (**AWFD**) and group 5 (**AWSE**), respectively, with values of 0.019 ± 0.005 d^{-1} (1.9 ± 0.6) % d^{-1}) and 0.019 ± 0.005 d^{-1} (1.9 ± 0.5 % d^{-1}), respectively, which were both significantly lower than groups 1 and 2, but not group 4.

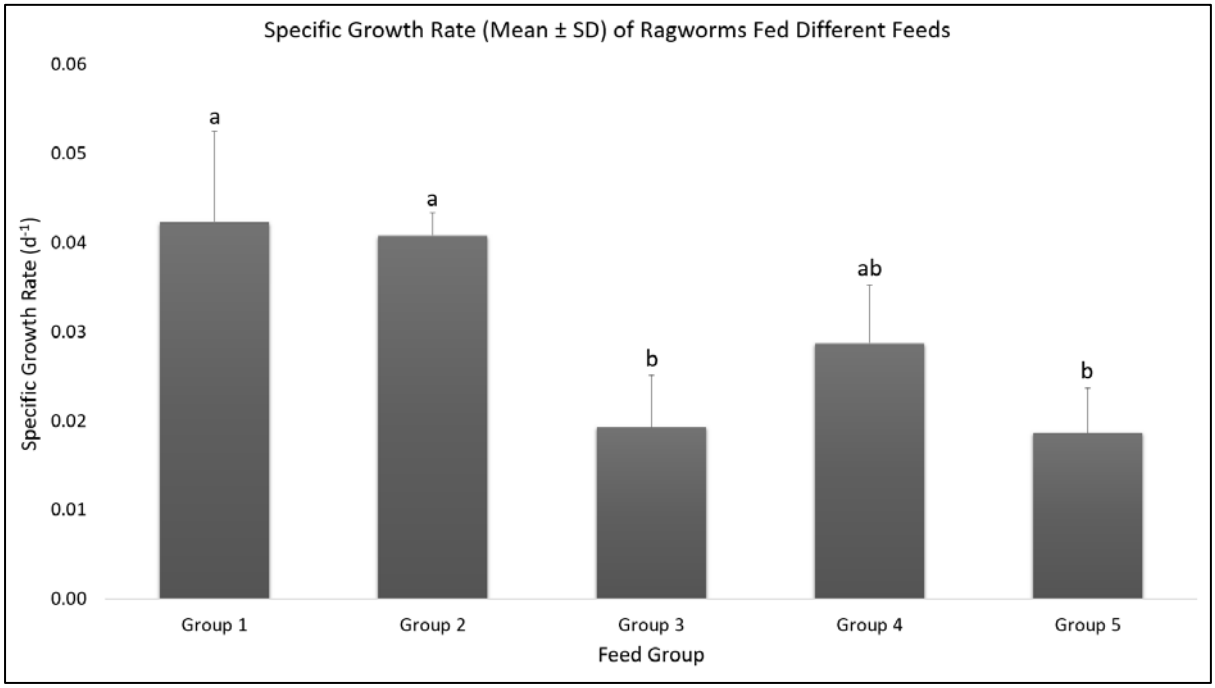

Figure 5.7 – Specific growth rates (mean ± SD) of ragworms fed CF or processed AW
Group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different letters indicate significant differences between groups ($P < 0.05$).

Group 1 (ragworms fed **CF**) and Group 2 (ragworms fed **AWCE**) exhibit the highest specific growth rates, suggesting these feeds are the most effective for promoting growth. Group 4 (ragworms fed **AWCH**) shows a moderately high specific growth rate, indicating moderate effectiveness. Groups 3 and 5 (ragworms fed **AWFD** and **AWSE**) have the lowest specific growth rates, indicating these feeds are the least effective for promoting growth. The significant differences between groups highlight the varying effectiveness of processing **AW** in different ways on the growth of ragworms and reflect what was observed in the nutritional contents of the feeds presented in Section 5.3.1 – Feeds. In other words, less intensive ways of treating the **AW** before feeding to the ragworms result in less growth.

5.3.2.2 Mortality

Figure 5.8 shows the percentage mortality (mean ± standard deviation) of ragworms fed the different feeds. No mortalities were recorded throughout the experiment in groups 1, 2 and 5. Group 3 had one mortality, and group 4 had 4 mortalities. All groups were statistically similar.

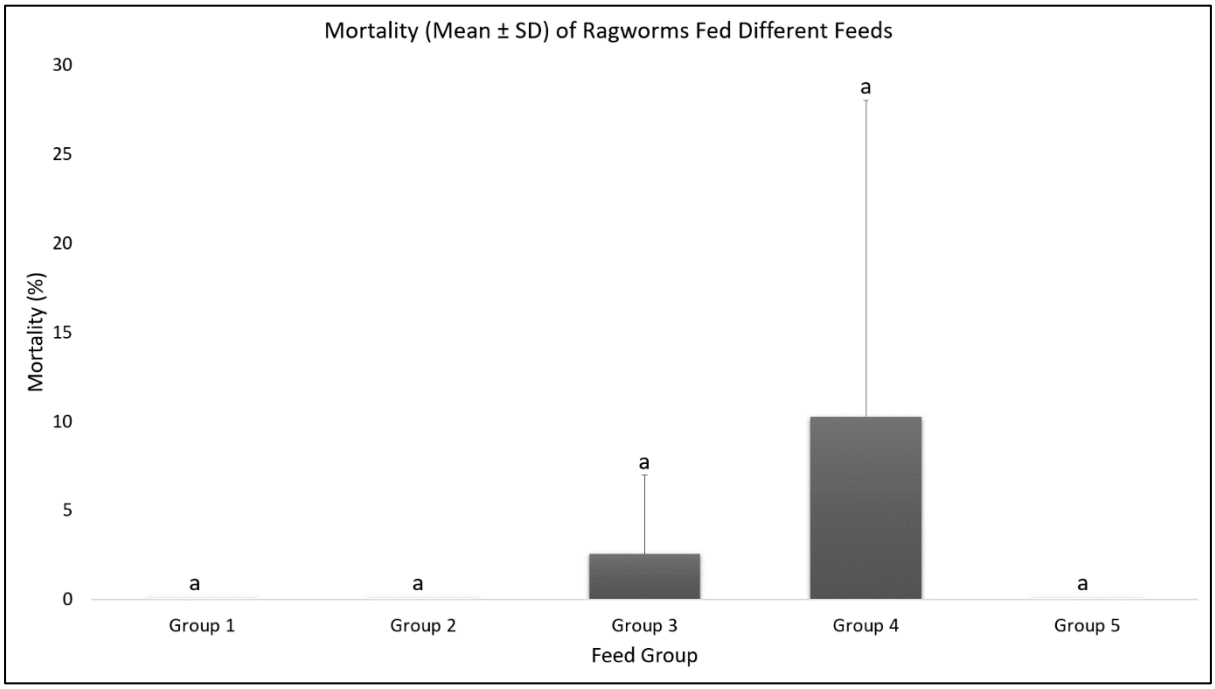

Figure 5.8 – Percentage mortality (mean ± SD) of ragworms fed CF or processed AW
Group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different letters indicate significant differences between groups ($P > 0.05$).

Overall, the mortality throughout the experiment was very low. The low mortality rates show that the experimental set-up was appropriate for rearing ragworms. The four mortalities in group 4 occurred in one replicate beaker in the second half of the experiment. Therefore, the mortalities attributed to group 4 are most likely due to an unexpected factor and not because of chitosan.

5.3.2.3 Total Organic Matter Contents

The ragworms' total organic matter (TOM) contents were analysed to understand ragworms fed with chitosan coagulated and flocculated **AW** quality as an aquafeed ingredient. The percentage TOM contents of the ragworms are displayed in Figure 5.9. Similar to the experiment described in chapter 4 and previous research (Seekamp, Reitan and Handå, 2017), the TOM contents of the ragworms were very similar, ranging between approximately 86.18 ± 5.70 and 90.08 ± 0.35%.

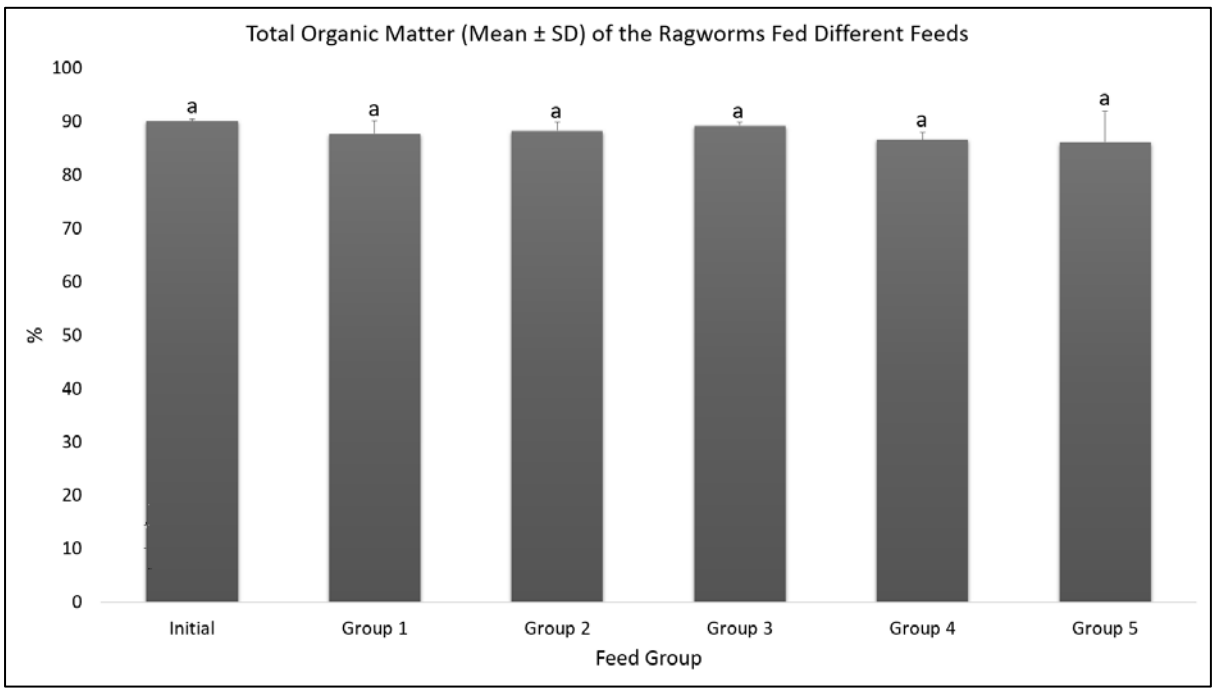

Figure 5.9 – Percentage total organic matter content (mean ± SD) of ragworms fed CF or processed AW Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different letters indicate significant differences between groups ($P > 0.05$).

The TOM content of the ragworms was similar to chapter 4, where TOM contents, regardless of feed, were statistically similar with values around 90%. Therefore, the TOM content of the ragworms was not impacted by how the feeds were processed.

5.3.2.4 Carbon and Nitrogen Contents

To understand ragworms fed **AWCH** quality as an aquafeed ingredient, the ragworms' carbon and nitrogen contents were also analysed (Figure 5.10). The initial group and group 1 (**CF**) had

significantly higher carbon contents (43.8 ± 0.98 & 45.59 ± 0.73%) than the other groups fed **AW** feeds, which ranged between 38.4 ± 0.91 and 40.26 ± 1.12% and were statistically similar.

The nitrogen contents in the ragworms were more varied than the carbon contents. Like the carbon contents, the nitrogen contents in the initial group and group 1 (9.6 ± 0.5 & 8.83 ± 0.38%) were higher than the groups fed **AW**, which ranged between 7.57 ± 0.24 and 8.7 ± 0.34%, but they did not differ significantly from group 3 (**AWFD**) and group 5 (**AWSE)**. On the other hand, groups 2 and 4 (**AWCE** and **AWCH**) had the lowest nitrogen contents, but group 2 was not significantly different to group 3 (**AWFD**) or group 5 (**AWSE**). Also, group 4 was not significantly different to group 2 or group 5.

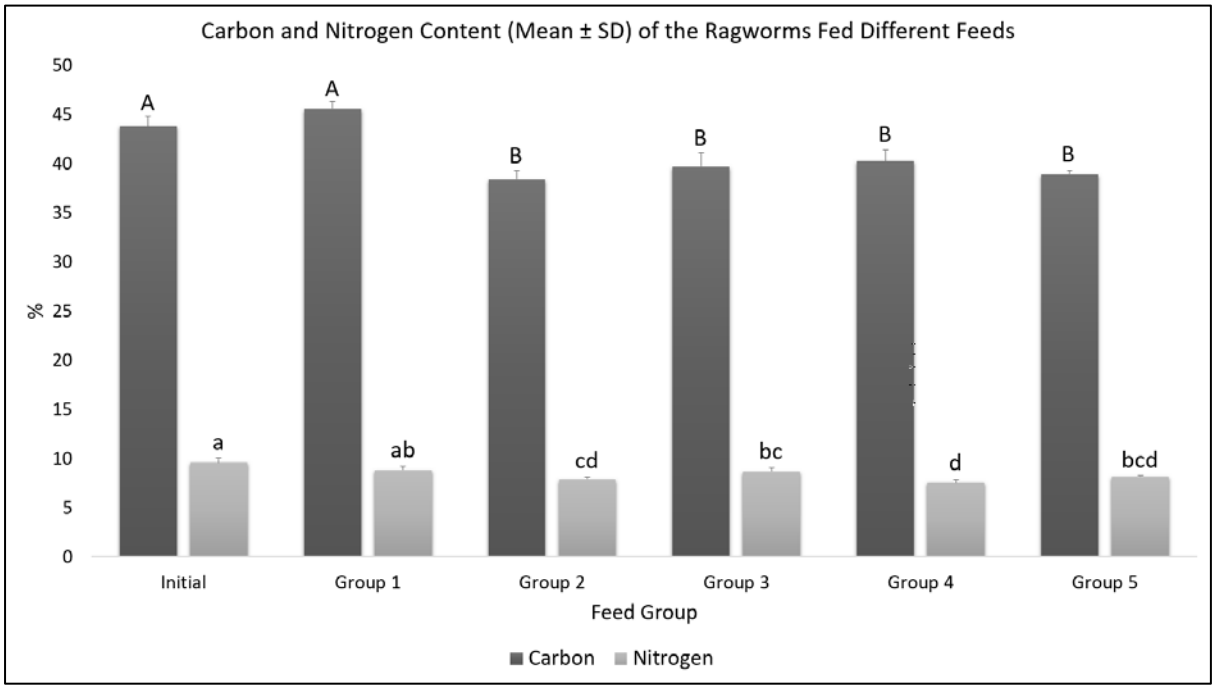

Figure 5.10 – Percentage carbon and nitrogen contents (mean ± SD) of the ragworms fed CF or processed AW

Initial = ragworms frozen and stored after the acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different uppercase letters indicate significant differences between carbon contents. Different lowercase letters indicate significant differences between nitrogen contents ($P > 0.05$).

The results show that ragworms fed **CF** (initial and group 1) had higher carbon and nitrogen contents than ragworms fed **AW** feeds. **CF** is a specifically formulated feed that includes all essential nutrients, vitamins, and trace elements that small (pond) fish require; therefore, the results are logical.

5.3.2.5 Protein Contents

Figure 5.11 shows the protein content (mean ± standard deviation) of the ragworms fed the different feeds. The initial group had the highest protein content with a concentration of 582.13 ± 9.21 mg g^{-1}, followed by group 1 (**CF**) (552.04 ± 23.59 mg g^{-1}) and group 3 (**AWFD**) (543.49 ± 21.03 mg g^{-1}). The initial group, group 1, and group 3, did not differ significantly. Then, groups 5, 2 and 4 followed (**AWSE**, **AWCE** and **AWCH**) respectively (510.94 ± 5.92 mg g^{-1}, 493.44 ± 12.26 mg g^{-1} & 472.87 ± 14.75 mg g^{-1}). Groups 5, 2 and 4 did not differ significantly from each other and group 5 also did not differ significantly from groups 1 or 3.

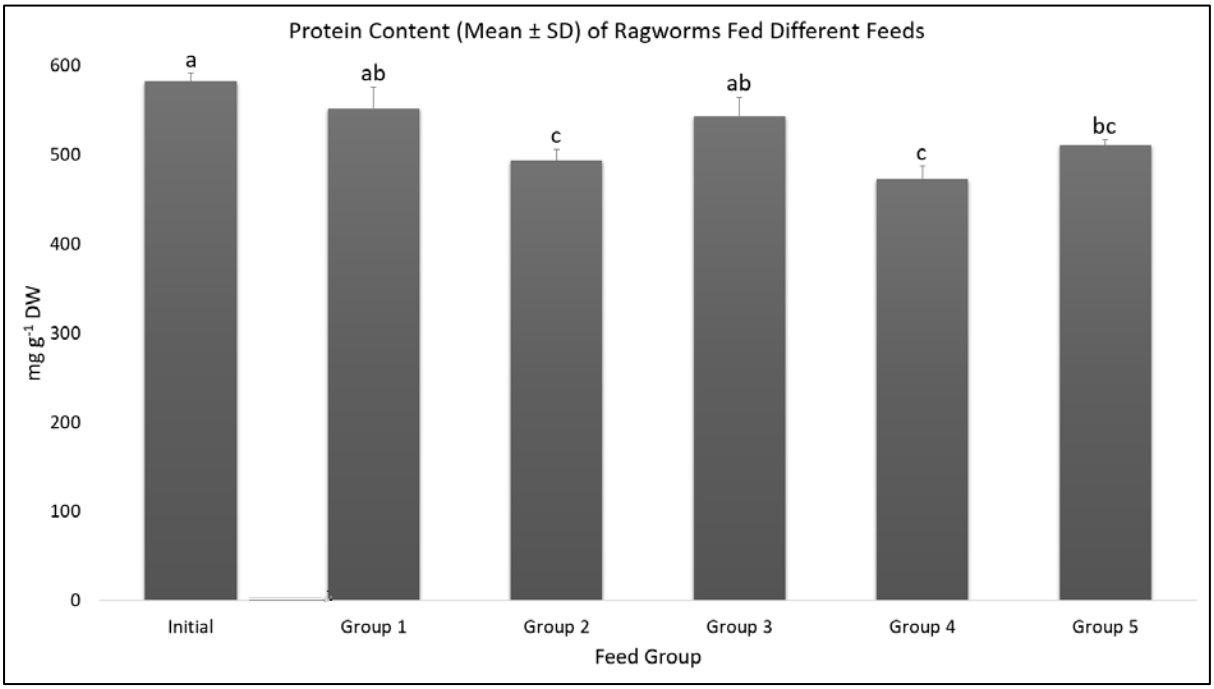

Figure 5.11 – Protein contents (mean ± SD) of the ragworms fed CF or processed AW
Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different letters indicate significant differences between groups ($P < 0.05$).

Group 1 (fed **CF**) and Group 3 (fed **AWFD**) are the most effective among the fed groups in maintaining protein levels (compared to the initial group), while Groups 2 (**AWCE**) and 4 (**AWCH**) show the least protein maintenance. Group 5 (**AWSE**) is intermediate in its effectiveness.

5.3.2.6 Amino Acid Profiles

Figure 5.12 illustrates the amino acid composition (% of total amino acids) of ragworms fed the different feeds. Group 5 (**AWSE**) and group 1 (**CF**) had the highest concentrations of AA (503.40 ± 8.42 mg g^{-1} and 494.56 ± 1.90 mg g^{-1}), which were statistically similar to each other. Group 2 (**AWCE**) and the initial group followed with values of 436.17 ± 0.37 and 429.75 ± 1.79 mg $g^{-1,}$ which were statistically similar. Lastly, group 4 (**AWCH**) and group 3 (**AWFD**) followed with values of 369.21 ± 19.38 and 358.66 ± 1.68 mg g^{-1}, which were also statistically similar.

Regarding EAA concentrations, the ragworms followed the same trend as the total AA concentrations. Group 5 (**AWSE**) had the highest mean EAA concentration (261.55 ± 0.53 mg g^{-1}), followed by group 1 (**CF**) (253.07 ± 1.42 mg g^{-1}), then the initial group (243.95 ± 0.36 mg g^{-1}), then group 2 (**AWCE**) (228.50 ± 1.42 mg g^{-1}), then group 4 (**AWCH**) (192.34 ± 10.45 mg g^{-1}), and finally group 3 (**AWFD**) (185.28 ± 9.92 mg g^{-1}). The initial group, group 1, and group 5, were statistically similar. The initial group was also statistically similar to group 2. Groups 3 and 4 were significantly lower than all other groups.

Ragworms in all groups contained all of the EAA for fish and shrimp (THR, LEU, MET, LYS, ARG, VAL, ILEU, HIS & PHE) (154) except for tryptophan (TYR). TYR cannot be identified using HPLC as hydrolysis damages it rendering it undetectable. The EAA with the highest percentage proportion varied between groups. In the initial group, it was ARG (≈9.48%). In groups 1 and 4, it was LYS (≈9.46 and ≈9.01%). In groups 2, 3 and 5, it was LEU (≈8.93, ≈8.82 and ≈9.06%).

Regarding NEAA concentrations, the ragworms followed the same trend as the total AA and EAA concentrations, except group 2 and the initial group switched positions. Group 5 (**AWSE**) had the highest mean NEAA concentration (241.84 ± 7.89 mg g^{-1}), followed by group 1 (**CF**) (241.49 ± 1.05 mg g^{-1}), then group 2, fed **AWCE** (207.67 ± 11.60 mg g^{-1}), then the initial group (185.80 ± 1.53 mg g^{-1}), then group 4 (**AWCH**) (176.87 ± 8.93 mg g^{-1}), and finally group 3 (**AWFD**) (173.38 ± 11.60 mg g^{-1}). Groups 5 and 1 were significantly higher than all other groups. Group 2 and the initial group were statistically similar. Groups 4 and 3 were significantly lower than all other groups, except for the initial group.

The NEAA detected in all ragworm samples were ASP, GLU, SER, GLY, ALA, TYR and ORN. The most prominent NEAA in percentage composition in all groups was GLU, between ≈14.11 and

≈16.33%. ASP and ALA were also significant in all groups, with an average of ≈9.23 and ≈7.92%, respectively.

Excluding the initial ragworm group, the percentage compositions of total EAA and NEAA were similar. The EAA ranged between ≈51.17 and ≈52.39%, and the NEAA ranged between ≈47.61 and ≈48.83%. The initial ragworm group was ≈56.76% and ≈43.24%, respectively.

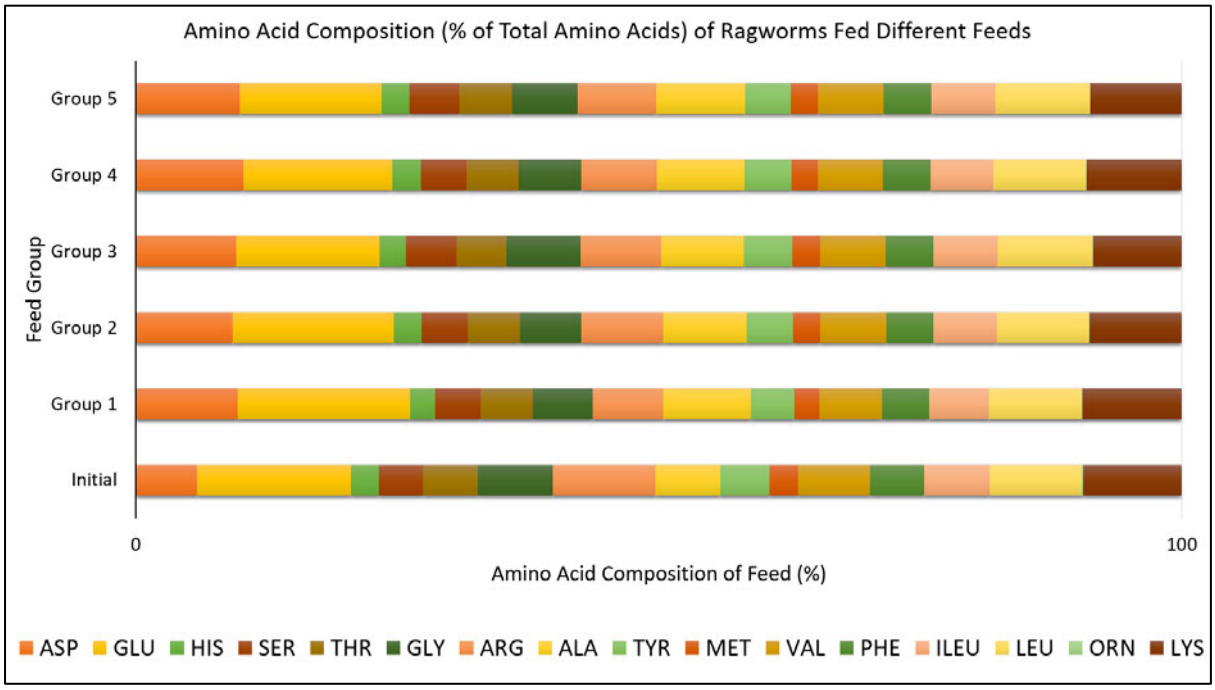

Figure 5.12 – Amino acid composition of the ragworms fed different feeds (% of total amino acids)
Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. ASP = aspartic acid; GLU = glutamic acid; HIS = histidine; SER = serine; THR = threonine; GLY = glycine; ARG = arginine; ALA = alanine; TYR = tyrosine; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; ORN = ornithine; LYS = lysine

These results suggest that ragworms can maintain a stable amino acid profile regardless of dietary variations, making them versatile in terms of their nutritional intake from different feed sources before their utilisation as an ingredient in aquafeeds. This is of particular interest regarding Group 4 (ragworms fed **AWCH**), if the intention is to use ragworms fed chitosan coagulated and flocculated aquaculture wastewater as ingredients in aquafeed.

5.3.2.7 Lipid Contents

Figure 5.13 illustrates the total lipid content (mean ± standard deviation) of the ragworms fed the different feeds. All ragworm feed groups had significantly higher lipid contents than the initial group of ragworms. Group 1 (**CF**) had the highest lipid content (100.9 ± 0.57 mg g^{-1}), followed by group 2 and group 4 (**AWCE** and **AWCH**) (80.75 ± 8.91 and 79.90 ± 3.68 mg g^{-1}). Groups 1, 2 and 4 were statistically similar. Group 3 and group 5 followed (**AWFD and AWSE**) (70.13 ± 8.91 and 62.71 ± 6.22 mg g^{-1}). Groups 3 and 5 were not significantly lower than groups 2 and 4 but were significantly lower than group 1.

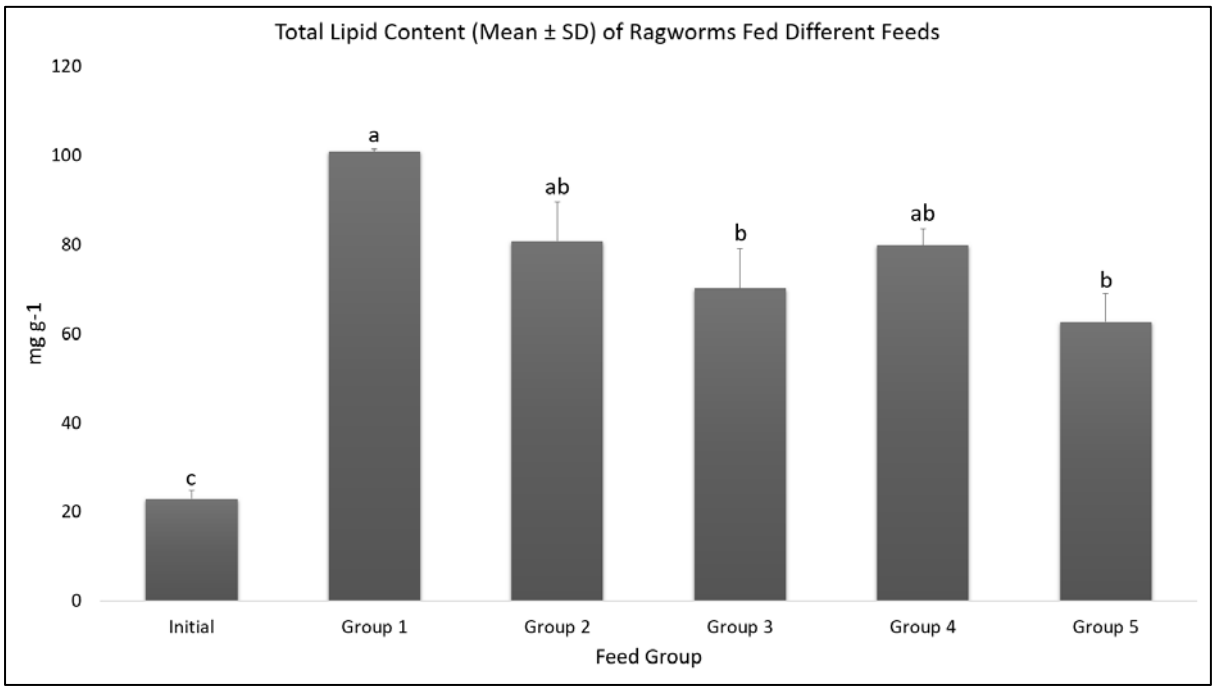

Figure 5.13 – Total lipid content (mean ± SD) of the ragworms fed CF or processed AW
Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. Different letters indicate significant differences between groups ($P < 0.05$).

The results demonstrate that feeding **CF** to ragworms will return a higher total lipid content in ragworms when compared to feeding them with **AW**. However, feeding the ragworms with either centrifuged (**AWCE**) or chitosan coagulated and flocculated (**AWCH**) **AW** showed that the total lipid contents were not massively impacted as they were statistically insignificant to group 1 (fed **CF**).

The ragworm results reflect the total lipid contents of their feeds (Figure 5.5) (Section 5.3.1.5 – Lipid Contents). In other words, the groups of ragworms that were fed with higher lipid-containing feeds returned higher lipid contents in their tissues after the experiment.

5.3.2.8 Fatty Acid Profiles

Table 5.2 summarises the fatty acid composition of the ragworms fed the different feeds, presented as mean ± SD (also presented as a percentage of total fatty acids).

The total FA concentration of ragworms fed **CF** was significantly higher than all other groups (74.49 ± 5.34 mg g^{-1}). Regardless of how it was processed, the groups fed **AW** had statistically similar concentrations of total FA ranging between 39.45 ± 3.91 mg g^{-1} in ragworms fed **AWSE** and 49.06 ± 3.31 mg g^{-1} in ragworms fed **AWCH**. All groups were significantly higher than the initial group of ragworms (10.02 ± 4.59 mg g^{-1}).

Concerning the SFA concentrations of the different ragworm groups, group 1 (**CF**) had the highest (20.31 ± 3.53 mg g^{-1}). **CF** was followed by group 2 (**AWCE**), group 4 (**AWCH**), group 3 (**AWFD**) and group 5 (**AWSE**) with concentrations of 19.45 ± 4.54, 16.50 ± 1.11, 15.43 ± 0.54 and 14.47 ± 1.44 mg g^{-1}, respectively, which were all statistically similar to **CF**. The initial group was significantly lower than all other groups (2.83 ± 1.30 mg g^{-1}). Regarding percentage composition, the ragworms fed **AW**, regardless of how it was processed, were relatively similar, with values ranging between ≈33.63 and ≈41.08%. The initial group and group 1 were similar, with percentage compositions of ≈28.24 and ≈27.26%, respectively.

Moreover, for the MUFA, group 1 (**CF**) had the highest concentration (27.35 ± 8.17 mg g^{-1}). Group 2 (**AWCE**), group 4 (**AWCH**), and group 3 (**AWFD**) were statistically similar to group 1 with values of 16.94 ± 3.95, 15.60 ± 1.05 and 15.56 ± 0.54 mg g^{-1}. Group 5 (**AWSE**) and the initial group were significantly lower than group 1, but not the other groups and had values of 13.51 ± 1.34 and 2.87 ± 1.31 mg g^{-1}, respectively. Regarding percentage composition, groups 1 – 5 were similar, with values ranging between ≈31.80 and ≈ 37.63%. The initial group had a lower value of ≈28.64%.

Regarding the PUFA concentrations, group 1 (**CF**) and group 4 (**AWCH**) ragworms had the highest concentrations and were statistically similar, with values of 26.84 ± 7.61 and 16.96 ±

1.14 mg g^{-1}, respectively. Group 5 (**AWSE**), group 2 (**AWCE**) and group 3 (**AWFD**) followed with values of 11.47 ± 1.14, 10.96 ± 2.56 and 10.36 ± 0.36 mg g^{-1}, respectively, which were statistically similar to group 4 (**AWCH**) but not group 1 (**CF**). Finally, the initial group had the lowest value of 4.32 ± 1.98 mg g^{-1}, significantly lower than group 1 (**CF**) and group 4 (**AWCH**). In percentage composition, the initial group had the highest value (≈43.12%). Group 1 (**CF**) and group 4 (**AWCH**) followed and were statistically similar (≈36.03 and ≈34.57%). Group 5 (A**WSE**), Group 3 (**AWFD**) and group 2 (**AWCE**) followed with values of ≈29.08, ≈25.05 and ≈23.14%, respectively.

The FA with the highest concentration varied between groups. In group 2 (**AWCE**), group 3 (**AWFD**), and group 4 (**AWCH**), the FA with the highest concentration was C16:0 with statistically similar values of 10.91 ± 2.54 (≈23.04%), 9.14 ± 0.32 (≈22.10%) and 9.67 ± 0.65 mg g^{-1} (≈19.71%), respectively. In group 1 (**CF**) and group 5 (**AWSE**), the FA with the highest concentration was C18:1 with statistically different values of 19.89 ± 7.26 (≈26.70%) and 8.09 ± 0.80 mg g^{-1} (≈20.52%). In the initial group, the FA with the highest concentration was linoleic acid (C18:2), with a value of 2.51 ± 1.15 mg g^{-1} (≈25.08%).

Regarding the essential omega-3 FA, EPA, all groups displayed a significantly higher concentration than the initial group, with values ranging between 3.16 ± 0.31 and 3.55 ± 0.24 mg g^{-1} and percentage compositions between ≈4.39 and ≈8.33%. In the initial group, the values were 0.40 ± 0.19 mg g^{-1} (≈4.03%). The values were varied in the other essential omega-3 FA, DHA. Only group 1 (**CF**) and group 2 (**AWCE**) had DHA concentrations significantly higher than the initial group. Groups 1 and 2 had values of 1.91 ± 0.09 (≈2.56%) and 0.61 ± 0.14 (≈1.29%) compared to 0.06 ± 0.03 mg g^{-1} (≈0.61%) in the initial group. Groups 3 (**AWFD**), group 4 (**AWCH**) and group 5 (**AWSE**) had values of 0.21 ± 0.01 (≈0.50%), 0.31 ± 0.02 (≈0.63%) and 0.25 ± 0.02 mg g^{-1} (≈0.62%), respectively.

Table 5.2 – Means ± SD (% of total fatty acids) fatty acid composition of ragworms fed different feeds

	Feed Group					
	Initial	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Σ FA (mg g^{-1})	10.02 ± 4.59^{c}	74.49 ± 5.34^{a}	47.34 ± 11.05^{b}	41.35 ± 1.44^{b}	49.06 ± 3.31^{b}	39.45 ± 3.91^{b}
FA	**FA Mean ± SD mg g^{-1} (% of Total FA)**					
C8:0	0.001 ± 0.00^{a} (≈0.01%)	0.01 ± 0.02^{a} (≈0.02%)	0.004 ± 0.00^{a} (≈0.01%)	0.006 ± 0.00^{a} (≈0.01%)	0.01 ± 0.00^{a} (≈0.01%)	0.004 ± 0.00^{a} (≈0.01%)
C10:0	0.003 ± 0.00^{a} (≈0.03%)	0.04 ± 0.04^{a} (≈0.06%)	0.03 ± 0.01^{a} (≈0.07%)	0.03 ± 0.00^{a} (≈0.07%)	0.01 ± 0.00^{a} (≈0.03%)	0.01 ± 0.00^{a} (≈0.03%)
C11:0	-	0.03 ± 0.03^{a} (≈0.04%)	0.01 ± 0.00^{a} (≈0.02%)	0.01 ± 0.00^{a} (≈0.01%)	0.01 ± 0.00^{a} (≈0.03%)	0.01 ± 0.00^{a} (≈0.03%)
C12:0	0.01 ± 0.00^{c} (≈0.08%)	0.09 ± 0.04^{ab} (≈0.13%)	0.13 ± 0.03^{a} (≈0.27%)	0.05 ± 0.00^{bc} (≈0.12%)	0.06 ± 0.00^{abc} (≈0.13%)	0.06 ± 0.01^{abc} (≈0.14%)
C13:0	-	0.04 ± 0.03^{a} (≈0.06%)	0.02 ± 0.01^{a} (≈0.05%)	0.01 ± 0.00^{a} (≈0.03)	0.02 ± 0.00^{a} (≈0.04%)	0.01 ± 0.00^{a} (≈0.03%)
C14:0	0.11 ± 0.05^{b} (≈1.15%)	1.34 ± 0.38^{a} (≈1.80%)	1.64 ± 0.38^{a} (≈3.47%)	1.15 ± 0.03^{ab} (≈2.79%)	0.90 ± 0.06^{ab} (≈1.84%)	0.79 ± 0.08^{ab} (≈2.00%)
C15:0	0.13 ± 0.06^{c} (≈1.27%)	0.95 ± 0.12^{a} (≈1.27%)	0.67 ± 0.16^{ab} (≈1.42%)	0.46 ± 0.01^{bc} (≈1.12%)	0.67 ± 0.05^{ab} (≈1.37%)	0.62 ± 0.06^{ab} (≈1.57%)
C16:0	1.80 ± 0.82^{b} (≈17.94%)	12.33 ± 2.38^{a} (≈16.55%)	10.91 ± 2.54^{a} (≈23.04%)	9.14 ± 0.32^{a} (≈22.10%)	9.67 ± 0.65^{a} (≈19.71%)	7.94 ± 0.79^{a} (≈20.13%)
C17:0	0.16 ± 0.08^{b} (≈1.65%)	1.00 ± 0.13^{a} (≈1.34%)	1.02 ± 0.24^{a} (≈2.14%)	0.83 ± 0.03^{a} (≈2.00%)	1.11 ± 0.07^{a} (≈2.26%)	1.19 ± 0.12^{a} (≈3.03%)
C18:0	0.60 ± 0.27^{b} (≈5.97%)	4.26 ± 0.33^{a} (≈5.72%)	4.79 ± 1.12^{a} (≈10.12%)	3.76 ± 0.14^{a} (≈9.10%)	3.97 ± 0.27^{a} (≈8.09%)	3.67 ± 0.36^{a} (≈9.30%)
C20:0	0.01 ± 0.01^{b} (≈0.14%)	0.15 ± 0.01^{a} (≈0.20%)	0.16 ± 0.04^{a} (≈0.33%)	0.10 ± 0.00^{a} (≈0.24%)	-	0.12 ± 0.01^{a} (≈0.30%)
C22:0	-	0.06 ± 0.02^{a} (≈0.08%)	0.07 ± 0.02^{a} (≈0.14%)	0.03 ± 0.00^{a} (≈0.07%)	0.06 ± 0.00^{a} (≈0.12%)	0.04 ± 0.00^{a} (≈0.11%)
Σ SFA	**2.83 ± 1.30^{b} (≈28.24%)**	**20.31 ± 3.53^{a} (≈27.26%)**	**19.45 ± 4.54^{a} (≈41.08%)**	**15.43 ± 0.54^{a} (≈37.31%)**	**16.50 ± 1.11^{a} (≈33.63%)**	**14.47 ± 1.44^{a} (≈36.67%)**
C14:1 (ω -5)	-	0.07 ± 0.06^{a} (≈0.10%)	0.03 ± 0.01^{a} (≈0.07%)	0.01 ± 0.00^{a} (≈0.02)	0.01 ± 0.00^{a} (≈0.03%)	0.02 ± 0.00^{a} (≈0.04%)
C16:1 (ω – 7)	0.23 ± 0.11^{c} (≈2.30%)	2.74 ± 0.25^{a} (≈3.68%)	1.87 ± 0.44^{ab} (≈3.95%)	1.54 ± 0.05^{b} (≈3.73%)	1.86 ± 0.13^{ab} (≈3.80%)	1.47 ± 0.15^{b} (≈3.73%)
C17:1 (ω -7)	0.05 ± 0.02^{a} (≈0.52%)	0.29 ± 0.34^{a} (≈0.39%)	0.16 ± 0.04^{a} (≈0.34%)	0.13 ± 0.00^{a} (≈0.31)	0.15 ± 0.01^{a} (≈0.30%)	0.10 ± 0.01^{a} (≈0.25%)

C18:1 (ω – 9)	2.18 ± 1.00^{b} (≈21.73%)	19.89 ± 7.26^{a} (≈26.70%)	10.08 ± 2.35^{ab} (≈21.28%)	8.57 ± 0.32^{ab} (≈20.73%)	9.33 ± 0.63^{ab} (≈19.01%)	8.09 ± 0.80^{b} (≈20.52%)
C20:1 (ω – 9)	0.35 ± 0.16^{b} (≈3.47%)	3.84 ± 0.16^{a} (≈5.16%)	3.95 ± 0.92^{a} (≈8.33%)	4.04 ± 0.15^{a} (≈9.78%)	3.75 ± 0.25^{a} (≈7.65%)	3.36 ± 0.33^{a} (≈8.51%)
C22:1 (ω – 9)	0.06 ± 0.03^{c} (≈0.62%)	0.50 ± 0.10^{ab} (≈0.68%)	0.82 ± 0.19^{a} (≈1.73%)	0.58 ± 0.02^{ab} (≈1.40%)	0.48 ± 0.03^{ab} (≈0.97%)	0.45 ± 0.04^{b} (≈1.14%)
C24:1 (ω -9)	-	-	0.03 ± 0.01^{a} (≈0.06%)	0.01 ± 0.00^{b} (≈0.01%)	0.02 ± 0.00^{ab} (≈0.04%)	0.02 ± 0.00^{ab} (≈0.06%)
Σ MUFA	**2.87 ± 1.31^{b} (≈28.64%)**	**27.35 ± 8.17^{a} (≈36.71%)**	**16.94 ± 3.95^{ab} (≈35.78%)**	**15.56 ± 0.54^{ab} (≈37.63%)**	**15.60 ± 1.05^{ab} (≈31.80%)**	**13.51 ± 1.34^{b} (≈34.25%)**
C18:2 (ω – 6)	2.51 ± 1.15^{a} (≈25.08%)	9.72 ± 6.36^{a} (≈13.05%)	3.21 ± 0.75^{a} (≈6.78%)	2.96 ± 0.10^{a} (≈7.15%)	7.05 ± 0.48^{a} (≈14.37%)	3.11 ± 0.31^{a} (≈7.89%)
C20:2 (ω – 6)	0.53 ± 0.24^{d} (≈5.32%)	5.43 ± 0.08^{a} (≈7.29%)	1.13 ± 0.26^{cd} (≈2.40%)	1.36 ± 0.04^{cd} (≈3.28%)	2.52 ± 0.17^{b} (≈5.13%)	1.79 ± 0.18^{c} (≈4.53%)
C20:3 (ω – 3)	0.37 ± 0.17^{b} (≈3.68%)	2.73 ± 0.20^{a} (≈3.66%)	0.34 ± 0.08^{b} (≈0.71%)	0.85 ± 0.00^{b} (≈2.06%)	0.45 ± 0.03^{b} (≈0.92%)	0.34 ± 0.03^{b} (≈0.86%)
C20:4 (ω – 6)	0.24 ± 0.11^{d} (≈2.36%)	2.08 ± 0.19^{a} (≈2.79%)	0.80 ± 0.19^{c} (≈1.68%)	0.65 ± 0.02^{cd} (≈1.58%)	1.35 ± 0.09^{b} (≈2.76%)	1.36 ± 0.14^{b} (≈3.45%)
C20:5 (EPA) (ω – 3)	0.40 ± 0.19^{b} (≈4.03%)	3.27 ± 0.51^{a} (≈4.39%)	3.33 ± 0.78^{a} (≈7.03%)	3.44 ± 0.12^{a} (≈8.33%)	3.55 ± 0.24^{a} (≈7.25%)	3.16 ± 0.31^{a} (≈8.00%)
C22:2 (ω – 6)	0.13 ± 0.06^{c} (≈1.26%)	0.97 ± 0.05^{a} (≈1.30%)	0.61 ± 0.14^{b} (≈1.29%)	0.48 ± 0.03^{a} (≈1.15%)	0.96 ± 0.06^{a} (≈1.95%)	0.82 ± 0.08^{ab} (≈2.08%)
C22:4 (ω – 6)	0.04 ± 0.02^{d} (≈0.45%)	0.48 ± 0.05^{a} (≈0.65%)	0.25 ± 0.06^{bc} (≈0.52%)	0.21 ± 0.01^{c} (≈0.51%)	0.44 ± 0.03^{a} (≈0.90%)	0.37 ± 0.04^{ab} (≈0.93%)
C22:5 (ω – 3)	0.03 ± 0.02^{c} (≈0.33%)	0.23 ± 0.04^{bc} (≈0.31%)	0.68 ± 0.16^{a} (≈1.44%)	0.74 ± 0.03^{a} (≈1.78%)	0.33 ± 0.02^{b} (≈0.68%)	0.28 ± 0.03^{bc} (≈0.71%)
C22:6 (DHA) (ω – 3)	0.06 ± 0.03^{c} (≈0.61%)	1.91 ± 0.09^{a} (≈2.56%)	0.61 ± 0.14^{b} (≈1.29%)	0.21 ± 0.01^{c} (≈0.50%)	0.31 ± 0.02^{c} (≈0.63%)	0.25 ± 0.02^{c} (≈0.62%)
Σ PUFA	**4.32 ± 1.98^{c} (≈43.12%)**	**26.84 ± 7.61^{a} (≈36.03%)**	**10.96 ± 2.56^{bc} (≈23.14%)**	**10.36 ± 0.36^{bc} (≈25.05%)**	**16.96 ± 1.14^{ab} (≈34.57%)**	**11.47 ± 1.14^{bc} (≈29.08%)**

Different superscript letters indicate significant differences in individual FA between feeds; a - d indicate descending concentrations ($p > 0.05$). Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. **SFA** = saturated fatty acids; **MUFA** = monounsaturated fatty acids; **PUFA** = polyunsaturated fatty acids. **Σ** = Total; **ω - n** = omega – position of first double bond from the terminal methyl group of chemical structure; **Cn:n** = number of carbons:number of double bonds.

The ragworms fed **AW** had very similar total fatty acid contents, however, those fed with chitosan coagulated and flocculated **AW** (**AWCH**) had a higher total PUFA with a percentage proportion similar to the ragworms fed **CF**. This suggests that the process of using chitosan to process **AW** before feeding to ragworms may be beneficial in terms of their fatty acid profile

making them a more suitable ingredient for inclusion in aquafeeds compared to other means of processing **AW**.

5.4 Discussion

5.4.1 Growth

The growth of ragworms was recorded to evaluate how each ragworm group responded to being fed **AW** that was processed differently or commercial fish feed (**CF**).

Overall, the ragworms grew well regardless of feed group, as demonstrated by the significantly higher weights at the experiment's end than the weights at the start. In chapter 4, this was observed in ragworms fed **CF**, and only one of the groups fed **AW**. The average ragworms' start weights in this experiment and chapter 4 were almost identical (69.61 and 69.94 mg, respectively). However, it is crucial to note that **AW** used in this experiment, albeit from the same aquaculture facility, was of a different batch, thus the nutritional composition was different. Therefore, at first look, it appears the batch of **AW** used in this experiment is better nutritionally than the batch used in the experiment described in chapter 4. The nutritional composition of the **AW** is described in greater detail in subsequent sections.

The growth results also show that ragworms grew well when fed **AWCH** (group 4). They grew more than ragworms fed **AWFD** and **AWSE** (groups 3 and 5). However, they did not grow as well as the ragworms fed **AWCE** (group 2). Moreover, they did not grow as well as the ragworms fed **CF** (group 1), which was included in the experiment as a control to compare ragworms fed **AWCH** to a nutritionally complete feed. Each method used to process the **AW** concentrates the feed to varying degrees, with a better concentration of the **AW**, resulting in better growth rates.

The mean SGR of ragworms fed **AW** in chapter 4 was 0.018 ± 0.010 d^{-1} (1.8 ± 0.6 % d^{-1}). In this experiment, the ragworms fed **AWFD** and **AWSE** (groups 3 and 5) had very similar SGRs of 0.019 ± 0.006 d^{-1} (1.9 ± 0.6 % d^{-1}) and 0.019 ± 0.010 d^{-1} (1.9 ± 0.5 % d^{-1}), respectively. The similar growth rates between experiments make sense as their feeds were both cleaner fish **AW** and processed in the case of group 3, the same way (freeze-drying). However, the SGR of ragworms fed **AWCH** was higher with a mean value of 0.029 ± 0.007 d^{-1} (2.9 ± 0.7 % d^{-1}),

further backing up the assumption that the more concentrated the feed, the better the growth performance. The ragworms fed **AWCE** (group 2), which was the most concentrated **AW** feed, had a much higher SGR than the ragworms fed **AW** in chapter 4, with a mean value of 0.041 ± 0.003 d^{-1} (4.2 ± 0.3 % d^{-1}).

Overall, by reviewing the growth performance of the ragworms fed **AW** that was processed in different ways, two conclusions pertinent to the hypothesis of this experiment can be made. Firstly, ragworms fed **AWCH** grew faster than ragworms fed **AW** using simple dewatering techniques (freeze-drying and settling). Secondly, ragworms fed **AWCH** do not grow to the same extent as ragworms fed centrifuged **AW**. However, the growth rates of ragworms fed **AWCH** were good, especially in comparison with similar research. For example, ragworms fed Atlantic salmon smolt (*Salmo salar*) **AW** had an average SGR of 0.012 d^{-1} (Wang *et al.*, 2019). Ragworms fed Beluga sturgeon (*Huso huso*) **AW** had an average SGR of 0.03 d^{-1} (O Pajand *et al.*, 2017). Ragworms fed European bass (*Dicentrarchus labrax*) and gilt-head sea bream (*Sparus aurata*) **AW** had SGR up to 0.02 d^{-1} (Bischoff, 2007). Therefore, the ragworms fed cleaner fish **AWCH** were higher than ragworms fed Atlantic salmon, Bass, and sea bream **AW** and similar to ragworms fed sturgeon **AW**. However, it is important to note that in the research mentioned, the duration of experiments varied.

Figure 5.14 shows the ragworms fed **AWCH** (group 4) before and after the experiment. At the end of the experiment, almost all of them were larger, denser, and deeper in colour.

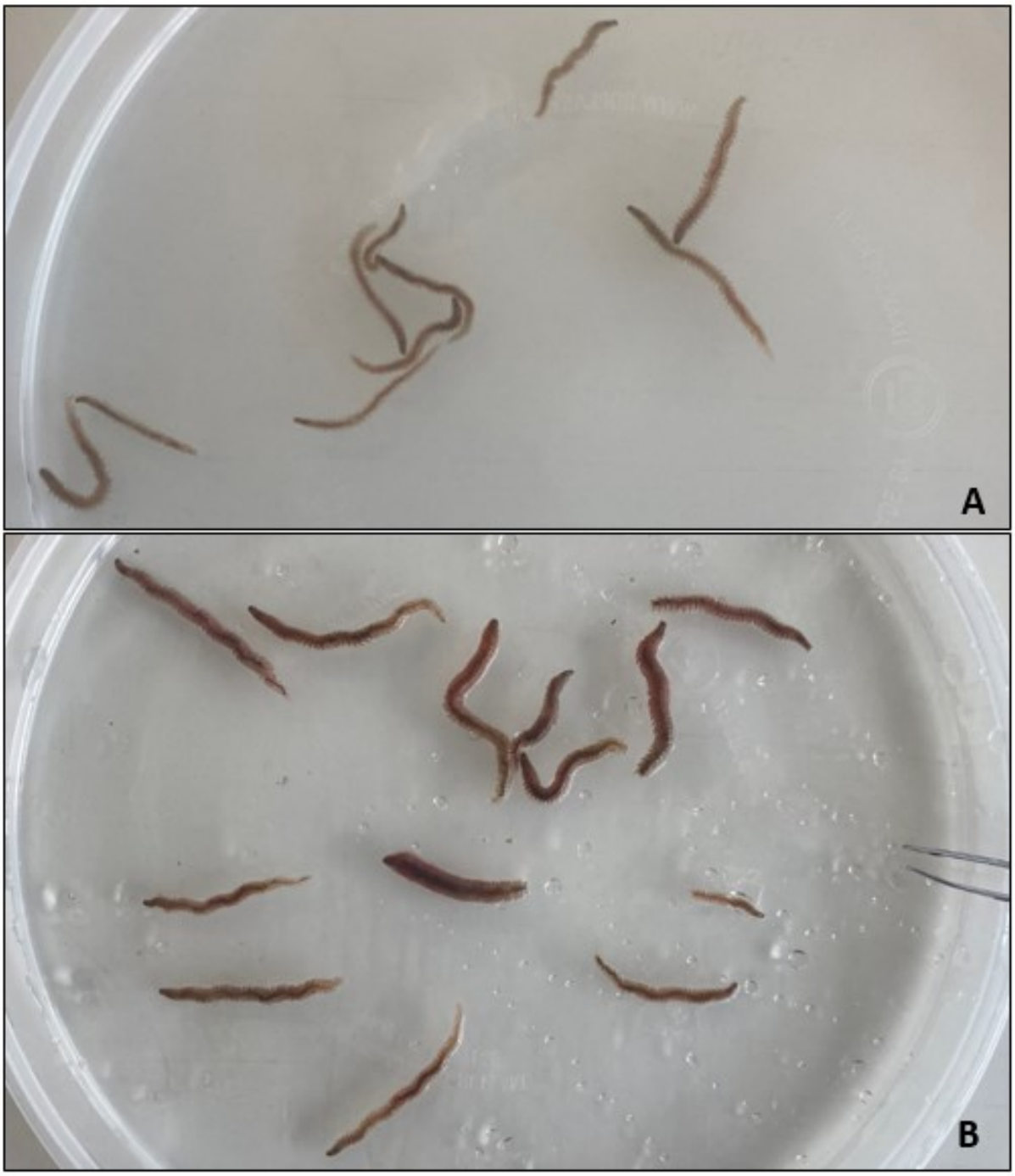

Figure 5.14 – Ragworms fed chitosan coagulated and flocculated aquaculture wastewater (group 4) (AWCH)
The top image shows the ragworms before the experiment (**A**), and the bottom image shows the ragworms after the experiment (**B**). Notice how much larger the ragworms in **B** are compared to the ragworms in **A**.

5.4.2 Mortality

Throughout the experiment, mortality was recorded to understand how the ragworms responded to being fed **AW** that has been processed in different ways.

No mortalities were recorded throughout the experiment in groups 1, 2 and 5. Group 3 had one mortality, and group 4 had 4 mortalities. All groups were statistically similar.

The reasons for the mortalities observed in one group 4 replicate culture beaker are unknown. Potential reasons, such as cannibalism, water quality issues, stress-related deaths, chitosan, or lack of food, can be ruled out because the mortalities were observed in one replicate culture beaker only. The replicate was not excluded due to the lack of significance between groups.

Thus, concerning the hypothesis of this experiment, it can be stated that feeding ragworms **AWCH** at a dose of 8 mg L^{-1} does not negatively impact the mortality of ragworms when compared to ragworms fed **AW** that was processed in different ways or ragworms fed **CF**. The mortality results agree with what was found in the experiment presented in chapter 4 (Section 4.3.2.2 – Mortality), where the mortalities in ragworms fed **AW** with chitosan incorporated did not differ significantly from ragworms fed **AW** without chitosan.

5.4.3 Total Organic Matter, Carbon and Nitrogen Contents of Feeds and Ragworms

The TOM content of the feeds partly explains the growth rates presented in Section 5.3.2.1 - Growth. The ragworms fed **CF** had the highest growth rate, followed by ragworms fed **AWCE**, **AWCH**, **AWSE** and **AWFD**. So, the feeds with higher TOM contents resulted in higher growth rates in ragworms. Therefore, it can be stated that coagulating and flocculating **AW** with chitosan create a feed with a higher energy content than freeze-drying or settling **AW**. However, it does not create a feed with a higher energy content than centrifuging **AW**.

Overall, referring to the hypothesis of this study, it can be concluded that using chitosan to coagulate and flocculate **AW** creates a feed with a higher TOM content than **AW** that has been freeze-dried or settled. However, the TOM content of chitosan-coagulated and flocculated **AW** is lower than that of centrifuged **AW**. Also, like chapter 4, the presence of chitosan does not impact the TOM content of the ragworms. Furthermore, processing the **AW** with different methods before feeding to ragworms does not impact their TOM content.

In addition to TOM, the feed's carbon and nitrogen contents were analysed. As discussed, a higher carbon content in the feed means more energy. Carbon is present in all macronutrients and is required to form carbohydrates, proteins, nucleic acid, and many other essential compounds. In addition, nitrogen is crucially important for living organisms as it is a component of amino acids, proteins, and DNA.

The carbon contents of the feeds, like the TOM contents, partly explain the growth rates of the ragworms discussed in Section 5.3.2.1 - Growth. The feeds with a higher carbon content have more energy than those with a lower carbon content. Therefore, the ragworms that consumed feeds with a higher carbon content had higher growth rates. Again, it can be stated that chitosan coagulation and flocculation of **AW** create a feed with a higher energy content than **AW** which has been processed by freeze-drying and settling. However, it does not create a feed with a higher energy content than centrifuging **AW**. As protein contains nitrogen, the values obtained here correspond to the protein contents of the feeds determined and discussed in greater detail in Section 5.4.4 (Protein Contents and Amino Acid Profiles).

Overall, relating to the hypothesis of the experiment, ragworms fed **AWCH** had statistically similar carbon contents to ragworms fed **AW** that were processed by centrifuging, freeze-drying, or settling. However, the nitrogen content of ragworms fed **AWCH** was lower than other groups fed **AW** processed in different ways but not significantly different to ragworms fed **AWCE** or **AWSE**. Therefore, ragworms fed **AWCH** quality as an aquafeed ingredient are similar to ragworms fed **AW** that were processed in different ways.

5.4.4 Protein Contents and Amino Acid Profiles

The protein contents were analysed to understand better how **AW**'s nutritional composition is impacted when coagulated and flocculated with chitosan.

The protein content of the feeds followed the same trend as the nitrogen contents presented in Section 5.3.1.2 – Carbon and Nitrogen Contents). Therefore, the correlation between nitrogen and protein contents is logical, as protein contains nitrogen, whereas carbohydrates and fats do not.

However, it was shown that the nitrogen contents of the ragworms were more varied than the feeds. Therefore, the ragworm protein contents should reflect what was observed in the nitrogen contents, which was the case (Figure 5.11). The ragworms that were given feeds with a higher protein content did not necessarily have higher protein contents, just like the ragworms that were given feeds with a higher nitrogen content did not have higher nitrogen contents.

Despite the ragworms in groups 2 and 4 (**AWCE** and **AWCH**) having feeds with higher protein contents than the other **AW** groups, their protein contents were lower. The lower protein contents are most likely due to their feed being more concentrated than others; therefore, they spent less time feeding, reducing muscle use. Increased muscle use builds more muscle, which consists of protein (Young and Schmidt-Nielsen, 1985). On the other hand, and discussed in greater detail later in Section 5.4.5 – Lipid Contents and Fatty Acid Profiles), the ragworms from groups 2 and 4 had higher lipid contents, meaning they also likely stored more energy as fat. In addition, their feeds had higher carbon contents meaning their food was more energy-dense, allowing more to be stored as fat. Carbohydrates (containing carbon, hydrogen, and oxygen) are an immediate energy source when broken down into glucose. Any excess glucose is converted into fatty acids and distributed in body tissues.

Overall, relating to the hypothesis of this experiment, coagulating and flocculating **AW** with chitosan creates a feed with a higher protein content than freeze-drying or settling **AW**. However, it does not create a feed with a higher protein content than centrifuging **AW**. When these feeds are fed to ragworms, the more concentrated **AWCE** and **AWCH** mean the ragworms use their muscles less and, therefore, put on less muscle compared to the other feeds, meaning they have a reduced protein content. However, the same was not observed in ragworms fed **CF**, but this was a specifically formulated feed that includes everything that growing organisms require; therefore, the ragworms should have high protein contents when provided with **CF**. Otherwise, it would not be a successful commercial feed.

Moreover, it is essential to note that the protein content of the **AW** used in this experiment was significantly lower than that of the **AW** used in the experiment described in chapter 4. The mean protein content of the batch of **AW** used in the experiment described in chapter 4 was 360.52 ± 7.24 mg g^{-1} compared to 73.66 ± 0.58 mg g^{-1} (**AWFD**) in this experiment. Furthermore, the protein content differs with different treatment methods to process the

AW. For example, 164.13 ± 5.31 mg g^{-1} for **AWCH** and 178.61 ± 1.85 mg g^{-1} for **AWCE**. Finding alternative aquafeed ingredients with a high protein content has been a focus of research for many years now (Ayadi, Rosentrater and Muthukumarappan, 2012). To create a suitable alternative aquafeed ingredient by feeding **AWCH** to ragworms, the **AW** must be a high-quality protein source so the ragworms can assimilate the protein, in turn producing an aquafeed ingredient that is a good source of protein.

Despite the protein contents of the feeds varying between batches and treatment methods, the protein contents of the ragworms were good, albeit lower than the ragworms analysed in the experiment described in chapter 4. In chapter 4, the ragworms fed different feeds had protein contents ranging between 55 and 64% of their total weight. In this experiment, protein contents were lower, ranging between 47 and 55%. Fish and shrimp have a high protein requirement ranging between 24 and 57% of their total dry weight of feed (Tacon, 1987). Therefore, despite the protein contents of the ragworms in this experiment being lower than the experiment described in chapter 4, they were still suitable to fulfil the protein requirements of many fish and shrimp species. The lower protein contents in the ragworms in this experiment compared to the experiment described in chapter 4 also highlights that the batch of **AW** used was not a great source of protein.

However, the quality of a protein source is not solely determined by the protein content. More critical is a favourable amino acid (AA) profile concerning the consumer's needs (Wang *et al.*, 2019). Favourable AA profiles are relevant to the feeds, and the ragworms as the ragworms will assimilate AA in the feed into their tissues. Therefore, the AA profiles of the feeds and the ragworms were analysed.

Relating the results of the feeds AA profiles to the hypothesis, it can be stated that coagulating and flocculating **AW** with chitosan, like TOM and protein contents, concentrates the **AW** more than freeze-drying or settling, but not centrifuging. In other words, the percentage composition of the AA in the **AW** feeds was similar, regardless of how it was processed. However, the total AA concentration increased as the **AW** was processed more effectively with chitosan or centrifuging.

Furthermore, the batch of **AW** used in this experiment had a lower AA concentration than the **AW** used in the experiment described in chapter 4. The same trend was seen in the total

protein contents between experiments. Protein primarily comprises AA, so a lower AA concentration is logical. In the chapter 4 experiment, the **AW** feeds total AA concentration ranged between 188.65 ± 1.79 and 195.22 ± 0.18 mg g^{-1}. In this experiment, the **AW** feeds total AA concentration ranged between 30.20 ± 0.05 and 137.59 ± 0.12 mg g^{-1}. It was discussed in chapter 4 that the batch of **AW** used was a high-quality source of protein for ragworms before their inclusion in aquafeeds. However, the batch of **AW** used in this experiment had a much lower total AA concentration and, therefore, was perhaps not of the same nutritional quality as the batch used in the chapter 4 experiment.

The AA profiles in the ragworms were well-balanced. However, the total concentrations of AA for ragworms fed **AWCH** and **AWFD** were lower than those fed **AW** in the experiment described in chapter 4. In this experiment, only group 1 (**CF**), group 2 (**AWCE**) and group 5 (**AWFD**) had total AA concentrations higher than the initial group of ragworms. Moreover, the AA concentration in the ragworms did not reflect the AA concentration in their feeds. For example, group 5 (**AWSE**) had the feed with the second lowest total AA concentration, but the ragworms had the highest total AA concentration out of all feed groups. However, as discussed previously regarding their protein content, their feed was not concentrated to the same extent. Therefore, they were likely using their muscles more when prospecting for food, which builds more muscle (Young and Schmidt-Nielsen, 1985).

In contrast, the ragworms fed **AWCE** (group 2) were given the **AW** feed with the highest AA concentration, and the ragworms also had a high AA concentration. On the other hand, the ragworms fed less concentrated feeds compared to **AWCE**, group 3 (**AWFD**) and group 4 (**AWCH**), which had lower AA concentrations. Again, this highlights the poorer nutritional quality of the **AW** used in this experiment compared with the **AW** used in chapter 4. The **AW** used in this experiment only became adequately nutritious in terms of AA if it was highly concentrated through centrifugation. However, just because the total AA concentration was lower for ragworms fed **AWCH** compared with other groups does not mean they have an inadequate nutritional composition for inclusion in aquafeeds. There are other ways their suitability can be evaluated.

To demonstrate the suitability of ragworms that have been fed **AWCH,** a comparison of limiting essential amino acids (LEAA) with ragworms fed **CF** and ragworms fed **AW** that has been processed in different ways is discussed below. LEAA are considered the most crucial

EAA as they improve the utilisation of other AA and reduce their oxidation rates and are defined as the AA that are in shortest supply in relation to need (Nunes *et al.*, 2014; Wang *et al.*, 2019). MET, LYS, ARG and THR are the most critical LEAA in fish (Wang *et al.*, 2019). Table 5.3 shows the LEAA requirements for seven fish species. Table 5.4 shows the LEAA content of the ragworms fed **CF** or processed **AW**.

Table 5.3 – LEAA percentage requirement of various fish species
[Adapted from (Tacon, 1987)]

LEAA Requirement (% of protein)	**Rainbow Trout**	**Common Carp**	**Japanese Eel**	**Chinook salmon**	**Channel Catfish**	**Chum Salmon**	**Mozambique Tilapia**
MET	≈1.8	≈2.1	≈2.1	≈1.5	≈1.3	N/A	≈1.33
LYS	≈5.3	≈5.7	≈4.8	≈5.0	≈5.1	≈4.8	≈4.1
ARG	≈3.5	≈3.3	≈3.9	≈6.0	≈4.3	N/A	≈4.0
THR	≈3.4	≈3.9	≈3.6	≈2.2	≈2.2	≈3.0	N/A
References	(Kaushik, 1979; Walton, Cowey and Adron, 1982, 1984; Ketola, 1983; Kim, Kayes and Amundson, 1983; Rumsey, Page and Scott, 1983)	(Nose, 1978; Ogino, 1980)	(Tacon, 1987)	(Nutrition and Council, 1983)	(Nutrition and Council, 1983)	(Akiyama *et al.*, 1985)	(Jackson and Capper, 1982)

Values are displayed as a percentage of dietary protein. LEAA = limiting essential amino acids, MET = methionine, LYS = lysine, ARG = arginine, THR = threonine.

Table 5.4 – LEAA percentage composition of ragworms fed different feeds

LEAA (% of protein)	Initial	Ragworms Fed CF	Ragworms Fed AWCE	Ragworms Fed AWFD	Ragworms Fed AWCH	Ragworms Fed AWSE
MET	≈1.95	≈1.94	≈2.63	≈2.76	≈2.55	≈2.53
LYS	≈6.88	≈6.21	≈8.65	≈8.81	≈9.01	≈8.71
ARG	≈7.00	≈6.03	≈7.74	≈5.50	≈7.21	≈7.36
THR	≈3.94	≈3.73	≈4.88	≈4.94	≈4.96	≈5.02

Values are displayed as a percentage of total protein. LEAA = limiting essential amino acids, MET = methionine, LYS = lysine, ARG = arginine, THR = threonine. **CF** = commercial fish feed; **AWCE** = centrifuged aquaculture wastewater; **AWFD** = freeze-dried aquaculture wastewater; **AWCH** = chitosan coagulated aquaculture wastewater; **AWSE** = settled aquaculture wastewater.

The Tables above show that the ragworms fed **AW** coagulated and flocculated with chitosan (**AWCH**) had LEAA in proportions of their total protein content suitable to meet the requirements of all 7 fish species described in Table 5.3. In addition, ragworms fed **AWCE** and **AWSE** could also satisfy the LEAA requirements of all 7 fish species. However, ragworms fed **CF** were deficient in THR and MET for common carp and MET for Japanese eel. Also, ragworms fed **AWFD** were deficient in ARG for Chinook salmon.

Another way to evaluate the suitability of ragworms as an alternative aquafeed ingredient after being fed chitosan coagulated and flocculated **AW** is to compare the concentrations of EAA with ragworms fed **CF** and ragworms fed **AW** processed in different ways (Figure 5.15).

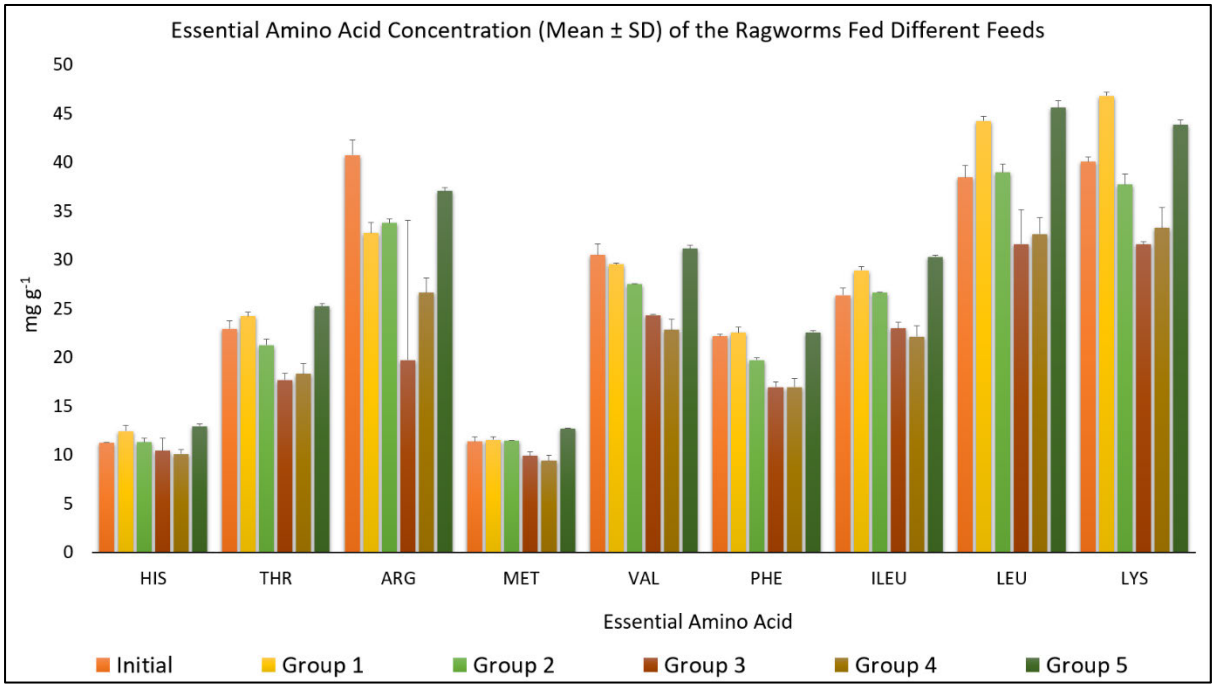

Figure 5.15 – Essential amino acid concentrations (Mean ± SD) of ragworms fed different feeds
Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. HIS = histidine; THR = threonine; ARG = arginine; MET = methionine; VAL = valine; PHE = phenylalanine; ILEU = isoleucine; LEU = leucine; LYS= lysine.

There are 10 EAA crucial in aquafeeds (HIS, THR, ARG, MET, VAL, PHE, ILEU, LEU, LYS, and TRY) (Tacon, 1987; Kaushik and Seiliez, 2010). The group 5 ragworms (**AWSE**) had the highest concentrations for 7 out of 9 measurable EAA; they also had the highest concentration of total AA. For the other 2 EAA, the initial group or group 2 was higher. Ragworms fed **AWFD** and **AWCH** consistently had the lowest concentrations of EAA. However, as described with the LEAA in Tables 5.1 and 5.2, a well-balanced AA profile is more important when considering the nutritional value of a potential aquafeed. Likewise, the NEAA concentrations in the ragworms fed **CF** or processed **AW** can be compared to evaluate ragworms fed **AWCH** suitability as an alternative aquafeed ingredient (Figure 5.16).

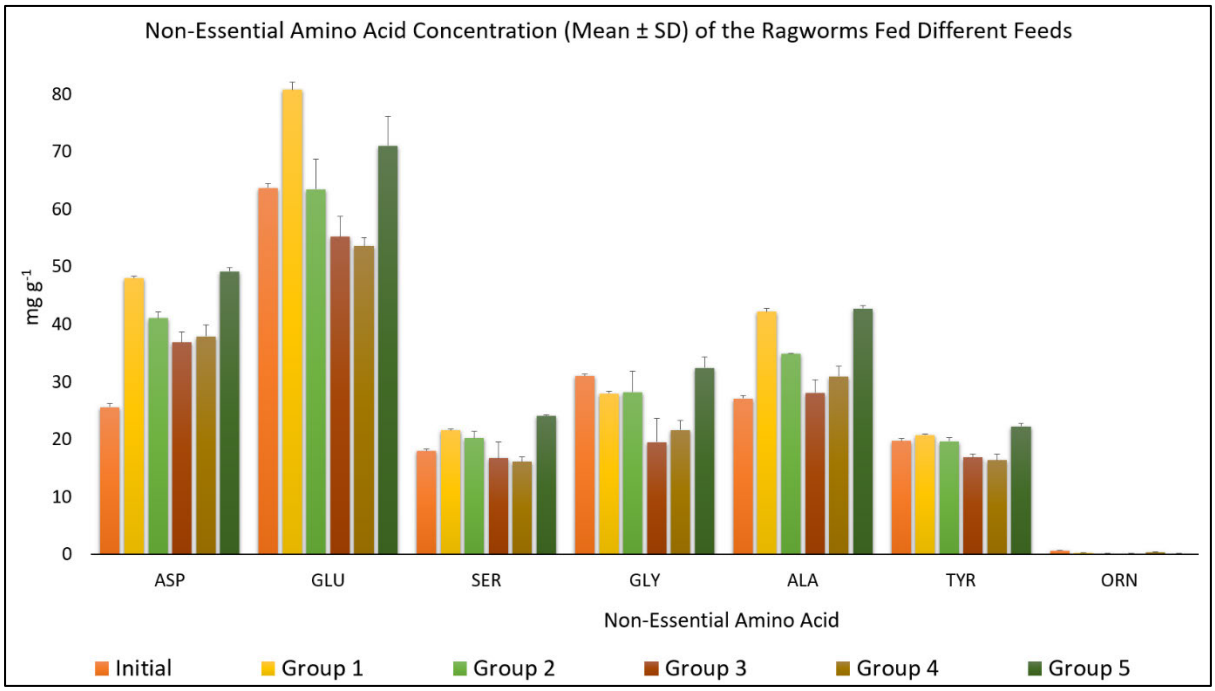

Figure 5.16 – Non-essential amino acid concentrations (Mean ± SD) of ragworms fed different feeds
Initial = ragworms frozen and stored after acclimation period; group 1 = ragworms fed **CF**; group 2 = ragworms fed **AWCE**; group 3 = ragworms fed **AWFD**; group 4 = ragworms fed **AWCH**; group 5 = ragworms fed **AWSE**. ASP = aspartic acid; GLU = glutamic acid; SER = serine; GLY = glycine; ALA = alanine; TYR = tyrosine; ORN = ornithine.

While NEAA are not essential, the presence of NEAA in ragworms still serves important functions related to metabolic activity and DNA synthesis as well as being demonstrated to attract and enhance feeding behaviour in fish (Carr *et al.*, 1996; Li *et al.*, 2009; Wang *et al.*, 2019). Group 5 ragworms (**AWSE**) had the highest concentration for 5 of the 6 NEAA; they also had the highest concentration of EAA and total AA. Group 1 ragworms (**CF**) had a higher concentration of GLU.

Overall, the ragworms fed **AWCH** had lower AA concentrations than other groups fed **AW** that were processed in different ways. However, their AA concentration was still sufficient to be used as feed for several fish species. Furthermore, they had well-balanced AA profiles demonstrated by their LEAA concentrations and percentage proportions which were appropriate to meet the requirements for several fish species. The same could not be said for ragworms fed **AW** that was freeze-dried, and surprisingly, ragworms fed **CF**. The ability of ragworms to assimilate AA when fed a diet that is not a great source of AA shows why ragworms are a highly regarded alternative aquafeed ingredient.

5.4.5 Lipid Contents and Fatty Acid Profiles

In addition to protein, commonly used aquafeeds derived from fish meal and fish oil have high lipid contents. Therefore, when creating an alternative aquafeed ingredient, such as ragworms fed **AWCH**, they must also have a suitable lipid content to satisfy the requirements of the aquaculture species they are intended to feed. Therefore, lipid contents were analysed to understand how the **AW** lipid content changes with different processing methods.

Fish and shrimp require lipids to oxidise AA. To do so without causing excessive lipid deposits in their tissues, a feed lipid content feed of around 10 to 20% is typically required (Rust, 2003). The ragworms fed **CF** (group 1) had a mean ± SD lipid content of 10.09 ± 0.02%. Ragworms fed **AWCE** (group 2), and **AWCH** (group 4) had lower contents (8.07 ± 1.36 and 7.99 ± 0.60%). Ragworms fed **AWFD** (group 3), and **AWSE** (group 5) followed (7.01 ± 0.66 and 6.27 ± 0.25%). Groups 1, 2 and 4 mean lipid contents were not significantly different, and groups 2, 3, 4 and 5 were not significantly different from each other.

Therefore, the results demonstrate that only ragworms fed **CF** (group 1) had the required lipid content of around 10 – 20%. However, the ragworms fed **AWCE** and **AWCH** (groups 2 and 4) were close behind with mean values of around 8% and were not significantly different from group 1. The fact that the values were lower than group 1 (**CF**) says more about **AW**'s lipid quality than ragworm or chitosan performance. The ragworms fed **AWFD** and **AWSE** (groups 3 and 5) had similar results to the experiment described in chapter 4, where values ranged between 5.41 ± 2.54 and 8.16 ± 0.35%. Also, the lipid content of the **AW**, regardless of how it was processed, was significantly lower than **CF**. This was not the case with the batch of **AW** used in the experiment described in chapter 4, where all feeds, including **CF**, were statistically similar. Again, like the conclusion drawn in chapter 4, although the **AW** used was demonstrated as a suitable source of protein and AA, the lipid content may not be sufficient for the ragworms to meet aquafeed requirements. However, suitable lipid contents in ragworms that are intended to be incorporated into aquafeed can be achieved through supplementation or combination with another source of **AW** with higher lipid content.

Furthermore, the lipid contents of ragworms can vary depending on the life cycle stage and the season. When approaching sexual maturation and the colder winter months, ragworms have been shown to become more effective at storing lipids (Luis and Passos, 1995; García-

Alonso and CT, 2008). However, this experiment was carried out with juvenile ragworms in July, so the ragworms were not approaching sexual maturation or the colder winter months.

As this experiment was carried out to investigate ragworms fed **AWCH** suitability as an aquafeed ingredient, it is essential to consider whether chitosan has affected lipid concentrations. It was seen in the experiment in chapter 4 (Section 4.3.2.7 – Lipid Contents), that ragworms fed **AW** with chitosan incorporated that their lipid contents increased as the concentration of chitosan in their feed increased, most notably the concentration of saturated fatty acids (SFA). Chitosan is positively charged and attracts negatively charged molecules, such as lipids, when solubilised in an acidic environment like the digestive tract. However, the dose of chitosan used in this experiment (8 mg L^{-1}) was lower than the concentrations that impacted ragworm lipid contents observed in chapter 4 (30 mg L^{-1}). Also, the lipid content of ragworms fed **AWCE** and **AWCH** was statistically similar. Therefore, chitosan impacting the lipid content of the ragworms fed **AWCH** can be ruled out.

When considering the suitability of aquafeeds in terms of lipids, the concentration of lipids is not the only thing to consider. What is arguably more significant is fatty acid (FA) profiles since fish represent a significant source of omega 3 FA in the human diet (Watters *et al.*, 2012). Furthermore, the fish obtain the omega 3s from their diet; therefore, the ragworms must have a sufficient omega-3 quantity to be considered an effective aquafeed. Therefore, the FA profiles of the feeds and the ragworms in the experiment were analysed.

Similar to past research and the experiment described in chapter 4 (Section 4.3.1.6 – Fatty Acid Profiles), compared to the initial group, the ragworms, regardless of feed, increased their total FA concentration throughout the experiment (Bischoff, 2007; A. Bischoff, Fink and Waller, 2009; Wang *et al.*, 2019). In the experiment described in chapter 4, the ragworms fed **CF** had a significantly higher total FA concentration than ragworms fed **AW**. The same was observed in this experiment. However, the total FA concentration of ragworms fed **AW** that was either centrifuged or coagulated and flocculated with chitosan was higher, but not significantly higher, than ragworms fed **AW** that was settled or freeze-dried. Therefore, this implies that the **CF** is a superior source of FA for ragworms compared to cleaner fish **AW**, regardless of how it has been processed. In addition, the nutritional quality of the **AW**, in terms of total FA content in ragworms, can be slightly improved with centrifugation or chitosan coagulation and flocculation.

When considering the total FA content of the feeds, centrifuging the **AW,** or coagulating and flocculating the **AW** with chitosan created feeds with statistically similar contents to **CF**. However, settled **AW** and freeze-dried **AW** were significantly lower. Therefore, this would suggest that centrifuging **AW** or coagulating and flocculating **AW** with chitosan creates a feed of similar nutritional quality, in terms of total FA, as **CF**. However, as with AA, the availability of nutrients, such as FA, is influenced by numerous factors, including feed composition and the presence or absence of other ingredients that can enhance or inhibit adsorption (Schuchardt and Hahn, 2013). Therefore, the ragworms fed **CF** had higher total FA contents, most likely because the FA in the **CF** is more bioavailable than the **AW** feeds and, therefore, a superior feed. The **CF** is a specifically formulated feed, so this finding is logical.

To further demonstrate the quality of the feeds used in the experiment, Group 1 (**CF**) ragworms had a total FA concentration of 74.49 ± 5.34 mg g^{-1}, similar to other research with ragworms fed **CF** (73.7 ± 6.2 mg g^{-1}) (Wang *et al.*, 2019). However, the total FA concentration of ragworms fed with **AW** feeds ranged between 39.45 ± 3.91 and 49.06 ± 3.31 mg g^{-1}, which was slightly higher than the ragworms fed **AW** in the experiment described in chapter 4 (25.74 ± 12.53 – 44.75 ± 10.78 mg g^{-1}), but slightly lower than research which fed ragworms Atlantic salmon smolt **AW** (56.9 ± 7.8 mg g^{-1}) (Wang *et al.*, 2019). Therefore, regarding total FA concentration, the cleaner fish **AW** used in this experiment is not of the same quality as the salmon smolt **AW**. Also, centrifuging **AW** or coagulating and flocculating **AW** with chitosan returns slightly higher total FA concentrations in ragworm tissues than freeze-drying or settling.

Interestingly, one crucial difference in ragworms fed **AWCH** (group 4) was a higher PUFA concentration compared with the other ragworm groups fed processed **AW**. All other individual FA concentrations were relatively similar. The PUFA content of group 4 (**AWCH**) was also statistically similar to ragworms fed **CF** (group 1). As a result, the ragworms fed **AWCH** also had similar percentage compositions of SFA, MUFA and PUFA as the ragworms fed **CF** (group 1). However, the high PUFA content appears to result from an increased linoleic acid (LA) (C18:2) concentration. While LA is not an essential PUFA for marine organisms like EPA or DHA, it is a precursor to arachidonic acid (ARA), which is considered essential in marine fish (Sargent *et al.*, 1999). In group 1 (**CF**), the higher PUFA content resulted from increased essential FA (EFA) DHA and EPA concentrations, which were also present in significantly

higher quantities in their feed. It is unclear why the ragworms fed **AWCH** had a higher concentration of LA, especially when LA in the **AWCH** was lower than **AWCE**. It may be an effect of chitosan in the diet, which may have an affinity for LA increasing its digestibility in the ragworms. Therefore, it can be concluded that ragworms fed with chitosan and flocculated **AW** have higher PUFA concentrations than ragworms fed centrifuged, settled, or freeze-dried **AW**.

Moreover, it was observed in the experiment described in chapter 4 that the SFA contents of ragworms increased as the concentration of chitosan in their feed increased. However, the chitosan concentration used to make the **AWCH** feed was lower than the concentrations used in the experiment described in chapter 4 that were observed to affect the ragworms. Also, the ragworms fed **AWCH** did not have significantly higher SFA concentrations than the other feed groups.

To determine ragworms fed with **AWCH** suitability as an aquafeed ingredient, Figure 5.17 displays their FA profile against the **CF** used to feed group 1. The ragworms contained 22 of the 24 FA identified in the **CF**, missing only the SFA arachidic acid (C20:0) and lignoceric acid (C24:0), which were present in low concentrations. Three FA were present in the ragworms that were not in the **CF**. The three were undecanoic acid (C11:0), heptadecenoic acid (C17:1) and docosatetraenoic acid (C22:4), which were present in low concentrations. The ragworms had higher concentrations for 12 FA. The **CF** had higher concentrations for 13 FA. However, most importantly, the **CF** had significantly higher concentrations of EFA, namely DHA and EPA.

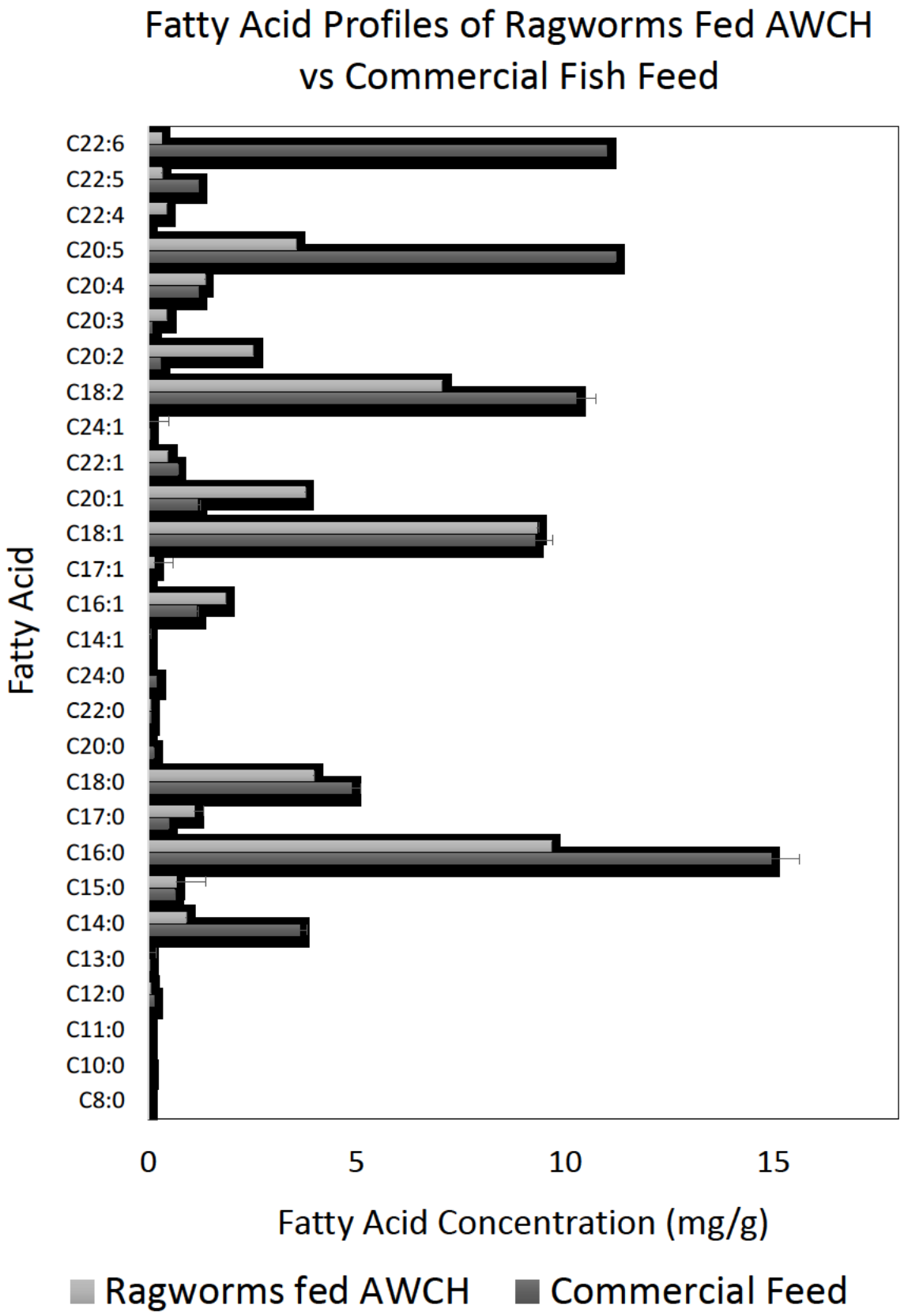

Figure 5.17 – The fatty acid profile of ragworms fed aquaculture wastewater coagulated and flocculated with chitosan versus the fatty acid profile of a commercial fish feed (Tetra® Pond Pellets Mini)

Despite the **CF** having significantly higher concentrations of EFA, the ragworms fed **AWCH** still had sufficient concentrations of EFA to meet the requirements of many fish species. Fish and shrimp display reduced growth and survival when fed diets deficient in EFA (Tacon, 1987). Table 5.5 below shows the dietary EFA requirement of different freshwater and marine fish as a percentage of their dry diet. The EFA requirements are displayed as highly unsaturated fatty acids (HUFA). HUFA are PUFA with 20 or more carbon atoms and at least 3 double bonds (Olsen, 2009).

Table 5.5 – Essential fatty acid requirement of fish species (% of dry diet)

Species	EFA Requirement	References
Rainbow trout (freshwater) (*Oncorhynchus mykiss*)	**1.0% HUFA (ω-3)**	(Castell *et al.*, 1972; Yu and Sinnhuber, 1972; Watanabe *et al.*, 1974; Takeuchi and Watanabe, 1977a; Tacon, 1987)
Chum salmon (freshwater) (*Oncorhynchus keta*)	**1.0% HUFA (ω-3)**	(TAKEUCHI, 1979; Takeuchi and Watanabe, 1982; Tacon, 1987)
Ayu (freshwater) (*Plecoglossus altivelis*)	**1.0% C20:5 (ω-3)**	(Kanazawa, 1985; Tacon, 1987)
Common carp (freshwater) (*Cyprinus carpio*)	**0.5-1.0% HUFA (ω-3)**	(Takeuchi and Watanabe, 1977b; Tacon, 1987)
Turbot (marine) (*Scophthalmus maximus*)	**0.6-1.0% HUFA (ω-3)**	(Gatesoupe and FJ, 1977; Leger *et al.*, 1979; Tacon, 1987)
Red seabream (marine) (*Pagrus major*)	**0.5 – 2.0% HUFA (ω-3)**	(Yone, 1978; Tacon, 1987)

(**ω-3**) = omega 3

The percentage composition of HUFA in ragworms fed **AWCH** was ≈2.97%, above the EFA requirements of the fish species displayed in Table 5.4. Therefore, despite the ragworms being fed **AW**, which is less nutritious than **CF**, they still contained EFA in sufficient quantities to satisfy the needs of many fish species. The EFA content of the ragworms fed **AWCH** further backs up why ragworms are highly regarded as an alternative aquafeed.

Overall, the ragworms fed **AWCH** included the necessary FA but not at the same concentration levels as **CF**. However, the concentrations of EFA in ragworms fed **AWCH** were suitable to satisfy the requirements of various fish species. Furthermore, the **CF** used is a specifically formulated feed including all nutrients, vitamins, and trace elements to support the growth of small fish, so the ragworms being of lesser quality is logical. Moreover, in ragworms fed **CF** (group 1), the percentage composition of HUFA was ≈3.5%; in ragworms fed **AWCE** (group 2), it was ≈2.14%. Therefore, the ragworms fed **AWCH** EFA content is not far off what was observed in ragworms fed a nutritionally complete feed and better than ragworms fed centrifuged **AW**. Therefore, relating to the hypothesis of this experiment, it can be stated that ragworms fed **AWCH** to be used as an alternative aquafeed ingredient have FA profiles suitable for the requirements of many fish species.

5.5 Conclusion

The findings of this study demonstrate that cleaner fish **AW** can be successfully coagulated and flocculated with chitosan before feeding to ragworms to create a suitable alternative aquafeed ingredient. Coagulating and flocculating **AW** with chitosan (**AWCH**) creates a more concentrated feed compared to freeze-drying **AW** (**AWFD**) or settling **AW** (**AWSE**). However, **AWCH** was not a more concentrated feed than centrifuged **AW** (**AWCE**). Despite **AWCH** being less concentrated than **AWCE**, the ragworms fed **AWCH** still performed well with reasonable growth rates and low mortality. In addition, their nutritional composition was suitable in terms of proteins and lipids for many fish species.

An important point to make is the expected additional labour and costs associated with centrifuging compared to coagulation and flocculation, which are generally higher. Centrifuging typically requires more energy, specialised equipment, and maintenance, as well

as skilled operators. In contrast, coagulation and flocculation involves adding chemicals to the water and allowing the particles to aggregate and settle, which is usually less labour-intensive and has lower operational costs. However, the specific differences in labour and costs can vary based on the scale of operation, the type of material being processed, and the efficiency of the systems in place.

Regarding growth performance, ragworms fed **AWCH** grew faster and more prominent than ragworms fed **AWFD** and **AWSE**. However, their growth performance did not match that of ragworms fed **CF** or **AWCE**. Nevertheless, despite lesser growth than **CF** and **AWCE**, the growth rates of ragworms fed **AWCH** were better than comparable research where ragworms were fed Atlantic salmon, bass, or seabream **AW** and similar to ragworms fed sturgeon **AW**. Therefore, it can be concluded that ragworms grow well when fed **AWCH**.

Regarding mortality, ragworms fed **AWCH** had the highest mortalities with 4. Only 1 other mortality was recorded in the other groups. However, the mortalities in the ragworms fed **AWCH** occurred in one replicate beaker and during the second half of the experiment. Therefore, most likely, an undetermined factor, such as pathogenic bacteria or a toxin in the replicate beaker was responsible for the mortalities. Therefore, it can be concluded that feeding **AWCH** to ragworms before processing into aquafeeds does not cause mortalities.

Furthermore, the total organic matter (TOM), carbon (C) and nitrogen (N) contents of the feeds and the ragworms were analysed. The feeds were analysed primarily to understand the nutritional quality of the feeds better, and why ragworms fed **CF**, **AWCE** or **AWCH** had superior growth rates compared to ragworms fed **AWFD** or **AWSE**. The AW feeds' TOM, C and N content increased with better processing methods in the ascending order of **AWFD**, **AWSE**, **AWCH** and **AWCE**. The **CF** had the highest TOM, C and N contents. The ragworms that grew better had feeds with higher TOM and C contents, which makes sense as they are more energy-dense. However, after analysing the ragworms, the TOM content was very similar regardless of feed group.

Furthermore, regardless of how it was processed, the C contents were similar in the ragworms fed **AW**, but the ragworms fed **CF** were significantly higher. The N contents of the ragworms were more varied, corresponding to the protein content discussed in subsequent protein content analysis. Therefore, it can be concluded that coagulating and flocculating **AW** with

chitosan will create a more energy-dense feed that will return higher growth rates in ragworms than settling or freeze-drying **AW** but not centrifuging **AW**.

Moreover, protein and lipid contents were analysed to understand the nutritional quality of the feeds and ragworms. Like the TOM, C and N contents, the **AW** feeds protein and lipid contents followed the same order (**AWFD**, **AWSE**, **AWCH**, and **AWCE**). Again, like the TOM, C and N contents, the **CF** had the highest protein and lipid contents. However, ragworms given feeds with higher protein and lipid contents did not necessarily have higher protein and lipid contents themselves. Presumably, ragworms fed a more concentrated feed use their muscles less when prospecting for food and therefore gain less muscle than ragworms that use them more frequently.

Furthermore, ragworms fed with more concentrated feed, which is more energy-dense, have the potential to store more energy as fat. Therefore, ragworms fed the more concentrated **AWCE** or **AWCH** had lower protein contents but higher lipid contents than ragworms fed **AWFD** or **AWSE**. The ragworms fed **CF** had the highest lipid and protein contents of all ragworm feed groups. However, they were given a specifically formulated feed that includes everything small marine animals require, so this finding is logical.

Additionally, the ragworms fed **AWCH**, along with all other feed groups, had protein contents able to satisfy a variety of fish and shrimp species. However, only ragworms fed **CF** had lipid contents suitable to satisfy fish requirements. However, as was discussed, this may have been misinterpreted as the method used to determine lipid contents were calculated on a wet weight basis. In contrast, other research calculates lipid content on a dry weight basis. When the ragworm contents were compared to research which also calculated lipid content on a wet-weight basis, the lipid contents of the ragworms in this experiment were higher. Therefore, the lipid contents may be higher than what the results suggest.

Also, to better understand the nutritional quality of the feeds and the ragworms, their amino acid (AA) profiles were analysed. Like the TOM, C, N, protein and lipid contents, the feeds AA concentrations increased with better processing methods in the order of **AWFD**, **AWSE**, **AWCH** and **AWCE**. Similarly, again, **CF** had the highest concentration of AA.

Unexpectedly, the ragworms fed **AWSE** had the highest concentration of total AA. However, as discussed above, this was one of the groups with a higher protein content; therefore, a

higher concentration of AA is logical. Pertinent to the study's hypothesis, ragworms fed **AWCH** had lower AA concentrations than the other groups fed **AW** processed in different ways. However, their AA concentrations were still suitable and well-balanced, as demonstrated by their limited essential AA (LEAA) concentrations, which were sufficient to meet the requirements of various fish species. The same could not be said for ragworms fed **AWFD**, and unexpectedly, ragworms fed **CF**. Therefore, the ragworms fed **AWCH**, **AWCE** and **AWSE**, in terms of AA profiles, were better nutritionally than ragworms fed **CF** or **AWSE**.

Finally, fatty acid (FA) profiles of the feeds and ragworms were also analysed to understand their nutritional quality better. In the feeds, as seen in other analyses, different processing methods concentrated the feeds more in the ascending order of **AWFD**, **AWSE**, **AWCH** and **AWCE**. However, **AWCE** had a higher total FA concentration than **CF** on this occasion. Then again, the **CF** was far superior in essential FA (EFA), reflected in the ragworm FA profiles.

Moreover, the ragworms fed **AWCH** had higher concentrations of polyunsaturated FA (PUFA), giving them a better FA nutritional profile than ragworms fed with the other **AW**-based feeds. Furthermore, ragworms fed **AWCH** were demonstrated to be able to satisfy the EFA requirements of 6 freshwater and marine fish species. Therefore, it can be stated that ragworms fed **AWCH** to be used as an alternative aquafeed ingredient have FA profiles that are suitable for the requirements of various fish species.

Overall, ragworms can be fed **AW** that has been processed with chitosan (**AWCH**) to create suitable alternative aquafeed ingredients. However, growth rates are slightly lower than ragworms fed centrifuged **AW** (**AWCE**) or **CF**. Furthermore, ragworms fed **AWCH** demonstrated favourable AA and FA profiles with concentrations and compositions suitable for multiple fish species. Therefore, feeding **AWCH** to ragworms is a viable option for creating alternative aquafeed ingredients that can help make help the aquaculture industry more sustainable by recycling waste into a feedstock to be put back into aquaculture as part of a circular economic system.

Chapter 6

Comparison of Aluminium Bioaccumulation in *Hediste diversicolor* When Fed Aluminium Enriched Aquaculture Wastewater or Chitosan Coagulated Aluminium Enriched Aquaculture Wastewater

6.1 Introduction

Although trace metals naturally occur in the Earth's crust, anthropogenic activity has mainly been responsible for their discharge into natural water sources (Urien *et al.*, 2017). As a result, the metals can take on chemical forms that are more mobile and subsequently more accessible to animals which inhabit these environments (Thévenot *et al.*, 2007). In aquatic habitats, the metals can be suspended in the water, as well as deposited on and in the sediment (Gagnon and Fisher, 1997). Consequently, metal accumulation and toxicity in aquatic animals may occur even at low concentrations due to persistent and widespread metallic exposure (Berthet *et al.*, 2003). Therefore, environmental surveys often include biomonitoring, which relies on measuring metal concentrations in species that collect pollutants from the environment in their tissues (Poirier *et al.*, 2006). For example, the species used throughout this thesis, *Hediste diversicolor*, has been suggested as an environmental management bioindicator since they are important for the sustainable functioning of estuarine ecosystems (Galloway *et al.*, 2004).

Upon reviewing an elemental analysis of aquaculture wastewater (**AW**) from a confidential aquaculture site, it was found to have a high aluminium concentration of 1754 mg kg^{-1}. To compare, the cleaner fish **AW** from MOWI® Anglesey (Wales) used in experiments described in chapters 4 and 5 had a mean (± standard deviation) aluminium concentration of 0.47 (± 0.04) mg kg^{-1}. How the aluminium ended up in the **AW** at such a high concentration was never determined. Instead, only speculation was made, such as the equipment used to extract the waste was made of aluminium or nearby industry releases aluminium into the same water body where the aquaculture facility is located.

Initially, the **AW** with the high aluminium concentration was to be used for the experiments described in chapters 4 and 5. Pennosan® were in collaboration with a company (company name confidential) that was working on a research and development project related to recovering and separating water from aquaculture sludge. However, the SARS-CoV-2 outbreak and resulting lockdown in 2020 terminated the project; therefore, access to that source was lost.

However, the presence of aluminium at such a high concentration sparked discussions regarding the potential toxicity of aluminium in **AW** intended to feed ragworms. As stated

earlier, ragworms can be used as bioindicators of pollution when environmental biomonitoring of natural habitats and many studies have investigated the accumulation of metals in ragworms other than aluminium (Ozoh and Jones, 1990; Berthet *et al.*, 2003; Poirier *et al.*, 2006; Kalman *et al.*, 2010). So, it is likely that ragworms will bioaccumulate aluminium when fed **AW**. In addition, there aren't many specific research papers on how aluminium affects polychaetes.

So, suppose **AW** is to be fed to ragworms before incorporating the ragworms into aquafeed; the metal source would come from their feed, not the water or sediment they inhabit. In that case, the **AW** intended to feed ragworms cannot have potentially toxic levels of aluminium as it can bioaccumulate in the ragworms. Through the phenomena known as biomagnification, organisms that accumulate a toxin through food consumption can result in a concentration that is higher than what would have occurred with only water and sediment exposure (Adriaens, Gruden and McCormick, 2007).

An infamous example of biomagnification was the outbreak of Minamata disease in Japan. Minamata is a neurological disease caused by mercury poisoning named after the city where thousands of people have been affected. The local population consumed shellfish that became enriched in mercury which bioaccumulated and biomagnified due to industrial wastewater released into the water the shellfish inhabited (Blenkharn, 2009).

In addition, since chitosan was investigated in previous chapters to coagulate and flocculate **AW** and the industrial partner is Pennosan®, who manufacture chitosan, it was decided that the role chitosan plays in ragworm aluminium bioaccumulation should be investigated too. Chitosan can bind and remove metal ions from wastewater (Varma, Deshpande and Kennedy, 2004). Therefore, to understand how aluminium is bioaccumulated in ragworms when they are fed **AW** with an elevated level of aluminium, with and without chitosan, three chapter aims were formulated:

1. Investigate feeding aquaculture wastewater with varying concentrations of aluminium to ragworms and measure concentrations in ragworm tissues after different exposure periods;

2. Investigate feeding aquaculture wastewater with an elevated level of aluminium that is coagulated and flocculated with chitosan to ragworms and compare with ragworms fed **AW** with an elevated level of aluminium that has not been coagulated and flocculated with chitosan;

3. Record growth and mortality of ragworms fed **AW** with varying concentrations of aluminium, with and without chitosan.

The objective of the chapter was to investigate the three aims stated above to answer the following hypothesis:

Feeding ragworms aquaculture wastewater with an elevated concentration of aluminium bioaccumulates faster in their tissues when coagulated and flocculated with chitosan.

Proving the hypothesis determines if ragworms fed **AW** with an elevated aluminium concentration bioaccumulates faster in ragworm tissues if it is coagulated and flocculated with chitosan. Additionally, it determines whether feeding **AW** with an elevated concentration of aluminium affects ragworms in terms of growth and mortality.

For this purpose, a 28-day experiment was set up with five ragworm feed groups. First, three feed groups were fed **AW**, individually spiked with varying concentrations of aluminium sulfate [$Al_2(SO_4)_3$]. Aluminium sulfate was chosen over other aluminium compounds because it is soluble in water. Next, one feed group was fed **AW** that was individually spiked with $Al_2(SO_4)_3$ and coagulated and flocculated with chitosan. Finally, one feed group was fed **AW** without aluminium as a control and one group was fed a commercial feed (**CF**) as another control. Each feed group consisted of three replicates.

Three ragworms from each replicate beaker were removed after 1, 7, 14 and 28 days, acid digested and analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES) to determine their aluminium concentration.

6.2 Heavy Metal Contamination in Ragworms

Heavy metal contamination is a severe concern for the health of ragworms living in contaminated environments. When ragworms are exposed to heavy metals, they can accumulate these toxic substances in their tissues, which can negatively impact their health and survival (Petrich and Reish, 1979). The effects of heavy metal contamination on ragworms can vary depending on the type and concentration of the metal and the route and duration of exposure. Some common heavy metals that contaminate aquatic environments and affect ragworms include lead (Philippe *et al.*, 2008), mercury (Han *et al.*, 2011), cadmium, zinc and copper (Berthet *et al.*, 2003).

It is difficult to determine the effect of metals to which ragworms are exposed in the wild. Metal concentrations vary in the environment between different sediment and water depths. Therefore, metal separation impacts how bioavailable metals are to benthic animals (Luoma and Bryan, 1982). Ragworms can absorb metals from ingesting sediment and the water they naturally inhabit. Therefore, it can be challenging to determine whether water concentration or sediment concentration impacts exposure more. The variety of feeding strategies ragworms use may also affect their exposure to metal pollutants.

However, past research has demonstrated that >98% of metals in the closely related polychaete *Nereis succinea* came from sediment ingestion (Wang, Stupakoff and Fisher, 1999). The omnivorous feeding habits of ragworms mean they consume whatever food is the most abundant in their ecosystem. If the main food supply is polluted, that is a significant exposure pathway (Bryan and HummerstonE, 1971; Collier and Pinn, 1998).

6.2.1 Aluminium Contamination in Ragworms

While aluminium is not considered highly toxic to humans, it can negatively impact the health of aquatic organisms, including ragworms. The effects of aluminium contamination on ragworms can depend on the concentration of aluminium in the environment, exposure route and duration of exposure. In high concentrations, aluminium can interfere with the ability of ragworms to filter nutrients from the water, which can lead to reduced acute effects on growth and survival (Petrich and Reish, 1979). In addition, aluminium can affect the behaviour

and reproduction of ragworms, and it can interfere with the development of their larvae (Gopalakrishnan, Thilagam and Raja, 2007).

6.2.2 Heavy Metal Contamination Resistance in Ragworms

Ragworms can have some natural resistance to heavy metal contamination, although the extent of this resistance can vary. In addition, some individuals may have specific mechanisms to detoxify or eliminate heavy metals from their tissues. In contrast, others may tolerate higher concentrations of these contaminants without experiencing adverse effects. However, it is essential to note that heavy metal contamination can still negatively impact the health, development and survival of ragworms, even if they have some level of resistance to these substances (Pedro *et al.*, 2022).

The effects of heavy metal contamination on ragworms can depend on various factors, including the type and concentration of the metal, the duration of exposure and the overall health and condition of the worms.

In ragworms, acute metal toxicity is influenced by the absorption rate since it controls how quickly the fatal dosage builds up. It is crucial to consider the rate of intake since it affects whether the organism's detoxifying systems can control internal concentrations. A complexing mechanism in ragworms is believed to detoxify contaminants and store them in their epidermis and nephridia. (Bryan and HummerstonE, 1971; McLusky, Bryant and Campbell, 1986).

Moreover, ragworms are at risk of mortality when metal concentrations exceed >0.1 mg L^{-1} mercury, >0.01 mg L^{-1} copper, >1 mg L^{-1} cadmium, >1 mg L^{-1} Zinc, >0.1 mg L^{-1} lead, >1 mg L^{-1} chromium, >1 mg L^{-1} arsenic or >10 mg L^{-1} nickel ('T.R. Crompton Toxicants in the aqueous ecosystem, xiii, 382p. Chichester: John Wiley and Sons, 1997. Price £7500.', 1997). No information was available for aluminium. In addition, ragworms have been reliably observed subsisting in environments where copper contamination ranges between 20 and > 4000 µm g^{-1} (Bryan and HummerstonE, 1971).

6.3 Aluminium General Description

6.3.1 Aluminium in Nature

Aluminium is a silver-coloured, lightweight metal that is highly reactive and never found in its pure form in nature. Aluminium makes up around 8% of the Earth's outermost layer and is the most prevalent metallic element. Aluminium naturally exists in the environment as silicates, oxides and hydroxides, in combination with other elements like sodium and fluoride, and complexes with organic matter (Gala-Gorchev, 1998).

Environmental aluminium can significantly impact aquaculture. High aluminium levels in water bodies can degrade water quality, adversely affecting fish health. If fish raised in aquaculture are exposed to elevated aluminium, it can pose health risks to consumers.

6.3.2 Aluminium Uses

Aluminium is a highly versatile metal and is used in a wide range of products. Aluminium is used in the production of metal alloys; the electric, building, automobile and aircraft sectors all apply aluminium as a structural component, as well as used in the manufacture of cooking equipment and food packaging (Gala-Gorchev, 1998). Antacids, deodorants and food additives all include aluminium compounds ('Toxicological Profile for Aluminum', 2002). Aluminium salts, most commonly aluminium sulfate, are often employed as coagulants in the water treatment industry to lower organic matter, prevent discolour, lower turbidity and inhibit microorganisms (Gala-Gorchev, 1998; Kvech and Edwards, 2002). Applying an aluminium coagulant to water is like the one described with chitosan (Section 4.2.4 – Making Feeds).

6.3.3 Aluminium Environmental Fate

Aluminium concentrations in potable water may rise due to using aluminium salts like aluminium sulfate as coagulants in water treatment. As a result, aluminium may accumulate in the distribution system in areas with significant residual concentrations. Changes in flow rate that disturb the deposits may result in higher amounts of aluminium at tap sources with

unattractive colour and high turbidity (Organization and Safety, no date). The concentrations of aluminium that cause these issues depend on the water treatment plant's operational aspects and several other water quality criteria.

Aluminium is mainly discharged into the environment through natural processes. Several factors impact aluminium mobility and consequent environmental transport. Examples include chemical speciation, hydrological flow routes, soil-water interactions, and the composition of the underlying geological elements. Acid environments, such as those created by acid mine drainage or acid rain, can raise the dissolved aluminium level of the surrounding waterways (Organization and Safety, 1997; 'Toxicological Profile for Aluminum', 2002).

Numerous distinct types of aluminium may be found in water. Based on aquated positive ions or hydroxylated aluminates, aluminium can create monomeric and polymeric hydroxy species, colloidal polymeric solutions and gels, and precipitates. Additionally, aluminium may combine with many organic molecules (such as humic or fulvic acids) and inorganic ligands (such as fluoride, chloride, and sulphate), most of which are soluble, but not all. The chemistry of aluminium in water is complicated, and several chemical factors, such as pH, affect which aluminium species are found in aqueous solutions. Aluminium has minimal solubility in the pH range of 5.5 to 6 in clean water. Concentrations of total dissolved aluminium increase at higher and lower pH values.

6.4 Chitosan and its Ability to Remove Aluminium from Water

There is evidence to suggest that chitosan can bind to and remove aluminium from water. For example, in one study, chitosan was shown to be effective for the adsorption of aluminium from textile wastewater (21.3 mg of aluminium per gram of chitosan) (Simionato *et al.*, 2006). However, the effectiveness of chitosan in removing aluminium from wastewater can depend on various factors, such as the concentration of aluminium in the water, the form of chitosan used and pH. The action of chitosan binding to aluminium and the subsequent concentrating of the **AW** would imply that feeding chitosan-coagulated **AW** may increase the exposure of the aluminium to ragworms.

6.5 Materials and Methods

This Section discusses experimental methodology specific to this chapter and not covered in chapter 3. Ragworm weights, growth and mortality were calculated as described in Section 3.7 (Determining Ragworm Weights and Growth Rates) and Section 3.7.3 (Determining Ragworm Mortalities). Statistical analysis was carried out as described in Section 3.9 (Statistical Analysis).

This experiment started 1st August 2022 lasting for 28 days.

6.5.1 Feed Groups

The following bullet points describe the feed provided to the ragworms in their respective groups. The bold lettering refers to what they are referred to in subsequent sections.

- **CF** – Commercial Feed (**CF**) [no chitosan] [no aluminium];
- **Group 1** – Aquaculture wastewater (**AW**) [no chitosan added] [no aluminium added];
- **Group 2** – Aquaculture wastewater 100 mg L^{-1} $Al_2(SO_4)_3$ (**AW + Low Al**) [no chitosan];
- **Group 3** – Aquaculture wastewater 350 mg L^{-1} $Al_2(SO_4)_3$ (**AW + Med Al**) [no chitosan];
- **Group 4** – Aquaculture wastewater 1760 mg L^{-1} $Al_2(SO_4)_3$ (**AW + High Al**) [no chitosan];
- **Group 5** – Aquaculture wastewater 1760 mg L^{-1} $Al_2(SO_4)_3$ coagulated and flocculated with chitosan (**AW + High Al + C**).

Three glass culture beakers containing 15 ragworms each (replicates) were set up for each feed group (45 ragworms per group) (225 ragworms in total). After a 5-day acclimation period where they were fed a commercial fish feed exclusively (Tetra® Pond Pellets Mini), alive and intact ragworms were selected for the experiment and randomly stocked to the beakers. They were not selected by sex, size, maturation, weight, or other.

6.5.2 Making Feeds

The cleaner fish **AW** from MOWI® Anglesey (Wales) used in this experiment was from the same batch used in the experiment described in chapter 5. The pH of the **AW** used for all feed groups was dropped from ≈7.9 to ≈6.5 using 2 M hydrochloric acid for two reasons. Firstly, the pH must be slightly acidic, or chitosan does not function effectively as a coagulating and flocculating agent. Secondly, different pHs can form different aluminium complexes in water. Since the chitosan coagulated and flocculated **AW** required pH adjustment, a decision was made to adjust the pH of all feeds so they were the same. For example, at pH 9, in drinking water, aluminium and magnesium form complex solid phases (Kvech and Edwards, 2002). Therefore, the pH was standardised to avoid differential properties in the feed between feed groups.

Before pH adjustment, the water was defrosted and homogenised. Then, appropriate doses of aluminium sulfate ($Al2(SO_4)_3$) were added to the respective beaker containing 1 L of **AW**. None was added in the case of group 1. Except for group 5, the beakers were sealed and frozen for 24 hours. Next, they were freeze-dried for 24 hrs to decrease the water content and make it easier to apply as a feed for ragworms.

For group 5 (**AW + 1.8 mg L^{-1} Al + C**), the **AW**, now containing an elevated aluminium concentration due to spiking with $Al2(SO_4)_3$, was then ready for coagulation and flocculation with chitosan. Therefore, the chitosan coagulation and flocculation process described in chapter 5 (Section 5.2.2 – Making Feeds), was also applied to group 5 in this experiment.

Now, because the **AW** must be treated to make it easier to apply as a feed, either by freeze-drying for 24 h or by coagulating and flocculating with chitosan, the water content is decreased and therefore, the concentration of the aluminium in the feed increases. For that reason, the concentration of the feeds before treatment was obtained in the same way as the aluminium concentrations in the ragworms tissues were obtained, described subsequently (Section 6.5.5 – Acid Digestion of Ragworms and Section 6.5.6 – Aluminium Detection and Quantification using Inductively Coupled Plasma Optical Emissions Spectroscopy). The aluminium concentrations of the feed are included in Appendix Q, Table Q1.

6.5.3 Feeding Protocol

The provision of feed was designed to ensure that it was not a factor limiting the development of the ragworms. Monday to Friday, 2 g of respective feed was added to each replicate beaker, 1 g in the morning and 1 g at the end of the day. An extra 4 g was added on a Friday afternoon to cover the weekend. However, when ragworms were removed for elemental analysis, the quantity of feed added was reduced in proportion to the number of ragworms removed, so they were exposed to the same concentration of aluminium throughout the experiment. Control ragworms fed commercial feed (CF) were given 0.5 g of feed per day and the amount was reduced in correlation with the number of worms removed also.

6.5.4 Ragworm Removal Protocol after Period of Exposure

As stated in the introduction, three ragworms from each replicate beaker were removed after 1, 7, 14 and 28 days of exposure to experimental conditions. To ensure feed contents within ragworm guts and potential aluminium adsorbed on the outside of the ragworms did not impact aluminium concentrations in the subsequently described ICP-OES analysis, the following procedure was followed:

Ragworms from individual replicate culture beakers were randomly removed, where they were immediately transferred to another beaker consisting of clean culture water to purge their gut contents for 4 h (Palmer *et al.*, 2014). Then, the ragworms were placed into a beaker of deionised water (Milli-Q® Ultrapure Water) for 1 hr to remove extracellular salts and ensure the guts were clear. Then, ragworms were rinsed in 2 mM ethylene-diamine tetra-acetic acid (EDTA) and 0.5 mM EDTA. Next, the ragworms were rinsed again with deionised water to remove any aluminium adsorbed onto the surface of the ragworm bodies (Lebrun *et al.*, 2011; Urien *et al.*, 2017). Finally, ragworms were frozen at -20 °C for 24 hrs before freeze-drying (Modulyo® 4K Freeze-Dryer) and ball milled to be ready for acid digestion.

6.5.5 Acid Digestion of Ragworms

An adapted method for the acid digestion of plant material using nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) was used to acid digest the ragworms before elemental analysis using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Firstly, 0.1 g of freeze-dried and ball-milled ragworm sample was placed into a test tube. Then, in a fume cupboard, 2 mL of concentrated HNO_3 was added to the test tube and left to stand overnight. Next, 250 µL of 30% H_2O_2 was added and heated to 50 °C using a heat block. An observable reaction is seen at this stage. Once the reaction subsided, another 250 µL of 30% H_2O_2 was added to the test tube. This process was repeated until 2 mL of H_2O_2 was added. Next, the test tube was covered using acid-washed marble, heated to 120 °C using the heat block, and held for 2 hrs. After 2 hrs, the marble was removed, and the sample was concentrated to approximately 1 mL. The process was sped up by turning the heat block to 150 °C. Finally, the resulting liquid was transferred to a pre-weighed 15 mL centrifuge tube, and the sample was rinsed with 3 aliquots of 3 mL deionised water, giving a final volume of approximately 10 mL. The tube was then reweighed, and the volume was determined from the mass (presuming a density of 1 mg mL^{-1}).

In addition, a Standard Reference Material (SRM) (bovine liver) (NIST 1577a) was also acid-digested following the method above. The purpose of the SRM was to evaluate the reliability of the analytical methods for determining aluminium in ragworm tissues.

Finally, the same method was utilised to determine the aluminium concentrations in the feeds after they had been freeze-dried or coagulated and flocculated with chitosan.

6.5.6 Aluminium Detection and Quantification using Inductively Coupled Plasma Optical Emission Spectroscopy

Aluminium concentrations for each ragworm and feed sample were measured on a Varian Vista-PRO Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). ICP-OES is a type of spectroscopy that uses an argon plasma at a temperature between 5000 and 7000 K to produce excited atoms and ions that emit electromagnetic radiations at wavelengths of light characteristic of a particular element, in this case, aluminium. Using an inert gas like

argon decreases the number of oxides and nitrides that can be generated. Several wavelengths were chosen to get accurate intensities at various concentrations, as each wavelength has a specific sensitivity. In addition, some wavelengths can be affected by interferences and analysing several wavelengths can help counteract the effect of interferences.

A minimum of three procedural blanks were used per run, and each acid-digested ragworm sample was loaded into 4 ml tubes in an auto-sampling rack. Each sample was measured 6 times to calculate the standard deviation and error.

Instrument calibration was performed against 5 calibration solutions of increasing concentration prepared from a multi-element ICP standard in a matrix of 2% nitric acid (HNO_3).

Blanks were analysed and found to be low. Limits of detection (LOD) for each wavelength were calculated using the following equation:

$$Limit\ of\ Detection = 3\ \times Standard\ Deviation\ of\ the\ Blank\ \times Calibration\ Slope$$

The calibration slope was calculated using the following equation:

$$Calibration\ Slope = \frac{Maximum\ Concentration - Minimum\ Concentration}{Maximum\ Intensity - Average\ Blank\ Intensity}$$

6.6 Results and Discussion

The results of the experiment pertinent to the hypothesis are presented below. The results are described first with an accompanying Figure or Table, and the discussion text follows each Figure. Where a result is described as significant or insignificant, an analysis of variance test (ANOVA) has been carried out to determine this. In addition, results relating to culture water conditions, namely temperature, conductivity, pH, and dissolved oxygen (DO), are included as appendices (appendices M, N, O, & P).

6.6.1 Ragworm Growth When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium

The growth of ragworms was recorded to evaluate how ragworms responded to being fed **AW** with varying concentrations of aluminium and compared to ragworms fed **AW** without aluminium and ragworms fed a commercial feed (CF). The ragworms were weighed at the experiment's start and end. The mean wet weights ± standard deviation (SD) of each ragworm feed group and the start and end of the experiment are shown in Figure 6.1.

All ragworm feed group final mean wet weights were significantly higher than their initial mean wet weights. The initial mean wet weights ranged between 52.6 ± 0.03 and 124.7 ± 0.05 mg. The initial mean wet weight of group 5 (**AW + High Al + C**) was significantly higher than all other groups except for group 2 (**AW + Low Al**). The initial mean wet weight of group 1 (**AW**) was significantly lower than all other groups except for ragworms fed **CF**. The initial mean wet weight of ragworms fed **CF** were not significantly different from groups 2, 3 and 4 (**AW + Low Al, AW + Med Al and AW + High Al**).

The final mean wet weights ranged between 126.8 ± 98 and 251.6 ± 155.7 mg. Group 1 (**AW**) ragworms had the highest final mean wet weight but were not significantly different to any other group except for group 4 (**AW + High Al**). Group 4 ragworms (**AW + High Al**) had the lowest final mean wet weights but were not significantly different compared to groups 2, 3 and 5 (**AW + Low Al, AW + Med Al and AW + High Al**).

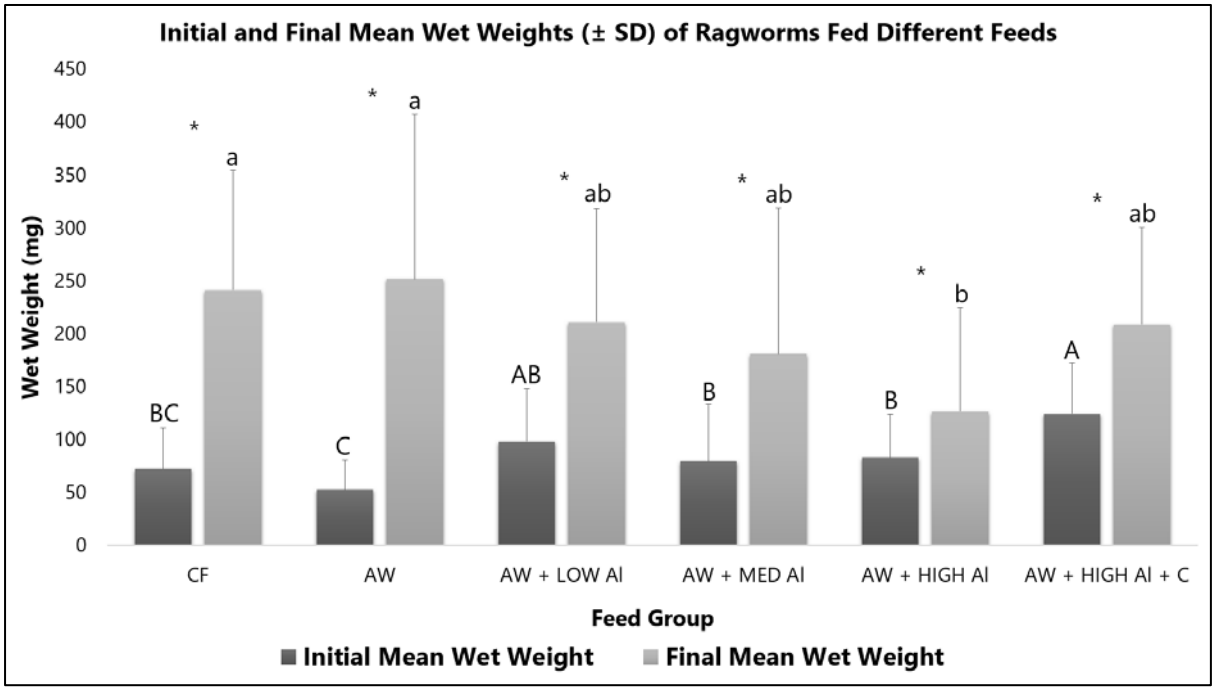

Figure 6.1 – Initial and final mean wet weights ± SD of ragworms fed CF or AW with varying concentrations of aluminium and chitosan

CF = ragworms fed CF; **AW** = ragworms fed AW; **AW + LOW Al** = ragworms fed AW with a low dose of aluminium; **AW + MED Al** = ragworms fed AW with a medium dose of aluminium; **AW + HIGH Al** = ragworms fed AW with a high dose of aluminium; **AW + HIGH Al + C** = ragworms fed AW with a high dose of aluminium coagulated and flocculated with chitosan. Significant differences between initial and final mean wet weights within feed groups are marked with an asterisk (*). Significant differences in initial mean wet weights between groups are marked by different uppercase letters. Significant differences in final mean wet weights between groups are marked by different lowercase letters ($P < 0.05$).

When comparing the initial and final mean wet weights between groups, the lower final mean wet weights of ragworms fed **AW + High Al** (group 4) suggest that the higher aluminium concentration in their feed has negatively impacted their growth. However, as their final mean wet weight was not significantly lower than other groups where aluminium was incorporated into their feed, it suggests that the aluminium has affected the other ragworms too, but not to the same extent as group 4.

In addition, ragworms fed **AW + High Al + C** (group 5), despite having the same concentration of aluminium added to the feed before coagulation and flocculation with chitosan, did not have the same negative impact on their growth as seen in ragworms fed **AW + High Al** (group 4).

6.6.2 Ragworm Specific Growth Rate When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium

Figure 6.2 shows each ragworm feed group's mean percentage growth increase as specific growth rates (SGR). The mean SGR for all feed groups ranged between 1.3 ± 2.8 and 5.7 ± 1.3 % d^{-1}. The highest mean SGR was observed in ragworms fed **AW** (group 1) but was not significantly higher than ragworms fed **CF**, **AW + Low Al** (group 2), and **AW + Med Al** (group 3). The lowest mean SGR was recorded in ragworms fed **AW + High Al** (group 4) but was not significantly lower than any other group except for ragworms fed **AW** (group 1).

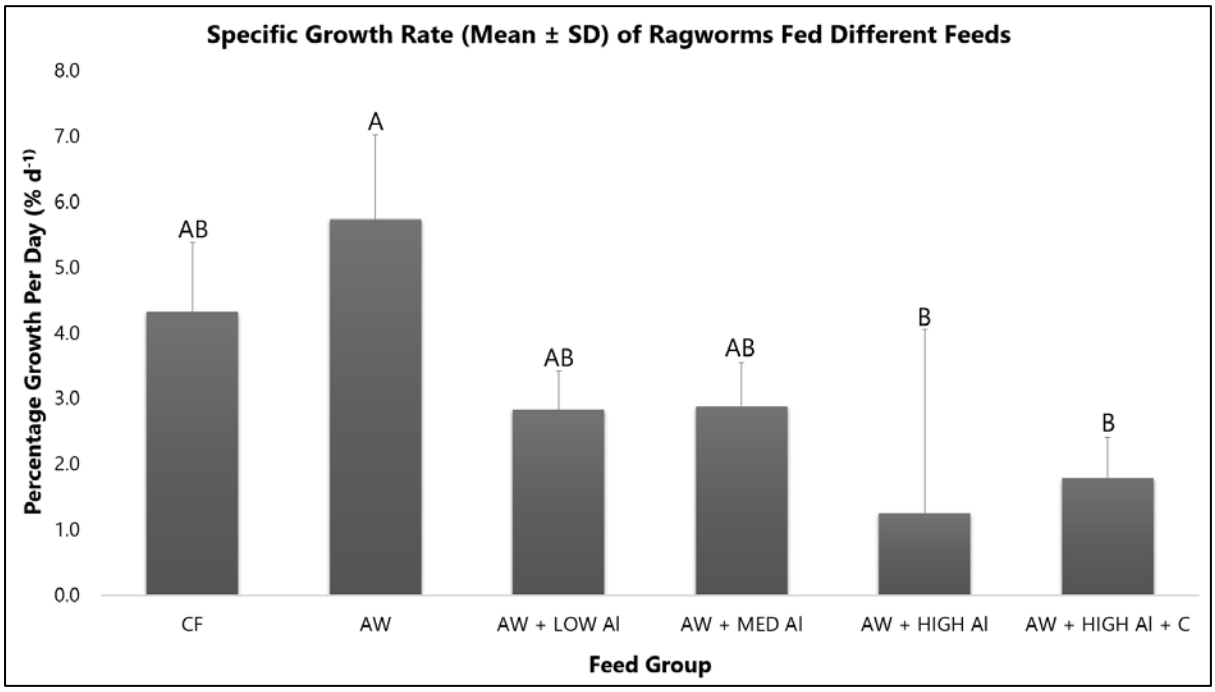

Figure 6.2 – Specific growth rate (mean ± SD) of ragworms fed CF or AW with varying concentrations of aluminium and chitosan
CF = ragworms fed CF; **AW** = ragworms fed AW; **AW + LOW Al** = ragworms fed AW with a low dose of aluminium; **AW + MED Al** = ragworms fed AW with a medium dose of aluminium; **AW + HIGH Al** = ragworms fed AW with a high dose of aluminium; **AW + HIGH Al + C** = ragworms fed AW with a high dose of aluminium coagulated and flocculated with chitosan. Different letters indicate significant differences ($P < 0.05$).

The fact that lower mean SGR was recorded in both ragworm feed groups with the highest dose of aluminium incorporated into their feed (**AW + High Al and AW + High Al + C**) further backs up the suggestion made in the previous Section (Section 6.6.1 – Ragworm Growth When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium), that aluminium has negatively impacted the growth of the ragworms. Furthermore, there is a clear trend in the decrease of mean SGR when the concentration of aluminium in the feed increases. Since the

development of contaminant tolerance consumes energy, less energy is available for other biological activities like growth (Durou, Mouneyrac and Amiard-Triquet, 2008; Mouneyrac, Perrein-Ettajani and Amiard-Triquet, 2010; Pedro *et al.*, 2022)

In addition, previous research has demonstrated that low aluminium concentrations can affect marine organisms' growth rates. For example, 10% effect concentrations (EC_{10}) in marine snails and diatoms (*Nassarius dorsatus* and *Ceratoneis closterium*) have been shown to reduce their growth rates (Gillmore *et al.*, 2016; Trenfield *et al.*, 2016; Botté *et al.*, 2022).

Moreover, numerous studies on fish have been done on the toxicity of aluminium. Fish and invertebrates may be stressed at moderate pH (5.5 to 7) because of aluminium adsorption onto gill surfaces causing subsequent asphyxiation (Sparling, Lowe and Campbell, 1997) and metabolic stress reducing growth rates. However, aquatic invertebrates such as polychaetes are typically less sensitive than fish to the toxicity of aluminium (Sparling, Lowe and Campbell, 1997).

6.6.3 Ragworm Mortality When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium

Throughout the experiment, mortalities were recorded to understand whether the chosen aluminium concentrations and exposure periods caused excessive fatalities in the ragworms. The mean percentage mortality ± standard deviation (SD) of each feed group is shown in Figure 6.3. Dead ragworms were removed immediately when found during the experiment.

Overall, the mortality throughout the experiment was low. No mortalities were recorded for ragworms fed **CF** over the 28 days. The mean percentage mortality in ragworms fed **AW** with varying concentrations of aluminium ranged between 2.2 ± 3.85 and 4.4 ± 7.7%. All mean percentage mortality results were statistically insignificant.

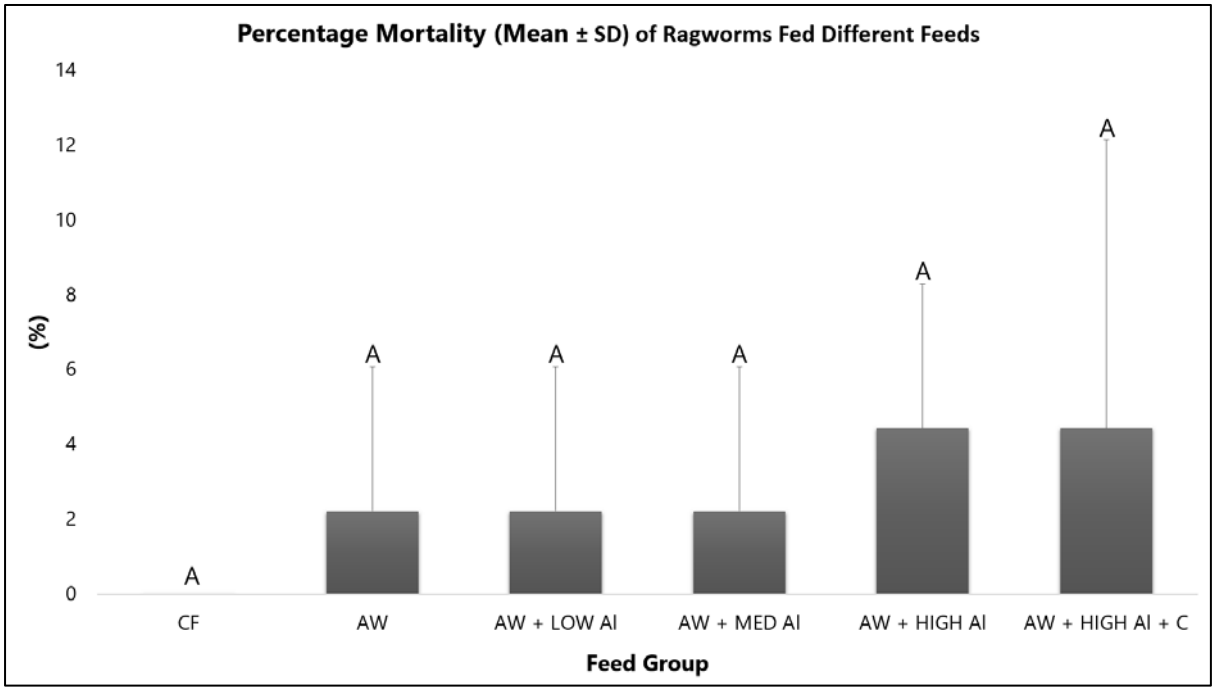

Figure 6.3 – Percentage mortality (mean ± SD) of ragworms fed CF or AW with varying concentrations of aluminium and chitosan
CF = ragworms fed CF; **AW** = ragworms fed AW; **AW + LOW Al** = ragworms fed AW with a low dose of aluminium; **AW + MED Al** = ragworms fed AW with a medium dose of aluminium; **AW + HIGH Al** = ragworms fed AW with a high dose of aluminium; **AW + HIGH Al + C** = ragworms fed AW with a high dose of aluminium coagulated and flocculated with chitosan. Different letters indicate significant differences ($P > 0.05$).

While aluminium concentrations in feed negatively impacted growth rates in ragworms at higher concentrations, higher aluminium concentrations in the feed appear not to have had a significant effect on mortality over the 28-day experiment. The low mortality suggests that the concentrations of aluminium in the feeds were below levels which can cause mortality in ragworms. In addition, the ragworms fed chitosan-coagulated, aluminium-contaminated **AW** did not affect mortality rates.

6.6.4 Ragworm Aluminium Accumulation When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium and Chitosan

Table 6.1 below displays four aluminium concentration readings for each feed group. Aluminium concentrations were recorded in ragworms after 1 day, 7 days, 14 days, and 28 days. Aluminium concentrations of ragworms fed CF were recorded at the start and the end of the experiment. In addition, the standard reference material (SRM) was analysed along with ragworm samples after 1 day and 28 days to ensure readings of the ragworm samples were accurate.

After 1 day and after 7 days, the results regardless of feed group did not differ significantly. After 14 days, similarly, ANOVA tests revealed that there were no significant differences between feed groups, most likely as a result of the wide-ranging standard deviations. However, the means suggest that there are variations between the groups. For example, there is a clear trend that as the aluminium concentration in the feed increases, the mean aluminium concentration in the ragworms increases. There is one exception where the ragworms fed **AW + High Al + C** (group 5) had lower aluminium concentrations after 14 days compared to **AW + High Al** (group 4). The same trend is observed after 28 days, however, significant differences between feed groups are not observed.

After 28 days, ragworms fed **AW + High Al** were significantly higher than all other groups except for ragworms fed **AW + High Al + C** (group 5) and **AW + Med Al** (group 3). In addition, the ragworms fed **Aw + High Al + C** and the ragworms fed **AW + Med Al** were not significantly higher than the other groups. However, as the means suggest, the ragworms fed **AW** with aluminium incorporated did have higher aluminium concentrations in their tissues. The ragworms fed **CF** had the same aluminium concentration at the start and end of the experiment.

Table 6.1 – Ragworm Aluminium Accumulation Over 28 Days

	AW	AW + LOW Al	AW + MED Al	AW + HIGH Al	AW + HIGH Al + C	CF	SRM
Aluminium Concentration After 1 Day (mg kg^{-1})							
Mean	1.00^{a}	0.48^{a}	2.37^{a}	1.61^{a}	2.48^{a}	2.02^{a}	1.12^{a}
SD	0.46	0.54	1.33	1.63	1.79	0.6	0.24
Aluminium Concentration After 7 Days (mg kg^{-1})							
Mean	2.54^{a}	3.38^{a}	2.96^{a}	3.98^{a}	2.91^{a}	-	-
SD	2.68	2.08	2.65	1.64	1.34	-	-
Aluminium Concentration After 14 Days (mg kg^{-1})							
Mean	2.47^{a}	8.74^{a}	11.24^{a}	31.63^{a}	13.44^{a}	-	-
SD	2.25	7.59	3.88	46.12	5.86	-	-
Aluminium Concentration After 28 Days (mg kg^{-1})							
Mean	6.03^{b}	26.36^{b}	37.02^{ab}	99.57^{a}	41.70^{ab}	2.02^{b}	1.2^{b}
SD	3.53	10.99	32.79	36.36	29.65	0.15	1.57

CF = ragworms fed CF; **SRM** = standard reference material; **AW** = ragworms fed AW; **AW + LOW Al** = ragworms fed AW with a low dose of aluminium; **AW + MED Al** = ragworms fed AW with a medium dose of aluminium; **AW + HIGH Al** = ragworms fed AW with a high dose of aluminium; **AW + HIGH Al + C** = ragworms fed AW with a high dose of aluminium coagulated and flocculated with chitosan. Different superscript letters indicate significant differences between groups (P > 0.05).

The results in Table 6.1 show that higher aluminium concentrations in the feed resulted in higher aluminium concentrations in the ragworms. Therefore, it can be said that ragworms bioaccumulate aluminium from their feed in a dose-dependent manner. The ragworms fed

AW with varying concentrations started to accumulate in their tissues. In contrast, the ragworms fed **AW** without the addition of aluminium, did not start to accumulate in their tissues until around 15 days. The aluminium concentrations of ragworms fed **CF** remained at a baseline level.

Increased metal concentrations in ragworm tissues as a result of increased exposure cannot be said for all metals. For example, zinc concentrations are not enhanced in ragworms in areas where zinc exposure is elevated suggesting zinc regulation mechanisms exist in ragworms (Berthet *et al.*, 2003). However, zinc is somewhat beneficial to ragworms as it forms part of their jaws (Lichtenegger *et al.*, 2003). On the other hand, there are no documented benefits for aluminium consumption by ragworms.

Furthermore, as reflected in the standard deviations, not all ragworms accumulate aluminium at the same rate, which agrees with similar research investigating other metals, such as copper (Ozoh and Jones, 1990). The differences in individual sizes of ragworms can also affect metal bioaccumulation, as suggested by other researchers (Cong *et al.*, 2014; Thit, Banta and Selck, 2015). Therefore, the means are a more accurate representation of the impact of aluminium bioaccumulation in ragworms.

Moreover, metal contamination tolerance in ragworms appears to be individually defined (Watson *et al.*, 2021) and in a broader sense, population-specific due to exposure to region-specific contaminants (Breton and Prentiss, 2019). In other words, if ragworms are exposed to a contaminant in their natural environment since birth, they will be more tolerant than a group of ragworms from a different environment not exposed to that contaminant.

On another note, ragworms fed **AW + High Al + C** had a lower mean aluminium concentration compared to ragworms fed **AW + High Al** (without chitosan), suggesting chitosan has had a positive impact on aluminium tolerance in ragworms. As suggested in the growth results (Section 6.6.1 – Ragworm Growth When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium and Section 6.6.2 – Ragworm Specific Growth Rate When Fed Aquaculture Wastewater with Varying Concentrations of Aluminium), most likely, this is an effect of the chitosan making the aluminium in the feed less bioavailable to the ragworm when ingested. The chitosan will bind to aluminium, perhaps making the aluminium less available during digestion, or the chitosan aids in the mechanisms in which ragworms tolerate

contaminant accumulation. Alternatively, as the **AW** has been coagulated and flocculated with chitosan, it is therefore a more concentrated feed than freeze-drying the **AW**. So, the aluminium concentration increases, but so too does the nutritional components of the feed which in turn can make the ragworms healthier and more able to tolerate the aluminium in their feed.

Finally, the ragworms fed **AW** without the addition of aluminium, still built up relatively high aluminium concentrations in their tissues compared to ragworms fed **CF**. The **AW** on its own had a mean aluminium concentration of 0.47 mg kg^{-1} (Appendix Q) highlighting that exposure to this relatively low concentration over a period of 28 days can result in built-up concentrations in the ragworms. Similar research has reported that low concentrations of copper resulted in elevated copper concentrations in the closely related *Alitta virens* (king ragworm) against the control (Watson *et al.*, 2021).

6.7 Conclusions

Results from this experiment suggest that the composition of the **AW** must be elementally analysed before it can be deemed safe to feed to ragworms. The main purpose of the elemental analysis would be to function as a pre-screen and identify potentially toxic contaminants, such as aluminium. As proven in this experiment, aluminium bioaccumulates in ragworms quite readily, even at relatively low concentrations. If the ragworms are fed to fish in an aquaculture system, the aluminium can potentially bioaccumulate in the fish and ultimately end up in humans when those fish are consumed.

Additionally, the results of this experiment demonstrated that chitosan lowered the rate of bioaccumulation of aluminium in ragworm tissues and reduced some of the detrimental impacts related to growth. However, the addition of chitosan did not prevent the concentration of aluminium in ragworm tissues from bioaccumulating to a high concentration. Therefore, this finding disproves the hypothesis stated in the introduction, where at the planning stages of this experiment, it was expected that the presence of chitosan would increase aluminium concentrations in ragworm tissues.

Furthermore, the results imply that the bioaccumulation of aluminium in ragworms is dose-dependent. However, as stated in the previous Section (Section 6.6 – Results and Discussion),

there are differences between individual ragworms in terms of their bioaccumulated aluminium concentrations, reflecting what has been observed in other research investigating other metals (Ozoh and Jones, 1990).

Chapter 7

Conclusions and Future Work

7.1 Conclusions

Conventional capture fisheries are becoming increasingly unreliable due to overfishing and improper management of fish stocks. The reliance on aquaculture to make up for the increased demand for fish products is increasing. However, most of the high-quality feed used in aquaculture systems comes from conventional capture fisheries. In addition, aquaculture systems produce wastewater that can negatively impact natural ecosystems and intensive systems produce large amounts of waste in the form of sludge.

The use of ragworms to bioremediate waste from aquaculture, while concomitantly producing an alternative ingredient of aquafeed is a potentially attractive solution to help reduce some of the unsustainable criticisms of aquaculture. Ragworms have fast growth rates, and favourable nutritional profiles and can assimilate nutrients from the waste (e.g., proteins and lipids). The use of chitosan to coagulate and flocculate aquaculture wastewater has also been proven to be an effective way of capturing solids that are dissolved or suspended within.

Moreover, as aquaculture waste is continually produced, ragworms can be continuously reared on the waste to create a renewable and commercially valuable resource. Aquaculture wastewater is the water that has been used to culture aquatic animals in aquaculture facilities and is normally high in organic matter (uneaten feed, faecal matter, and other biological wastes from the cultured animals) and nutrients (mainly nitrogen and phosphorous). However, several issues can prevent ragworm aquaculture wastewater bioremediation from becoming a more widely used technology for producing aquafeed ingredients. For instance, sources of aquaculture wastewater can contain toxic contaminants (e.g., heavy metals) which can be harmful to ragworms, potentially making ragworms unsuitable to be utilised as aquafeed ingredients. To an extent, there is a lack of understanding regarding the connections between biological, chemical, and physical parameters of ragworm bioremediation of aquaculture wastewater and the role chitosan plays. Standardised cultivation techniques and assessment of sources of aquaculture wastewater before it is fed to ragworms are required. In addition, a greater understanding of the biological aspects of metal bioaccumulation in ragworms is essential. Within this scope, the main objectives of the thesis were:

- To determine whether the presence of chitosan at different concentrations in feed (aquaculture wastewater) is harmful to the development of ragworms in terms of growth, mortality, and nutritional composition;

- To determine whether coagulating and flocculating aquaculture wastewater with chitosan and feeding to ragworms produces ragworms that can be used as a nutritionally suitable, alternative aquafeed ingredient;

- To examine how ragworms bioaccumulate an under-researched toxic contaminant (aluminium) from their feed (aquaculture wastewater) and the effect chitosan has on the process.

In this project, polychaetes of the species *Hediste diversicolor* (ragworms) were selected for investigation as they are widespread and can naturally exist in contaminated habitats. In addition, their nutritional profiles are reported in the literature to be suitable for a range of aquacultured species. Furthermore, ragworms in previous research have been regarded as nutritionally viable when reared on sources of aquaculture wastewater. Therefore, the properties of ragworms suggest they have potential in the applications of aquaculture wastewater bioremediation while producing a source of alternative aquafeed ingredients. However, until now, the potential benefit of interjecting chitosan into this process has not been investigated.

In chapter 4, chitosan was added to a feed source of aquaculture wastewater at different concentrations and fed to ragworms and compared against ragworms fed aquaculture wastewater without chitosan and ragworms fed with a nutritionally complete commercial feed. The different chitosan concentrations were 10, 20 and 30 mg L^{-1}. As expected, the ragworms fed the commercial feed had the best growth rates, lowest mortality rates and best fatty acid profiles. However, surprisingly, the amino acid profiles of the ragworms fed aquaculture wastewater with and without chitosan were more favourable than ragworms fed the commercial feed suggesting the source of aquaculture wastewater used in the experiment was a high-quality protein source. The growth results showed that ragworms fed aquaculture wastewater with the highest concentration of chitosan (30 mg L^{-1}) had the lowest growth rates compared with the other groups (0.5 ± 0.8 % d^{-1} compared to between 1.0 ± 1.0 % d^{-1} and 4.8 ± 2.4 % d^{-1}). Furthermore, the ragworms fed aquaculture wastewater with the

highest concentration of chitosan (30 mg L^{-1}), also had the highest mean mortality percentage (23.33% compared to between 0 and 20%). Moreover, regarding the nutritional profiles of the ragworms, those fed aquaculture with the highest concentration of chitosan (30 mg L^{-1}) had a higher proportion of saturated fatty acids and a lower proportion of polyunsaturated fatty acids suggesting chitosan at the higher concentration interfered with the assimilation of fatty acids in the ragworms. Fortunately, 30 mg L^{-1} is much higher than what is typically required to coagulate and flocculate a source of aquaculture wastewater. Therefore, the detrimental effects recorded in ragworms fed aquaculture wastewater with a 30 mg L^{-1} chitosan concentration would unlikely be seen in a real-life application.

In chapter 5, chitosan was used to coagulate and flocculate a source of aquaculture wastewater and fed to ragworms and compared against other methods of treating aquaculture wastewater before feeding to ragworms (freeze-drying, settling, and centrifuging). In addition, the ragworms were also compared against a group of ragworms fed a nutritionally complete commercial feed. In terms of growth rates, ragworms fed chitosan coagulated and flocculated aquaculture wastewater were lower than ragworms fed the commercial feed and the centrifuged aquaculture wastewater (2.9 ± 0.7 % d^{-1} compared to 4.2 ± 0.3 % d^{-1} and 4.3 ± 1.1 % d^{-1}), but higher than ragworms fed settled or freeze-dried aquaculture wastewater (1.9 ± 0.5 % d^{-1} and 1.9 ± 0.6 % d^{-1}). Despite lower growth rates, the ragworms fed chitosan coagulated and flocculated aquaculture wastewater demonstrated favourable amino acid and fatty acid profiles with concentrations and compositions suitable for multiple fish species. Regarding fatty acid profiles, the ragworms fed chitosan coagulated and flocculated aquaculture wastewater had higher concentrations of polyunsaturated fatty acids (16.96 ± 1.14 mg g^{-1}) compared to ragworms fed aquaculture wastewater that was processed in different ways. In addition, the ragworms fed chitosan coagulated and flocculated aquaculture wastewater had limiting essential amino acid (LEAA) concentrations suitable to meet the requirements of 7 fish species, outperforming ragworms fed commercial feed in this analysis. The findings of this chapter are perhaps the most important in the thesis as they demonstrate that ragworms fed chitosan coagulated and flocculated aquaculture wastewater are nutritionally viable to be fed to fish reared in aquaculture systems in the form of aquafeeds.

In chapter 6, upon review of the elemental analysis of a source of aquaculture wastewater, it was shown to have a very high concentration of aluminium (1754 mg kg^{-1}). Access to that source of aquaculture wastewater was lost as a result of the SARS-CoV-2 outbreak and the resulting lockdown in 2020. However, a decision was made to spike another source of aluminium wastewater with aluminium to investigate how quickly the aluminium bioaccumulates in ragworm tissues and the effect chitosan has on the process. Different concentrations of aluminium were spiked into aquaculture wastewater and freeze-dried for 24 hours before feeding it to different groups of ragworms. One group were fed a source of aluminium-spiked aquaculture wastewater that was subsequently coagulated and flocculated with chitosan. One group was fed aquaculture wastewater without spiking with aluminium and another group was fed a commercial feed. Growth rates in ragworms were observed to decrease as the concentration of aluminium in the feed increased. However, the same was not observed in ragworms fed the chitosan coagulated and flocculated aluminium spiked aquaculture wastewater. Most likely, this is because the chitosan coagulated and flocculated aquaculture wastewater is more concentrated than freeze-drying. So, at the same time, the concentration of aluminium in the feed increases but so too do the nutritional components, therefore, improving the health of the ragworms. Regarding the bioaccumulation of aluminium in ragworm tissues, this occurred in a dose-dependent manner. In other words, a higher dose of aluminium in the feed resulted in a higher aluminium concentration in the ragworm tissues, except for the ragworms fed the chitosan coagulated and flocculated aluminium spiked aquaculture wastewater. Again, this is most likely due to the ragworms being fed a nutritionally better feed and are therefore healthier and able to tolerate the aluminium more effectively. Overall, the results of the experiment demonstrated the importance of analysing aquaculture wastewater before it is fed to ragworms. This is essential as it was shown in the aluminium wastewater feed that was not spiked with aluminium still contained aluminium at a relatively low concentration (0.47 ± 0.04 mg kg^{-1}). In turn, the aluminium from this feed bioaccumulated in the ragworms fed it (6.03 ± 3.53 mg kg^{-1}). This shows that if this source of aquaculture wastewater were chosen to be a feed source for ragworms before processing them into aquafeed, then a pre-treatment step would be required to remove the aluminium.

7.2 Future Work

The research findings suggest that chitosan and ragworms used in conjunction have the potential to bioremediate aquaculture wastewater while concomitantly producing alternative, nutritionally suitable aquafeed ingredients. The next logical step would be to research how the studied ingredient (ragworms reared on chitosan coagulated and flocculated aquaculture wastewater) performs within fish nutrition and digestibility trials (a proof-of-concept study).

Before ragworms and chitosan may feasibly be considered for application, more research and development are required. Areas identified that are outside the scope of this thesis but require more exploration are described chapter by chapter below and a summary of what is needed to make the aquaculture industry more sustainable follows.

7.2.1 Chapter 4 – Controlled Chitosan Toxicity Study in Ragworms

More experimentation is required to understand, with greater confidence, the impact of chitosan toxicity in ragworms when it is included in their feed. Therefore, an idea for future work within this area would be to run a controlled chitosan toxicity study in ragworms like the ones carried out with rats and mice described in Section 2.4.7 (chitosan toxicity in animals).

A larger scale experiment where juvenile ragworms are reared under optimal conditions and fed a nutritionally optimal feed with different chitosan concentrations incorporated and controls would be best. The experiment should include periodic analysis of ragworm characteristics that are required for their utilisation as aquafeed, such as growth rates, mortality, and nutritional composition. In addition, ragworms originating from the same broodstock should be used to minimise the influence of biological variety and ragworms maintained in a biosecure flow-through system to minimise the potential of pathogens entering the system.

Moreover, the study should last over a period longer than 28 days to establish whether long-term exposure to chitosan, even at low concentrations, is detrimental to ragworms. For instance, it has been shown that ragworms can reach commercial size within four months

(Nesto *et al.*, 2012). Therefore, an experiment lasting at least this length of time would be optimal.

7.2.2 Chapter 5 – Feeding Chitosan Coagulated and Flocculated Aquaculture Wastewater to Create Alternative Aquafeed Ingredients – An Economic Feasibility Study

To extend the applicability of feeding chitosan-coagulated and flocculated aquaculture wastewater to ragworms to create alternative aquafeed ingredients, an in-depth cost analysis should be carried out. The aim would be to compare the costs of different treatment methods (freeze-drying, settling, centrifuging and chitosan coagulation and flocculation). In addition, the scale would have to be established to estimate costs related to aquaculture wastewater transport, aquaculture wastewater processing, electricity, water, equipment, packaging, and shipping of the produced ragworm aquafeeds, amongst other parameters, would all have to be considered.

Once projected costs for the creation of alternative aquafeed ingredients in the form of ragworms reared on chitosan-coagulated and flocculated aquaculture wastewater have been established, they can be compared against the costs of making traditionally used aquafeeds. At this point, aquaculture facilities managers can then determine whether switching to a more sustainable product is economically feasible.

7.2.3 Chapter 6 – Ragworm Aluminium Depuration Experiment

The scope for future work surrounding the issue of metal contamination in aquaculture wastewater that is intended to feed marine animals before processing into aquafeed is broad. Two suggestions for future experiments related to aluminium, aquaculture wastewater, chitosan and ragworms are presented below:

1. From the results of this experiment, the bioaccumulation of aluminium in ragworm tissues when fed a source of AW that has an elevated aluminium content is dose-dependent. However, aluminium depuration mechanisms and timescales have not been investigated. For example, it may be the case that ragworms can depurate toxic aluminium from their tissues

if given enough time and if they have the mechanisms to do so, would make them safe again to process into aquafeed. Perhaps the ragworms can be fed a contaminated source of AW for one month while developing and the following month, they switch to an alternative, commercially available feed allowing time for depuration while keeping them viable. Using the contaminated AW for part of the maturation process may lower costs compared to feeding the ragworms the commercially available feed the whole time and still contribute to making aquaculture more sustainable.

2. Investigate the mechanisms involved in ragworms bioaccumulating aluminium from their feed. In addition, investigate how chitosan interacts with these mechanisms and discover why in the experiment described above, the ragworms fed the aluminium-contaminated AW that had been coagulated and flocculated with chitosan had lower aluminium concentrations in their tissues.

7.2.4 Making Aquaculture More Sustainable

Addressing numerous environmental, social, and economic variables is necessary to increase the sustainability of aquaculture. Here are a few holistic suggestions for doing so:

1. Encourage research and development to enhance aquaculture's sustainability, like the research presented in this thesis. The idea of sustainable aquaculture is increasingly understood through technical research to investigate environmental, economic and social aspects of spreading sustainable aquaculture knowledge throughout the world (Frankic and Hershner, 2003);

2. Put into practice feed techniques that employ feed derived from sustainable and renewable sources (such as ragworms reared on **AW** that have been processed with chitosan) to cut feed waste and raise feed conversion ratios (Naylor *et al.*, 2009) and reduce reliance on wild-caught fish to produce aquafeed;

3. Integrate multi-trophic aquaculture systems (IMTA), which contain plant, animal, and microbial species to build a healthy and sustainable system. The by-products of one cultured species are passed to another to produce valuable products and promote environmental

bioremediation (Mohsen and Yang, 2021). Increasing product diversity also lessens reliance on singular species and markets (Das and Mandal, 2022);

4. Utilise more environmentally friendly farming techniques, like closed-containment systems, to reduce the waste released and disease transmission. With improved market acceptance and scalability, recirculating aquaculture systems (RAS) development has significantly risen during the last two decades (Espinal and Matulić, 2019);

5. Produce more substantial and disease-resistant fish through selective breeding and genetic improvement initiatives. One of the most significant issues with aquaculture production is fish and shellfish disease. Therefore, every effort should be taken to raise the survival rate and thereby lower economic losses (Gjedrem, 2015);

6. Increase accountability and transparency in aquaculture through certification and labelling initiatives to encourage and support sustainability. Positive connotations are associated with openness in value chains. The more information, the better for chain sustainability, consumer empowerment and civil society (Mol, 2015).

7.3 Concluding Remarks

Aquaculture wastewaters are incredibly complex mixtures with several interrelated biological, chemical, and physical variables, which all interact with ragworms and chitosan in different ways, which are both already very complicated subjects. Results obtained from this study (feeding chitosan coagulated aquaculture wastewater to ragworms for aquaculture wastewater bioremediation and producing alternative aquafeed ingredients) were promising. The ragworms fed chitosan coagulated and flocculated aquaculture wastewater exhibited good rates of growth and developed suitable nutritional profiles for a variety of fish species (chapter 5). In addition, the presence of chitosan at concentrations required to effectively coagulate and flocculate the aquaculture wastewater returned no significant detrimental effects to the ragworms (chapter 4). However, potential contaminants in aquaculture wastewaters, most importantly toxic heavy metals, must be identified before feeding to ragworms. As seen in the case of aluminium, contaminants can bioaccumulate in ragworms

very quickly which would make them unsuitable to be processed into aquafeed intended to feed animals reared in aquaculture systems (chapter 6).

A controlled and consistent source of aquaculture wastewater before feeding to ragworms would be required, including constant analysis of the wastewater and ragworms to ensure there are not any harmful contaminants. A ragworm aquaculture system located on-site at a current aquaculture facility or ragworms included in an integrated multitrophic aquaculture system would be the ideal logical scenario. However, this setup would require input from biological and chemical scientists as well as civil and environmental engineers. Not to mention funding from relevant industrial partners, funding councils and academic institutions to make sure the system is viable economically.

Overall, this is a fascinating concept with a lot of promise, but it also poses significant obstacles to overcome. Like many other technologies, it needs further research and development.

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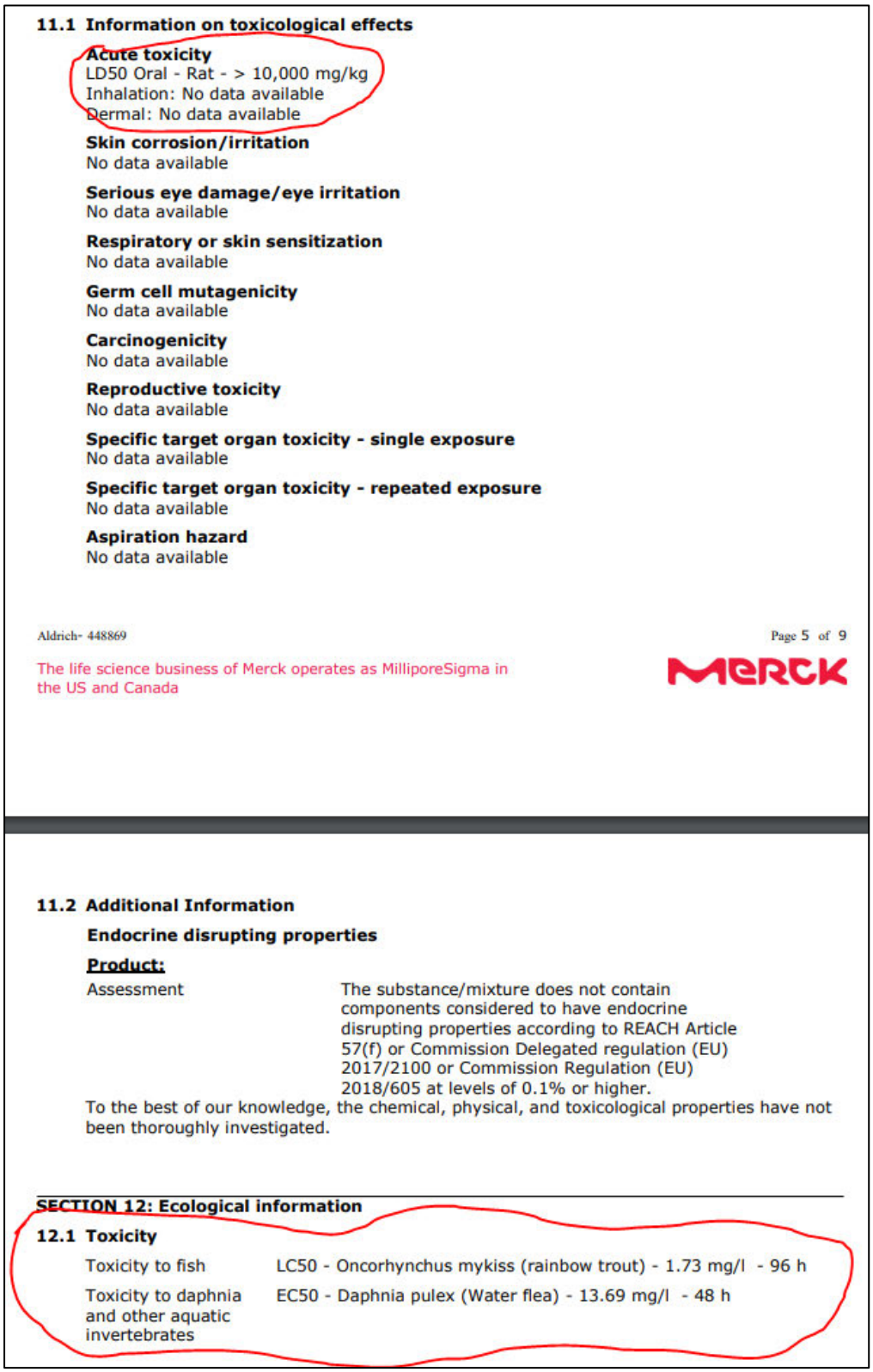
Appendices

Appendix A - Sigma-Aldrich Chitosan Safety Data Sheet



11.1 Information on toxicological effects

Acute toxicity
LD50 Oral - Rat - > 10,000 mg/kg
Inhalation: No data available
Dermal: No data available

Skin corrosion/irritation
No data available

Serious eye damage/eye irritation
No data available

Respiratory or skin sensitization
No data available

Germ cell mutagenicity
No data available

Carcinogenicity
No data available

Reproductive toxicity
No data available

Specific target organ toxicity - single exposure
No data available

Specific target organ toxicity - repeated exposure
No data available

Aspiration hazard
No data available

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The life science business of Merck operates as MilliporeSigma in the US and Canada

11.2 Additional Information

Endocrine disrupting properties

Product:

Assessment	The substance/mixture does not contain components considered to have endocrine disrupting properties according to REACH Article 57(f) or Commission Delegated regulation (EU) 2017/2100 or Commission Regulation (EU) 2018/605 at levels of 0.1% or higher.

To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

SECTION 12: Ecological information

12.1 Toxicity

Toxicity to fish	LC50 - Oncorhynchus mykiss (rainbow trout) - 1.73 mg/l - 96 h
Toxicity to daphnia and other aquatic invertebrates	EC50 - Daphnia pulex (Water flea) - 13.69 mg/l - 48 h

Figure A1 – Screenshot of pages 5 and 6 of the Sigma-Aldrich Chitosan Safety Data Sheet
Available at: https://www.sigmaaldrich.com/GB/en/sds/aldrich/448869

Appendix B - ThermoFisher Scientific Chitosan Safety Data Sheet

ThermoFisher SCIENTIFIC

SAFETY DATA SHEET

Creation Date 26-Aug-2009 **Revision Date** 26-Dec-2021 **Revision Number** 5

1. Identification

Product Name Chitosan

Cat No. : AC428850000; AC428850500; AC428851000; AC428855000

CAS No 9012-76-4
Synonyms Poly(beta-(1,4)-2-amino-2-deoxy-D-glucose); Poly(beta-(1,4)-D-glucosamine)

Recommended Use Laboratory chemicals.
Uses advised against Food, drug, pesticide or biocidal product use.

Details of the supplier of the safety data sheet

Company
Fisher Scientific Company
One Reagent Lane
Fair Lawn, NJ 07410
Tel: (201) 796-7100

Acros Organics
One Reagent Lane
Fair Lawn, NJ 07410

Emergency Telephone Number For information **US** call: 001-800-ACROS-01 / **Europe** call: +32 14 57 52 11
Emergency Number **US:**001-201-796-7100 / **Europe:** +32 14 57 52 99
CHEMTREC Tel. No.**US:**001-800-424-9300 / **Europe:**001-703-527-3887

2. Hazard(s) identification

Classification
Classification under 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200)

This chemical is not considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200)

Label Elements
None required

Hazards not otherwise classified (HNOC)
None identified

Figure B1 – Screenshot of page 1 of the ThermoFisher Scientific Chitosan Safety Data Sheet
Available at:
https://www.fishersci.com/store/msds?partNumber=AC428850500&productDescription=CHITOSAN%2C+MOLECULAR+WEIG+50GR&vendorId=VN00032119&countryCode=US&language=en

Appendix C - Chitosan Dose

The following equation was used to calculate the chitosan dose to add to 900 ml **AW** to achieve the concentrations for groups 3, 4 and 5 described in Section 4.2.3 – Feed Groups:

$$V1C1 = V2C2$$

Where: **V1** = Volume of Stock Required

C1 = Stock Concentration (0.1 g/L)

V2 = Final Solution Volume (0.9 L)

C2 = Concentration in Final Volume (0.001 g/L)

For group 3:

$$V1 = \frac{0.001\ g\ L^{-1} \times 0.9\ L}{0.1\ g\ L^{-1}}$$

$$V1 = 0.009\ L$$

$$V1 = 9\ mL$$

For group 4:

$$V1 = \frac{0.002\ g\ L^{-1} \times 0.9\ L}{0.1\ g\ L^{-1}}$$

$$V1 = 0.018\ L$$

$$V1 = 18\ mL$$

For group 5:

$$V1 = \frac{0.003\ g\ L^{-1} \times 0.9\ L}{0.1\ g\ L^{-1}}$$

$$V1 = 0.027\ L$$

$$V1 = 27\ mL$$

Appendix D - Turbidity

Table D1 – Mean ± SD turbidity of culture water for each feed group

Feed Group	Mean ± SD Turbidity of Culture Water (NTU)
Group 1a	147.22 ± 52.74[a]
Group 1b	5.18 ± 3.18[b]
Group 2	7.49 ± 5.62[b]
Group 3	6.81 ± 5.75[b]
Group 4	7.95 ± 7.67[b]
Group 5	10.69 ± 5.64[b]

NTU = Nephelometric Turbidity Units. Mean turbidity of clean artificial seawater = ≈0.478 NTU. Means that do not share the same superscript letter are significantly different (P > 0.05).

All results for turbidity were similar except for group 1a. The far higher turbidity recorded in group 1a can be explained by the addition of too much **CF** causing the water quality to deteriorate. The turbidity values reflect what was observed in the pH values (Appendix G). The highest mean turbidity value was in group 1a (147.22 ± 52.74 NTU). The lowest was group 1b (5.18 ± 3.18 NTU).

Appendix E - Temperature

Table E1 – Mean temperature ± SD for each feed group

Feed Group	Mean Temperature ± SD (°C)
Group 1a	20.16 ± 1.25[a]
Group 1b	21.7 ± 1.04[b]
Group 2	19.94 ± 2.88[a]
Group 3	19.96 ± 1.26[a]
Group 4	19.93 ± 1.24[a]
Group 5	20.02 ± 1.31[a]

Means that do not share the same superscript letter are significantly different (P > 0.05).

All results for temperature were similar, except for group 1b, which was significantly different. The higher temperature of group 1b culture water can be explained by a slight increase in the ambient temperature of the laboratory room where the experiment was conducted, as it was later into the summer months. The highest mean value ± SD was obtained in group 1b (21.7 ± 1.04 °C), and the lowest mean value ± SD obtained was in group 4 (19.93 ± 1.24 °C).

Appendix F - Conductivity

Table F1 – Mean conductivity ± SD for each feed group

Feed Group	Mean Conductivity ± SD (S/m)
Group 1a	20.58 ± 2.71[ab]
Group 1b	19.28 ± 3.01[b]
Group 2	21.42 ± 2.74[a]
Group 3	21.03 ± 2.50[a]
Group 4	20.98 ± 2.31[a]
Group 5	21.33 ± 2.28[a]

S/m = siemens per metre. Means that do not share the same superscript letter are significantly different (P > 0.05).

The mean results for conductivity were similar across all groups. The highest mean value ± SD was in group 2 (21.42 ± 2.74 S/m), and the lowest was in group 1b (19.28 ± 3.01 S/m). Group 1b was significantly lower than other groups, except for group 1a. Group 1b ragworms were fed half of what other groups were fed; therefore, a lower mean conductivity throughout the experiment makes sense. Less is being added to the culture water and, therefore, fewer salts resulting in lower conductivity.

Appendix G - pH

Table G1 – Mean pH ± SD for each feed group

Feed Group	Mean pH ± SD
Group 1a	7.37 ± 0.51[a]
Group 1b	8.07 ± 0.18[b]
Group 2	8.08 ± 0.13[b]
Group 3	8.12 ± 0.12[b]
Group 4	8.13 ± 0.13[b]
Group 5	8.12 ± 0.12[b]

Means that do not share the same superscript letter are significantly different (P > 0.05).

As discussed in Section 4.2.7 – A Problem with Group 1, the water quality of group 1a culture beakers was affected by the addition of too many commercial fish feed pellets (1 g). Table G1 shows that the mean pH in group 1a was significantly lower than the other groups, which were all similar. Excluding group 1a, the highest pH value ± SD was group 4 (8.13 ± 0.13), and the lowest was group 1b (8.07 ± 0.18). The mean values in all groups, excluding group 1a, are all within 0.06 of each other.

Previous research has shown that ragworms exposed to a low pH of 7.3 over 28 days experienced stress indicators such as impacted osmotic regulation, higher carbonic anhydrase activity, lower energy reserves and a higher metabolic rate (Freitas *et al.*, 2016). The ragworms in group 1a were exposed to an average pH of 7.37 ± 0.51 throughout the experiment.

Appendix H - Aquaculture Wastewater Elemental Analysis

Table H1 – Cleaner fish aquaculture wastewater elemental analysis

Element	Concentration (mg kg^{-1}) [standard deviation]
Ag	**0.00** [± 0.00)
Al	**0.47** [± 0.04]
B	**0.24** [±0.06]
Ba	**0.09** [±0.01]
Ca	**149.39** [±10.61]
Cd	**0.00** [± 0.00)
Ce	**0.00** [± 0.00)
Co	**0.00** [± 0.00)
Cr	**0.01** [± 0.00)
Cu	**0.32** [± 0.05]
Fe	**4.72** [± 0.33]
Ga	**0.00** [± 0.00)
Hg	**0.00** [± 0.00)
K	**5.93** [± 1.27]
Mg	**16.67** [± 3.88]
Mn	**0.19** [± 0.01]
Na	**124.29** [± 30.14]
Ni	**0.01** [± 0.00]
P	**67.21** [± 7.72]
Pb	**0.18** [± 0.12]
S	**49.41** [± 8.14]
Si	**1.19** [± 0.70]
Sr	**1.55** [± 0.28]

Ti	**0.04** [± 0.01]
Zn	**1.51** [± 0.09]
Zr	**0.00** [± 0.00)

Number of repeats = 4. Ag = silver; Al = aluminium; B = boron; Ba = barium; Ca = calcium; Cd = cadmium; Ce = cerium; Co = cobalt; Cr = chromium; Cu = copper; Fe = iron; Ga = gallium; Hg = mercury; K = potassium; Mg = manganese; Na = sodium; Ni = nickel; P = phosphorus; Pb = lead; S = sulphur; Si = silicon; Sr = strontium; Ti = titanium; Zn = zinc; Zr = zirconium.

Appendix I - Temperature

Table I1 – Mean temperature ± SD for each feed group

Feed Group	Mean Temperature ± SD (°C)
Group 1	21.72 ± 1.04[a]
Group 2	21.53 ± 1.03[a]
Group 3	21.57 ± 1.06[a]
Group 4	21.57 ± 1.06[a]
Group 5	21.47 ± 1.08[a]

Means that do not share the same superscript letter are significantly different (P > 0.05).

The mean temperature results in all groups were statistically similar. The highest temperature value was observed in group 1 (21.72 ± 1.04 °C) and the lowest in group 5 (21.47 ± 1.08 °C).

Appendix J - Conductivity

Table J1 – Mean conductivity ± SD for each feed group

Feed Group	Mean Conductivity ± SD (S/m)
Group 1	19.28 ± 3.01[b]
Group 2	20.08 ± 3.22[ab]
Group 3	20.25 ± 3.20[ab]
Group 4	21.24 ± 3.09[a]
Group 5	20.85 ± 3.13[a]

S/m = siemens per metre. Means that do not share the same superscript letter are significantly different (P > 0.05).

All mean conductivity values were statistically similar except for group 1. Group 1 was significantly lower than groups 2 and 3, but not groups 4 and 5. Group 1 ragworms were fed **CF** which is specifically formulated to help maintain culture water quality, so the lower conductivity makes sense. The highest mean conductivity value was recorded in group 4 (21.24 ± 3.09 S/m) and the lowest in group 1 (19.28 ± 3.01 S/m).

Appendix K - pH

Table K1 – Mean pH ± SD for each feed group

Feed Group	Mean pH ± SD
Group 1	8.07 ± 0.18[b]
Group 2	8.07 ± 0.10[b]
Group 3	8.13 ± 0.13[a]
Group 4	8.12 ± 0.08[ab]
Group 5	8.15 ± 0.08[a]

Means that do not share the same superscript letter are significantly different (P > 0.05).

The pH values were more statistically varied than the temperature, conductivity and DO results. The highest mean pH value was observed in group 5 (8.15 ± 0.08) and the lowest in groups 1 and 2 (8.07 ± 0.18 and 8.07 ± 0.10). Groups 1 and 2 were significantly lower than groups 3 and 5. Group 4 was not significantly higher or lower than any other group. Despite statistical tests showing significant differences between groups, all groups were within 0.08 of each other. When the SD is considered, the values are, in reality, very similar.

Appendix L - Dissolved Oxygen

Table L1 – Mean dissolved oxygen (DO) ± SD for each feed group

Feed Group	Mean DO ± SD (mg L^{-1})
Group 1	6.9 ± 0.75^{b}
Group 2	7.5 ± 0.52^{a}
Group 3	7.3 ± 0.65^{a}
Group 4	7.5 ± 0.61^{a}
Group 5	7.4 ± 0.46^{a}

Means that do not share the same superscript letter are significantly different ($P > 0.05$).

All mean DO values were statistically similar in all groups, except for group 1 which was significantly lower. The groups with the highest mean DO values were groups 2 and 4 (7.5 ± 0.52 and 7.5 ± 0.61 mg L^{-1}) and the lowest was group 1 (6.9 ± 0.75 mg L^{-1}).

Despite statistical tests showing that group 1 was significantly lower than the other groups when the SD is considered, the values are more similar. Due to the nature of the experimental set-up, where tubes provide oxygenation to the culture beaker, there may be areas where the DO content is higher or lower than other areas. However, as the range was between 6.9 and 7.5 mg L^{-1}, the impact of the variation of oxygenation in certain areas is negligible.

Appendix M - Temperature

Table M1 – Mean temperature ± SD for each feed group

Feed Group	Mean Temperature ± SD (°C)
Group 1	21.70 ± 1.38[a]
Group 2	21.55 ± 1.36[a]
Group 3	21.69 ± 1.29[a]
Group 4	21.75 ± 1.31[a]
Group 5	21.39 ± 1.40[a]

Means that do not share the same superscript letter are significantly different (P > 0.05).

The mean temperature results in all groups were statistically similar. The highest temperature value was observed in group 4 (21.75 ± 0.06 °C) and the lowest in group 5 (21.39 ± 0.14 °C).

Appendix N - Conductivity

Table N1 – Mean conductivity ± SD for each feed group

Feed Group	Mean Conductivity ± SD (S/m)
Group 1	21.64 ± 3.08[a]
Group 2	21.42 ± 2.59[a]
Group 3	21.00 ± 2.71[a]
Group 4	21.01 ± 2.74[a]
Group 5	20.72 ± 2.80[a]

S/m = siemens per metre. Means that do not share the same superscript letter are significantly different (P > 0.05).

All mean conductivity results in all groups were statistically similar. The highest conductivity value was recorded in group 1 (21.64 ± 0.21 S/m) and the lowest in group 5 (20.72 ± 0.48 S/m).

Appendix O - pH

Table O1 – Mean pH ± SD for each feed group

Feed Group	Mean pH ± SD
Group 1	8.25 ± 0.08[a]
Group 2	8.23 ± 0.06[a]
Group 3	8.24 ± 0.06[a]
Group 4	8.24 ± 0.07[a]
Group 5	8.16 ± 0.06[b]

Means that do not share the same superscript letter are significantly different (P > 0.05).

The highest mean pH value was observed in group 1 (8.25 ± 0.08) and the lowest in group 5 (8.16 ± 0.06). Group 5 was significantly lower than other groups. Despite statistical tests showing significant differences between group 5 and the others, they were very similar.

Appendix P - Dissolved Oxygen

Table P1 – Mean dissolved oxygen (DO) ± SD for each feed group

Feed Group	Mean DO ± SD (mg L^{-1})
Group 1	7.26 ± 0.54^{b}
Group 2	7.62 ± 0.51^{a}
Group 3	7.61 ± 0.53^{a}
Group 4	7.53 ± 0.59^{a}
Group 5	7.52 ± 0.44^{a}

Means that do not share the same superscript letter are significantly different ($P > 0.05$).

All mean DO values were statistically similar in all groups, except for group 1 which was significantly lower. The group with the highest mean DO value was group 2 (7.62 ± 0.51) and the lowest was group 1 (7.26 ± 0.54 mg L^{-1}).

Despite statistical tests showing that group 1 was significantly lower than the other groups when the SD is considered, the values are more similar. Due to the nature of the experimental set-up, where tubes provide oxygenation to the culture beaker, there may be areas where the DO content is higher or lower than other areas. A similar trend was observed in chapter 5 (Appendix L).

Appendix Q - Aluminium Concentrations in Feeds after Processing

Table Q1 – Aluminium concentrations in feed after processing by freeze-drying or coagulating and flocculating with chitosan

Feed Group	Aluminium Concentration (mg kg^{-1})
AW	0.47 ± 0.04
AW + Low Al	523.58 ± 1.39
AW + Med Al	865.03 ± 2.19
AW + High Al	2065.49 ± 2.47
AW + High Al + C	2614.48 ± 9.45

AW = aquaculture wastewater; **AW + Low Al** = aquaculture wastewater with a low dose of aluminium; **AW + Med Al** = aquaculture wastewater with a medium dose of aluminium; **AW + High Al** = aquaculture wastewater with a high dose of aluminium; **AW + High Al + C** = aquaculture wastewater with a high dose of aluminium that has been coagulated and flocculated with chitosan.

Appendix R - Conference Participation

Conferences Attended:

- Climate Launchpad (European Institute of Innovation and Technology [EIT] Climate-Knowledge and Innovation communities [KICs] [EIT Climate-KIC]) – McEwan Hall/Teviot Building at The University of Edinburgh – 01/11/2018 to 02/11/2018 – Volunteer;

- 28th Annual University of Edinburgh School of Geosciences Graduate School Conference – MacDonald Cardrona Hotel, Peebles, Scotland – 25/01/2019 to 27/01/2019 – Poster Presentation;

- Industrial Biotechnology Innovation Centre (IBioIC) Annual Conference 2019: Industrial Biotechnology for a Sustainable Future – Glasgow – 30/01/2019 to 31/01/2019 – Poster Presentation;

- The University of Edinburgh School of Engineering Postgraduate Research Conference 2019 – John McIntyre Centre, Pollok Halls of Residence, The University of Edinburgh, Edinburgh – 18/04/2019 – Attended;

- 29th Annual University of Edinburgh School of Geosciences Graduate School Conference – DoubleTree by Hilton, Westerwood, Glasgow – 07/02/2020 to 09/02/2020 – Presentation;

- The University of Edinburgh School of Engineering Postgraduate Research Conference 2020 – Online Conference – 11/09/20 – Presentation and poster presentation;

- The University of Edinburgh School of Engineering Postgraduate Research Conference – Online Conference – 16/04/2021 – Attended

Resource Recovery Using Chitosan and Ragworms

Discovering the Potential of Ragworms to Bioremediate Wastewater with Chitosan

Blair Mackie

Supervisors:
- Dr Andrea Semiao (The University of Edinburgh)
- Dr Adam Hughes (The Scottish Association for Marine Science)
- Dr Jonathan Hughes (Pennosan)

THE UNIVERSITY of EDINBURGH

Pennosan

SAMS

Project Hypothesis

'Ragworms and chitosan can bioremediate aquaculture and freshwater effluents allowing for the recovery of valuable resources'

Figure 1. Polychaete (Ragworm)

Figure 2. Chitosan Powder

- **Ragworms** (*Figure 1*):
  - Marine segmented annelid worms.
  - Capable of survival in challenging environments.
  - Capable of bioremediation in wastewater.
  - Studied for use in recirculating aquaculture systems.
    - Consume wastewater pollutants for nourishment.
- **Chitosan** (*Figure 2*):
  - Linear polysaccharide molecule with coagulating properties.
  - Derived from hard outer skeletons of crustaceans.
  - Sold commercially for water clarification and other applications.
- **Idea** (*Figure 3*):
  - Combine coagulation efficiency of chitosan with bioremediation capacity of polychaetes.
    - Test with various effluents.
    - Assess polychaete biomass growth for potential use as high quality fish feed.

Figure 3. Circular Economy/Feed from Waste through Chitosan Coagulation and Polychaete Bioremediation of Wastewater

Objectives

Task A
- Investigate mechanisms of chitosan coagulation with various effluents.

Task B
- Understand ragworm characteristics with different effluents.
  - Does chitosan affect ragworms' digestion of nutrients?

Task C
- Assess ragworm bioremediation capacity of chitosan coagulated effluents.
- Lab scale system.

Previous Research

- Model system studies conducted by Master student Conor Dawson at University of Edinburgh.
- Preliminary research highlighted potential success of this methodology (*Table 1*).

Table 1. Main Aims of Preliminary Research

Aim	Achieved?
Ragworms and chitosan can be used to treat simulated effluents	Yes
Chitosan improves ragworms' ability to feed and survive	Yes
Chitosan does not inhibit digestion by Ragworms	Yes

Anticipated Outcomes

- Feed from waste solution for the bioremediation of aquaculture wastewater and other wastewater effluents.
- Ragworm biomass growth through wastewater bioremediation to be subsequently used as high quality fish feed.

References/Further Reading

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- Costa PFR, Oliveira RF, Da Fonseca LC. Feeding Ecology of Nereis diversicolor (O. F. Müller) (Annelida, Polychaeta) on Estuarine and Lagoon Environments in the Southwest Coast of Portugal. Panam J Aquat Sci. 2006;
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NERC SCIENCE OF THE ENVIRONMENT

Figure R1 - Poster presented at the 28th Annual University of Edinburgh School of Geosciences Graduate School Conference and Industrial Biotechnology Innovation Centre (IBioIC) Annual Conference 2019.

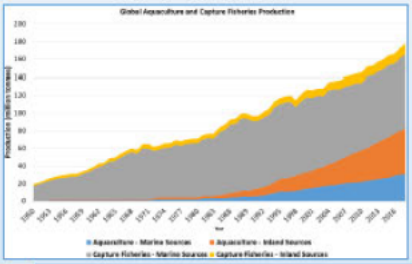
The Circular Economy in Action: Aquaculture Wastewater Bioremediation Using Chitosan and Ragworms

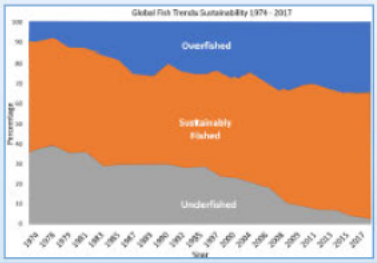
THE UNIVERSITY of EDINBURGH
Institute for Infrastructure and Environment
Pennosan
E3 DTP NERC SCIENCE OF THE ENVIRONMENT

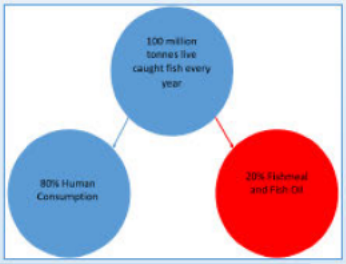
Blair Mackie, Andrea J C Semião, Jonathan Hughes

(LinkedIn QR Code)

Motivation: Aquaculture facilities require the ability to treat wastewater produced. Aquaculture uses wild fish to feed farmed fish, killing more than it produces. Aquaculture production has grown over 500% since 1990 and will continue to expand as the global population increases and the state of conventional capture fishing becomes increasingly unsustainable. However, as the feed used in aquaculture comes from caught fish, alternatives are required to produce aquaculture feed.







Aim: Achieve the ability to effectively bioremediate aquaculture wastewater using chitosan and ragworms while concomitantly providing an alternative source of ragworm based aquaculture feed to help avoid current unsustainable overfishing practices to produce aquaculture feed.

1. Investigate chitosan properties in aquaculture wastewaters.
2. Investigate ragworm behaviour and characteristics in wastewater.
3. Aquaculture wastewater bioremediation with ragworms and chitosan (laboratory scale).
4. Pilot scale testing (if possible).
5. (If time and/or pilot scale testing not possible) Assess potential for feeding ragworms with sludge derived from chitosan coagulated freshwater.

Experiments:

1. Assess Chitosan Properties in Aquaculture Wastewater.
   - Experiments to compare percentage turbidity removal in aquaculture wastewater across 4 different chitosan samples which have different properties.
   - The purpose of the experiments was to get used to creating chitosan formulations and understand the mechanisms of chitosan.
   - Add dose of chitosan, stir in a paddle stirrer (100 rpm for 30 sec, 40 rpm for 30 min), settle (15 min), measure turbidity.

2. Investigate Ragworm Behaviour and Characteristics.
   - Chitosan forms flocs, therefore experiments carried out to see if ragworms can feed on pellet feed ground to 4 different sizes.
   - 10 ragworms fed each size (3.25, 2, 0.85 and 0.425 mm) for 15 days (+7 day acclimatisation period).
   - Covid-19 halted experiment and will be repeated.
   - Results below show 6 days of data.

Results:

Chitosan sample	Optimum Dose (mg/L)	Percentage Removal of Turbidity (%)
1	20	72.5
2	14	79
3	14	10.4
4	19	88

Food size group	Ragworm Number	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0.425mm	1	0.3	0.28	0.28	0.27	0.26	0.2751
	2	0.43	0.41	0.35	0.34	0.32	0.341
	3	0.23	0.2	0.22	0.19	0.2	0.195
	4	0.47	0.44	0.44	0.42	0.41	0.45
	5	0.27	0.25	0.24	0.24	0.228	0.251
	6	0.19	0.16	0.16	0.16	0.155	0.181
0.85mm	7	0.27	0				
	8	0.57	0.47	0.41	0.41	0.402	0.424
	9	0.3	0.29	0.29	0.272	0.283	0.301
	10	0.41	0.35	0.36	0.34	0.338	0.407
	11	0.23	0.2	0.21	0.208	0.192	0.2285
	12	0.23	0.2	0.2	0.215	0.206	0.2225
2mm	13	0.44	0.41	0.35	0.345	0.345	0.37
	14	0.37	0.34	0.33	0.31	0.317	0.354
	15	0.41	0.39	0.34	0.343	0.343	0.357
	16	0.37	0.33	0.3	0.297	0.281	0.2995
	17	0.2	0.2	0.16	0		
	18	0.24	0.22	0.2	0.198	0.193	0.2005
3.25mm	19	0.31	0.27	0.27	0.272	0.282	0.332
	20	0.27	0.26	0.23	0.235	0.232	0.2555
	21	0.26	0.27	0.23	0.233	0.228	0.2086
	22	0.28	0.26	0.24	0.241	0.231	0.2645
	23	0.46	0.44	0.36	0.38	0.377	0.3755
	24	0.1	0.08	0.08	0.0877	0.0975	0.1133

Conclusion:

- Past research and the results displayed here demonstrate chitosan is effective at coagulating pollutants in aquaculture wastewater.
- Juvenile ragworms are capable of consuming flocs in the size range formed by chitosan flocculation as demonstrated by their growth.

Future Work:

- Chitosan coagulated feed versus non-chitosan coagulated feed versus chitosan on its own.
  - Measure ragworm growth to determine if chitosan provides any sustenance.
- Repeat with aquaculture wastewater sources. Measure wastewater quality before and after as well as ragworm growth.

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Figure R2 – Poster presented at The University of Edinburgh School of Engineering Postgraduate Research Conference 2020.