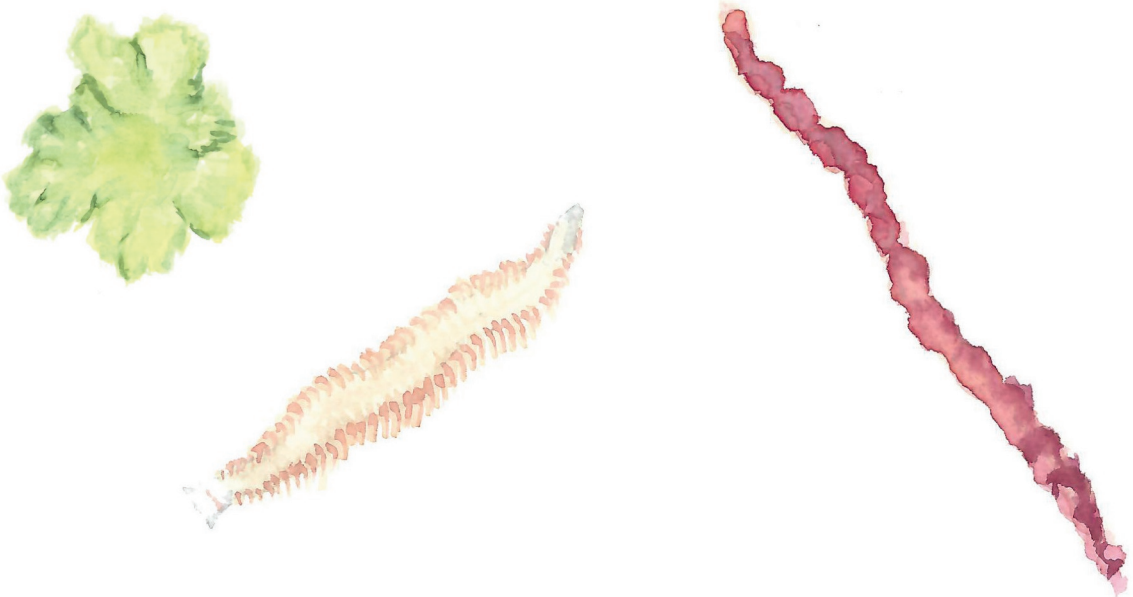
Integrated Multi-Trophic Aquaculture

Nutrient retention efficiency and valorisation of waste nutrients



Marit AJ Nederlof

Propositions

1. Maximum waste retention is highly different between different IMTA systems. (this thesis)
2. Polychaetes upgrade particulate waste from fish farms into a high-quality resource. (this thesis)
3. The Covid-19 pandemic tells us that it is time to halt biodiversity loss.
4. In a resource-efficient human food production system feed-food competition does not exist.
5. The health benefits of horse riding outweigh the risk of falling off.
6. For a resource-efficient economy, each vegetarian needs a buddy eating a veal burger or chicken soup once in a while.
7. Digital meetings bring our ability to communicate with body language in danger.

Propositions belonging to the thesis, entitled

Integrated Multi-Trophic Aquaculture. Nutrient retention efficiency and valorisation of waste nutrients

Marit AJ Nederlof
Wageningen, 25 March 2022

Integrated Multi-Trophic Aquaculture

Nutrient retention efficiency and valorisation of waste nutrients

Marit AJ Nederlof

Thesis committee

Promotors

Prof. Emeritus Dr A.C. Smaal
Special Professor, Sustainable Shellfish Culture
Wageningen University & Research

Dr M.C.J. Verdegem
Associate professor, Aquaculture and Fisheries Group
Wageningen University & Research

Co-promotor

Dr H.M. Jansen
Researcher Sustainable Aquaculture, Aquaculture and Fisheries Group
Wageningen University & Research

Other members

Prof. Dr I.J.M. de Boer, Wageningen University & Research
Dr P. Kamermans, Wageningen University & Research
Dr R. Osinga, Wageningen University & Research
Dr M.D. Callier, Institut Français de Recherche pour l'Exploitation de la Mer (Ifremer), Palavas-les-Flots, France

This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

Integrated Multi-Trophic Aquaculture

Nutrient retention efficiency and valorisation of waste nutrients

Marit AJ Nederlof

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 25 March 2022
at 1.30 p.m. in the Aula.

Marit Aleida Jantien Nederlof
Integrated Multi-Trophic Aquaculture – Nutrient retention efficiency and valorisation of waste nutrients,
186 pages.

PhD thesis, Wageningen University, Wageningen, The Netherlands (2022)
With references, with summaries in English and in Dutch

ISBN: 978-94-6447-108-3
DOI: https://doi.org/10.18174/564056

Voor Kas

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Chapter 1

General introduction

1 Aquaculture challenges

With a rising human population, the demand for fish and seafood is likely to increase. Captured fisheries is rather stagnant since the late 1980s and it is predicted that the aquaculture sector will expand to meet its growing demands (Ottinger et al. 2016, FAO 2020). Aquaculture is already one of the fastest growing animal food production sectors worldwide, with an average annual growth rate of 5.3% in the period 2001-2018 (FAO 2020). Although aquaculture contributes to global food security, and fish and seafood play an important role in healthy human diets, expansion and intensification of the sector is not without consequences and is associated with several ecological concerns (Bergqvist & Gunnarsson 2011, Ottinger et al. 2016). Aquaculture relies on natural resources, like water, energy, raw materials, space, and expansion of the sector will inevitable put more pressure on these resources. Increase of the aquaculture sector also contributes to the growing demand for high-quality feed, as 50% of the global aquaculture production concerns fed species (FAO/FishStatJ 2021). Ingredients traditionally used in aquafeeds, like fishmeal and fish-oil, are unsustainable, as the majority of global fishmeal and fish-oil production consists of food-grade fish (Cashion et al. 2017), resulting in feed-food competition. An increase in aquaculture production will also increase waste production, with potential detrimental effects on the environment due to the discharge of metabolic waste, uneaten feed and faeces (Bostock et al. 2010, Holmer 2010). In land-based systems, water purification techniques can be used to convert waste into less hazardous forms. This includes the conversion of ammonia into nitrate and the capture and conversion of solid waste into a novel resource like a fertilizer. Nevertheless, a large fraction of these (valuable) waste nutrients nowadays ends up in the environment, resulting in adverse effects (Schneider 2006). This highlights the need for the development of sustainable aquaculture approaches, which allow to keep up with the growing demand for food and resources with no or minimal adverse impacts on the environment.

2 Integrated Multi-Trophic Aquaculture (IMTA)

The concept of Integrated Multi-Trophic Aquaculture (IMTA) has developed, and is now applied as a sustainable aquaculture approach (Chopin 2013b). In IMTA, *fed species* cultivation (i.e. dependent on external feed supply) is linked to *extractive species* cultivation (i.e. extract nutrients from their environment), in such a way that waste resulting from fed species cultivation (e.g. feed spillage, metabolic waste and faeces) can be recycled in extractive species biomass (Chopin et al. 2001). In other words, in these systems waste is seen as a food source for additional species, thereby valorising the wastes from fed aquaculture. Choosing the right species combinations in IMTA systems enables a more efficient use of resources, since more biomass can be produced with the same feed input compared to monocultures of fed species. This approach also results in a higher product diversification which may reduce risks and increase profits for the farmer (Ridler et al. 2007, Barrington et al. 2010). Additionally, Barrington et al. (2010) showed that IMTA has the potential to raise social acceptance and to grow niche products with a high market value. Combined, these positive aspects make the

public to view IMTA as an ecological, economic and social sustainable aquaculture practice (Chopin et al. 2012), that fits well in the ecosystem approach to aquaculture (EAA) (Costa-Pierce 2008). This thesis focuses on the ecological aspects of IMTA.

2.1 Different names, same concept: towards a more circular farming system

The term IMTA was introduced in the beginning of this century (Chopin et al. 2007), but the concept is not new. Integration of multiple species in aquaculture systems originates from Asia and the Middle East, and goes back to the origin of aquaculture (Strand et al. 2019). Tombs in Egypt show images of tilapia culture combined with agricultural activities, which dates back to 1550-1070 B.C., while in 2200-2100 B.C. the integration of fish with aquatic plants and vegetables was already practiced in China as was specified in the document *You Hou Bin* (Chopin 2013b, Strand et al. 2019). In recent years, various reports on co-cultivation and integration of different species in aquaculture systems, mainly in Asia, testify to the history and diversity of integrated aquaculture practices (Strand et al. 2019). It should be noted that, although having the same origin, IMTA differs from polycultures in the sense that in IMTA systems species of different trophic levels are combined, while in polyculture, species of the same trophic level are co-cultured (Soto 2009).

Contrasting to Asia, today's aquaculture in Western countries is typically characterized by highly industrialized monoculture practices, and interest in the integrated approach only started some decades ago after the work of Ryther et al. (1975) who used micro- and macroalgae combined with bivalves to treat domestic wastewater effluents. Since then a diversity of integrated practices developed, for which several terms have been used. Integration of freshwater recirculating aquaculture systems (RAS) with horticulture is for example referred to as aquaponics (Love et al. 2015, Goddek et al. 2016), but FIMTA (Freshwater Integrated Multi-Trophic Aquaculture) has also been suggested (Murray 2017). For marine systems the term integrated mariculture has been used (Soto 2009, Gökalp et al. 2020), or IMTA, which can refer to land-based systems or systems at sea (Abreu et al. 2011, Handå et al. 2012b, Shpigel et al. 2016). Even though diverse names have been proposed, all rely on the same concept: the recycling of waste nutrients by combing different species, with the overall aim to transform linear monocultures into more circular farming systems, producing additional valuable crops that benefit from the waste nutrients. In this thesis we consider marine aquaculture production systems which include fed species integrated with extractive species as IMTA.

2.2 Challenges for IMTA development

The IMTA concept is simple, easy to visualize and is propagated as an ecological and economic win/win situation, due to the possibility to reduce waste and simultaneously increase system productivity (Chopin et al. 2012, Granada et al. 2016). The concept accords well in the circular economy, propagated among others

by the European Commission, in which nutrient losses are minimized and resource use efficiency is maximized (European Commission 2015). Furthermore, it is a flexible concept that can be applied to different aquaculture production systems (i.e. recirculation and flow-through, land-based and open-water systems) (Chopin 2013a), in which a wide variety of fed and extractive species combinations is possible (Soto 2009).

In spite of all the appealing qualities of IMTA, and its long history in Asia, commercial application in Western countries remains limited. Several constraints and bottlenecks have been identified, that could explain the challenges to develop commercial IMTA in Western countries (Hughes 2016, Hughes & Black 2016). Hughes and Black (2016) mentioned the difference in relative importance of extractive species aquaculture between Europe and Asia. Seaweed and shellfish cultivation is already an important share of the Asian aquaculture industry and compared to Europe, extractive species aquaculture has a relative higher value in Asia. In addition, this industry is suited for extensive production, usually characteristic for extractive species cultivation, that is often less-mechanised, more labour intensive, and requires more space, compared to the highly industrialized and intensive fish farming practices which dominate in Europe. Spatial issues, increased complexity and economic constraints related to the addition of extractive species to fed species cultivation have therefore been mentioned as bottlenecks for IMTA development in Western countries (Hughes & Black 2016). There are also challenges of legislative and regulatory nature. While there is a lack of specific IMTA regulations, several existing regulations form a barrier for IMTA development (Hughes 2016). In Norway there are for instance restrictions on co-cultivation of multiple species on the same site (Rebours et al. 2021), EU regulations put restrictions on the use of animal sourced waste products in the food chain (European Commission 2001) and in Europe, regulations to reduce environmental impacts from marine cage aquaculture focus on the benthic zone, while benthic IMTA is still in its infancy (Hughes & Black 2016). Hughes and Black (2016) and Hughes (2016) also highlighted the lack of a quantitative definition of the environmental benefits of IMTA, which is now mostly conceptually described. Such quantification is required, not only to develop standards for future certifications and regulations, but also to understand factors that influence the bioremediation potential across a range of IMTA systems.

The bioremediation potential of IMTA is determined by the amount of waste nutrients retained and subsequently harvested in extractive species biomass. Non-retained nutrients by fed cultures can be categorized in inorganic and organic nutrients. The latter is subdivided in large organic particles that sink, and smaller organic particles that remain in the water column (Wang et al. 2013). Based on these waste flows three types of extractive species can be defined, each serving a different niche in the food web: (*i*) autotrophs that take up inorganic nutrients, (*ii*) filter feeders that consume organic particles suspended in the water column and (*iii*) deposit feeders that feed on organic particles that settle on the bottom (Soto 2009). Simply connecting extractive species to a fed culture does not guarantee

ecological sustainability, as numerous factors influence nutrient retention efficiencies in IMTA. These factors can be divided in biological and environmental factors (Fig. 1.1). Biological factors play a role in each type of IMTA system, and relate to nutrient requirements and eco-physiological responses of extractive species to fish waste. The role of environmental factors is system dependent; i.e. in systems at sea there are less options to control the environment than in land-based systems, where nutrient fluxes are (theoretically) easier to control. Nevertheless, land-based systems have their own challenges linking extractive species to fed species. For instance, Both et al. (2011) showed that feeding mussels with effluent from an Atlantic cod farm resulted in nutritionally stressed mussels, indicating that the quality of the Atlantic cod farm effluent was insufficient for mussel production. In RAS, nutrient accumulation may result in unfavourable conditions for extractive species. So, even though the applied concept is the same, each type of production system faces its own challenges for linking extractive species to fed species.

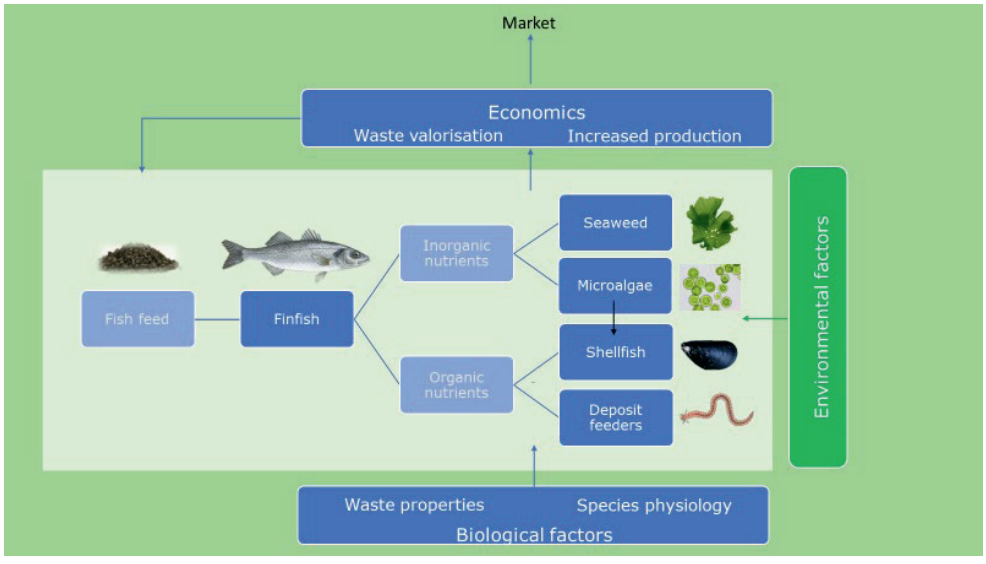

Figure 1.1
Diagram of nutrient fluxes within Integrated Multi-Trophic Aquaculture systems.

3 Aim and research questions of the thesis

The general concepts and principles related to IMTA have been explained thoroughly (Chopin et al. 2007, Soto 2009, Troell et al. 2009, Chopin 2013a, Hughes & Black 2016). Nevertheless, the environmental benefits of IMTA systems are mainly conceptually described and there is a lack of quantification of the environmental performance of IMTA operated either next to marine fish cages, in land-based flow-through systems or in RAS. Therefore, this thesis explores nutrient fluxes within different IMTA systems and evaluates biological and environmental factors influencing nutrient retention efficiencies in IMTA. Empirical studies were carried out to address specific knowledge gaps. More specifically, this thesis investigates nutrient retention efficiencies in marine IMTA systems by quantifying the fluxes involved in nitrogen (N), phosphorus (P) and carbon (C) retention, explores the impact of biological and environmental factors on retention efficiencies and explores waste valorisation potential of IMTA systems. Questions in this thesis focus on:

- What are realistic retention efficiencies of IMTA systems reported in literature?
- Does accumulation of fish waste nutrients in RAS effluents create unfavourable rearing conditions for seaweeds?
- In how far can polychaete species naturally observed below marine fish cages contribute to waste bioremediation in IMTA?
- What is the potential of polychaete species to convert and eventually valorise fish faeces?

Answers to these questions are needed to create more insight in the (re)cycling potential of fed nutrients by extractive species in IMTA and the potential volumes of extractive species that can be produced under different biological and environmental conditions in land-based and open sea IMTA systems.

4 Outline of the thesis

In *Chapter 2*, based on a literature review, the variability in nutrient dynamics within different IMTA systems (fish cages at open sea, land-based flow-through, recirculating aquaculture systems) is determined, with the aim to provide a generic framework to quantify nutrient retention efficiencies in IMTA systems. The collated literature is used to identify, and when possible, quantify biological and environmental factors that limit nutrient retention efficiencies of extractive species under different farming conditions.

Chapter 3 tests the hypothesis that nutrient accumulation in recirculating aquaculture systems might cause reduced seaweed performance due to unfavourable nutrient stoichiometry and rearing conditions. Therefore, growth, tissue content and nutrient removal rates of the seaweed *Ulva* spp. exposed to moderate to high nitrogen and phosphorus concentrations, are studied.

Chapter 4 & 5 focus on the benthic part of IMTA, as there is relatively little quantification available for extractive species targeting settleable particulate organic matter. Two polychaete species are selected, *Capitella* sp. and *Ophryotrocha craigsmithi*, that naturally occur in high densities under open-water fish cages and we study their bioremediation potential (*Chapter 4*) and their ability to upgrade fish waste into a high-quality marine resource (*Chapter 5*). We investigate their potential role for coupled and decoupled IMTA systems. In coupled IMTA, polychaetes are integrated with fed species within the same (eco)system, while in decoupled IMTA spatial connection between the fed and extractive species is not required. As for decoupled IMTA systems preservation of fish waste is recommended, the hypotheses that preservation of fish faeces affects the bioremediation potential (*Chapter 4*), and the production potential and body composition of the polychaete species (*Chapter 5*) are tested.

In the general discussion (*Chapter 6*), the results of the previous chapters are integrated in a larger framework, to address the ecological, economic and societal value of IMTA. In addition, ecosystem services of IMTA systems and potential contributions to circularity are discussed.



Chapter 2

Nutrient retention efficiencies in Integrated Multi-Trophic Aquaculture

Marit A.J. Nederlof, Marc C.J. Verdegem,
Aad C. Smaal, Henrice M. Jansen

Reviews in Aquaculture (2021), 00: 1-19

Abstract

One of the bottlenecks for commercial implementation of Integrated Multi-Trophic Aquaculture (IMTA) is the difficulty in quantifying its environmental performance. We reviewed a large body of literature to determine the variability in nutrient dynamics within different IMTA systems (open sea-cages, land-based flow-through, recirculating aquaculture systems), with the aim to provide a generic framework to quantify nutrient retention efficiencies in integrated aquaculture systems. Based on the eco-physiological requirements of the cultured species, as well as the response of 'extractive' species to waste from 'fed' species, the maximum retention efficiency was defined for a conceptual four-species marine IMTA system (fish-seaweed-bivalve-deposit feeder). This demonstrated that 79-94% of nitrogen, phosphorus and carbon supplied with fish feed could theoretically be retained. In practice, however, various biological and environmental factors may limit retention efficiencies and thereby influence the bioremediation of IMTA systems. These biological (waste production, stoichiometry in nutrient requirements) and environmental (temporal and spatial connectivity) factors were therefore evaluated against the theoretical reference frame and showed that efficiencies of 45-75% for closed systems and 40-50% for open systems are more realistic. This study is thereby the first to provide quantitative estimates for nutrient retention across IMTA systems, demonstrating that a substantial fraction of nutrients released from fish culture units can be retained by extractive species and subsequently harvested. Furthermore, by adapting this framework to the design and the condition prevailing for a specific IMTA system, it becomes a generic tool to analyse the system's bioremediation potential and explore options for further improvement.

1 Introduction

Aquaculture is the fastest growing food production sector globally (FAO 2020). With increasing pressure on freshwater resources and terrestrial space, a substantial expansion of marine aquaculture in particular is foreseen (Gentry et al. 2017). Rapid development of (marine) aquaculture dependent on formulated feed (i.e. fed species) is associated with various environmental concerns (Bergqvist & Gunnarsson 2011). One of these concerns is the release of fed nutrients, not retained for growth, as organic (i.e. uneaten feed and faeces) and inorganic (i.e. branchial and urinary losses) waste (Schneider et al. 2005, Wang et al. 2012). These wastes cause nutrient enrichment, which affects food web functioning, and a loss of valuable resources (Duarte et al. 2009, Bostock et al. 2010).

The expected growth of (fed) aquaculture requires the development of responsible and sustainable technologies, practices and approaches. Therefore, the integrated multi-trophic aquaculture (IMTA) approach has been developed. In IMTA systems, cultivation of fed species (e.g. fish and shrimp) is linked to cultivation of extractive species (e.g. autotrophs, filter- and deposit feeders), in such a way that the waste of fed species becomes a nutrient source for extractive species (Troell et al. 2003, Troell et al. 2009, Chopin et al. 2012, Chang et al. 2020). The idea behind the IMTA approach is that recycling of waste nutrients results in less nutrients being released into the environment, while overall productivity of the system increases (Chopin et al. 2012, SAPEA 2017). This approach fits well within the global ambition for circularity in food production, which strives to minimise energy and nutrient losses and maximise resource use efficiency, by closing the nutrient loop (European Commission 2015).

The general concepts and principles of the IMTA approach are straightforward, easy to visualise, and have been well explained in previous reviews (Troell et al. 2003, Neori et al. 2004, Soto 2009, Chopin 2013a). One of the pillars of the IMTA approach is to reduce nutrient losses to the environment, by harvesting nutrients retained in the biomass of extractive species, but it remains unclear under which conditions maximum nutrient retention efficiencies can be achieved. Nutrient removal efficiencies varying between 2–100% have been reported for extractive species (e.g. Troell et al. 2003, Schneider et al. 2005), whereby this large scope reflects a broad diversity in cultivation techniques, waste quality, measuring methods, culture intensity and species. In this review we use 'system openness' as the main criterium influencing nutrient retention efficiencies in IMTA systems. 'System openness' is here defined as the extent to which system functioning is influenced by the surrounding environment, and classifies three types of aquaculture production systems: (1) closed systems, where the environment can be controlled (e.g. recirculating aquaculture systems (RAS)); (2) open systems, where control over environmental influences is very limited (e.g. sea cages); and (3) semi-open systems, where environmental influences can partially be controlled (e.g. land-based flow-through systems or ponds).

The concept of integrated aquaculture has its roots in Asia (Chopin 2013b). In Western countries, development of integrated aquaculture is currently moving from a pilot to a commercial scale, but implementation is still limited (Hughes & Black 2016). Hughes and Black (2016) and Hughes (2016) reviewed several factors explaining this limited adoption of IMTA in Western countries, with a focus on Europe. One of the bottlenecks is the lack of a quantitative definition of the environmental performance of IMTA, as the benefits of IMTA are mostly conceptually described (Chopin 2013a, Hughes 2016). Know-how on the maximum bioremediation potential of IMTA systems would aid in formulating regulations, policies and certification criteria (Hughes 2016). Furthermore, where the fraction of waste nutrients harvested via extractive species biomass is relatively small, questions concerning the bioremediation potential of IMTA might arise (Cranford et al. 2013). To assess the amount of waste nutrients that can be recycled in extractive species biomass, there is thus a strong need to quantify waste flows through IMTA systems and to understand the factors that influence nutrient retention efficiencies. This will help in identifying options for enhancing bioremediation within IMTA systems.

In this review, we quantify nutrient retention efficiencies in IMTA systems according to system openness, while considering several biological and environmental limiting factors (Fig. 2.1). We aim to establish a generic quantitative framework to highlight which factors must be considered when estimating nutrient retention efficiencies. The framework consists of three steps: (*i*) firstly, a conceptual IMTA system was developed for which we quantified the maximum retention efficiency for the macronutrients nitrogen (N), phosphorus (P) and carbon (C), based on physiological requirements and responses of extractive species fed with fish waste (*Section 3*). These theoretical values were then evaluated against (*ii*) biological and (*iii*) environmental factors that place boundaries on the nutrient retention efficiencies that can be expected under practical farming conditions (*Section 4*). The framework presented, when adapted to local conditions and farm design and thus taking into account variability in biological and environmental factors between IMTA systems, can help to identify and optimise the bioremediation potential of integrated systems.

2 Method

This review summarises a large body of peer-reviewed literature on IMTA, with the focus on quantifying nutrient retention by extractive species fed fish waste, under different degrees of system openness. To quantify nutrient retention efficiencies, literature was collated on eco-physiological responses, including nutrient utilization processes, of extractive species fed fish waste. Nutrients retained in extractive species biomass can subsequently be harvested from the system, and these numbers can be used to define the overall bioremediation potential of the system. Peer-reviewed literature was collated from Google Scholar, using the keywords: IMTA, integrated aquaculture, integrated mariculture, bioremediation, biomitigation, waste retention, waste removal; combined with one of the following

keywords: extractive species, seaweed, macro-algae, bivalves, mussel, oyster, deposit feeder, polychaete, sea cucumber. Only studies providing quantitative data were included in the summary, resulting in a total of 25 papers for seaweeds, 17 papers for bivalves and 20 papers for deposit feeders (*section 3*). These, and additional papers, were used to identify factors that limit nutrient retention efficiencies of extractive species under different farming conditions, which were in turn used to establish a generic quantitative framework (*section 4*; Fig. 2.1).

3 Maximum nutrient retention efficiency based on a conceptual IMTA

In this section we define a conceptual marine IMTA that includes four functional groups, in order to quantify its theoretical maximum nutrient retention potential. The first group, the fed fish species, excretes faeces and metabolites that can be used as a nutrient and energy source by extractive species. For the conceptual IMTA it was chosen to focus on fish as fed species, but it should be noted that invertebrates, like shrimp, are also a major group of fed species (Chang et al. 2020). To estimate retention efficiencies, we first qualify and quantify fish waste (*section 3.1*), and subsequently summarise the eco-physiological responses of extractive species, with a focus on their responses when fed fish waste (*section 3.2*). Three groups of extractive species were chosen, each taking up a different fraction of the waste released by the fed fish: (1) an autotrophic species, which takes up inorganic nutrients; (2) a filter-feeder, which consumes particulate organic matter (POM) suspended in the water column; and (3) a deposit-feeder, which scavenges on POM that settles on the bottom (Soto 2009). Although biofloc technology also focusses on the recycling of waste nutrients into biomass (Bossier & Ekasari 2017), bioflocs are not included in this review, since the focus is on extractive species that will be harvested from the system for commercial purposes. Data summarised in *section 3.1* and *3.2* are used in step 1 of the framework, where we calculate the theoretical maximum nutrient retention potential of our conceptual IMTA (*Section 3.3*).

3.1 Fed species

Nutrient retention by the fed species is influenced by species, feeding level and management, diet composition, temperature and fish size (Kim et al. 1998, Lemarié et al. 1998, Lupatsch & Kissil 1998, Islam 2005, Schneider et al. 2005). Retention efficiencies reported for marine fish species range between 13-43% for N, 18-36% for P and 14-38% for C (Appendix Table S2.1). Fed nutrients that are not retained by the fed species become input for the extractive species.

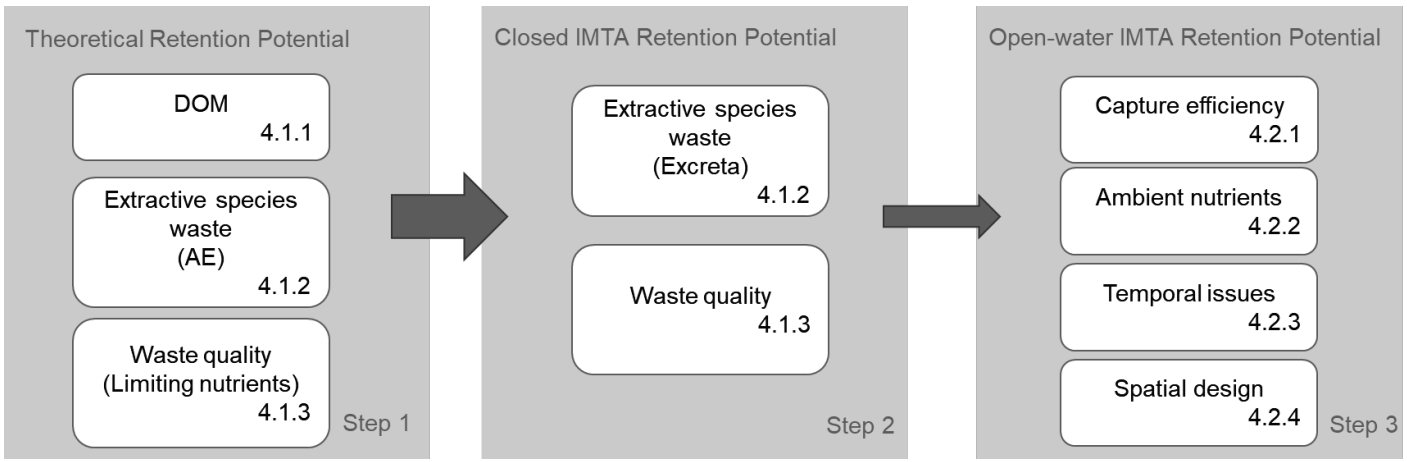

Figure 2.1
Generic framework which can be used to identify factors to consider when quantifying the nutrient retention potential of integrated aquaculture (IMTA) systems according to system openness. The framework consist of three steps. In step 1 the maximum theoretical retention potential of a conceptual IMTA (fish – seaweed – bivalve – deposit feeder) is calculated based on physiological requirements and responses of extractive species fed fish waste and under the assumptions that extractive species perform at their maximum and that ambient nutrients are absent. DOM, Dissolved Organic Matter; AE, Assimilation Efficiency. Step 1 acts as a reference frame to discuss factors that influence retention potentials under a range of practical conditions. Step 2 considers biological factors, which are the main factors to take into account when calculating the retention potential of closed IMTA systems. Step 3 considers environmental factors, which besides the biological factors, have to be taken into account to calculate the retention potential of open-water IMTA systems. Factors are described in the section corresponding with the number mentioned in each box. Quantifications per factor are given in Table 2.2.

3.1.1 Waste characteristics

Waste nutrients can be divided into inorganic and organic fractions. Fish excrete inorganic N as NH_3/NH_4^+, inorganic P as PO_4^{3-} and respire inorganic C (CO_2). Under aerobic conditions, NH_4^+ is converted to NO_3^- by nitrifying bacteria, with NO_2^- as an intermediate product. Together, these three forms of N are referred to as dissolved inorganic nitrogen (DIN). Mass balance models indicate that 39-63% N, 18-30% P and 39-70% C in feed are released as inorganic waste (Appendix Table S2.1).

Faeces and uneaten feed (3-5% of the feed in cage cultures remains uneaten; Reid et al. 2009, Bureau & Hua 2010) make up the POM waste fraction (Wang et al. 2012). In total, 5-45% N, 42-57% P and 6-44% C in feed are released as POM. Breakage and disaggregation of POM results in dissolved organic matter (DOM). The amount of POM that ends up as DOM depends on faecal and feed pellet stability, which is influenced by feed composition, feed processing methods and environmental conditions (Reid et al. 2009, Bureau & Hua 2010). On average, 1-7% N, 2-8% P and 1-6% C in feed become DOM, which indicates that 5-45% N, 42-54% P and 5-44% C remain as POM in the system (Appendix Table S2.1).
POM can be subdivided into small particles suspended in the water column, i.e. suspended solids or suspended particulate matter (SPM), and large particles that sink rapidly to the bottom, i.e. settled solids (Olsen & Olsen 2008). Wong and Piedrahita (2000) estimated that 30% of POM in a commercial rainbow trout farm consisted of suspended solids, while the remaining 70% were settled solids. Waste particle size is influenced by fish species and fish size (Reid et al. 2009) - with bigger fish producing larger particles (Chen et al. 1999)- and culture systems, as mechanical and hydraulic conditions differ between cages, pond and tank systems (Reid et al. 2009).

3.2 Extractive species

To study the bioremediation potential of extractive species, several methods have been used: (*i*) removal rate: measuring nutrient removal rates (e.g. clearance rate (CR), assimilation efficiency (AE) and feeding rates) (e.g. Lefebvre et al. 2000, Yu et al. 2014a, Fang et al. 2017); (*ii*) retention: comparing growth and nutrient retention in biomass measured over time, in and outside IMTA systems (e.g. Sanderson et al. 2012, Jiang et al. 2012, Yu et al. 2014b, Tolon et al. 2017); (*iii*) balance: measuring water flows and nutrient concentrations in sediments, inflow and outflow water, of extractive species cultures (e.g. Jones et al. 2001, Al-Hafedh et al. 2012, Marques et al. 2017); (i*v*) tracers: tracing shifts in stable isotope or fatty acid composition (e.g. Handå 2012, Yokoyama 2013a, Jiang et al. 2012); and (*v*) modelling: combining growth models with ecological and/or spatial models to simulate the bioremediation potential of extractive species (Reid et al. 2018). Below a summary is given on the outcome of the various approaches described, to estimate bioremediation potential of the extractive species included in our conceptual IMTA.

3.2.1 Seaweeds

Data collected on the bioremediation potential of seaweeds in IMTA systems are summarised in Appendix Table S2.2. The retention method is frequently used to define the bioremediation potential of seaweeds in open-water IMTA systems, while this method is less common in land-based systems. Several studies reported higher specific growth rates (SGR) and higher N content, some studies reported similar growth rates and N content, and some studies reported lower growth rates and N content for seaweeds cultivated in IMTA compared to seaweeds cultivated away from fish cages. The retention method does not distinguish between nutrients taken up from the environment and those of fish waste origin, but tracer studies do indicate that seaweeds cultivated in open-water IMTA take up N derived from fish feed (Halling 2004, Wang et al. 2014).

The balance method was mostly used in semi-open and closed systems to quantify waste extraction efficiency of seaweeds. All studies reporting waste extraction efficiencies looked at inorganic N (as DIN or total ammonia nitrogen; TAN), while few studies looked at inorganic P or C. This main focus on inorganic N can most likely be ascribed to the dominant release of inorganic N by fed species (Appendix Table S2.1), which plays a crucial role in eutrophication, and nitrogen often being the first limiting nutrient for seaweed growth, in particular in temperate regions (Troell et al. 2003). In cases where N loads are low, environmental conditions are close to optimal (in particular light and temperature) and fast growing seaweed genera (e.g. *Ulva* and *Gracilaria*) are cultivated, inorganic N extraction efficiencies of up to 100% have been reported (e.g. Cohen and Neori 1991, Jiménez del Río et al. 1996, Chow et al. 2001, Jones et al. 2002, Appendix Table S2.2). Inorganic P extraction efficiencies ranged from 3 to 95% (Jones et al. 2002, Hernández et al. 2005, Appendix Table S2.2), while the only study including C reported extraction efficiencies of 2-5% (Corey et al. 2014). The highest N extraction efficiencies were achieved in balance studies (up to 100%; Appendix Table S2.2), while N extraction based on the retention method was at maximum 56% (Neori et al. 2003). This suggests that, although often mentioned as negligible processes, nitrification and denitrification may contribute to the high efficiencies reported by balance studies, as removal of TAN and N_2 through nitrification and denitrification is attributed to the seaweed extraction potential when TAN or DIN concentrations are measured in the water. Krom et al. (1995) estimated that in their sea bream - seaweed integrated system, approximately 8% of inorganic N entering the seaweed compartment was removed by denitrification. Studies based on TAN/DIN concentrations in the water may therefore overestimate N extraction efficiency of the seaweeds. Lastly, it should be noted that although low nutrient loads result in high extraction efficiencies, as uptake rates of seaweeds follow a Michaelis-Menten saturation curve (Cohen & Neori 1991), the highest growth and tissue content can only be achieved under high nutrient loads (Buschmann et al. 2001).

3.2.2 Bivalves

In IMTA systems, the role of filter-feeder bivalves (hereafter referred to as bivalves) is to remove POM from the water column (i.e. suspended solids). Bivalves capture fish waste directly by removing feed-derived POM (i.e. fish faeces and feed fines), and also indirectly by removing plankton that has grown on feed-derived inorganic waste (Lefebvre et al. 2000). The degree of system openness determines the importance of these two different waste flows. Closed systems, and to a lesser extent semi-open systems, provide opportunities to manage waste flows towards either direct or indirect fractions. For example, microalgae can be cultivated on inorganic waste nutrients in separate cultivation units, before being fed to bivalves (Hussenot et al. 1998, Van Khoi & Fotedar 2012). In open systems, and the majority of semi-open systems, flows cannot be controlled and direct and indirect mitigation are intertwined. Bivalves prefer plankton to fish feed-derived POM (Lefebvre et al. 2000, Handå 2012), with the different waste flows influencing their bioremediation potential, for example by differences in removal rate (Appendix Table S2.3). Lefebvre et al. (2000) showed that AE of oysters (*Crassostrea gigas*) fed with a phytoplankton diet was higher (66%) than when fed feed-derived POM (56%). Results are less conclusive for mussels; Reid et al. (2010) reported comparable AE for *Mytilus edulis* and *M. trossulus* fed salmon feed, salmon faeces or algae diets; while based on growth and fatty acid profiles, Handå (2012) showed that *M. edulis* assimilated and utilised salmon feed more efficiently than salmon faeces.

Studies using either the retention or the balance method to define the bioremediation potential of bivalves in IMTA are summarised in Appendix Table S2.4. The majority of these studies used the retention method, and show contradictory results. These contradictory results might be explained by differences in ambient food quality and quantity between these studies; in open systems, located in areas or during seasons of low ambient food concentration or quality (i.e. organic content), integration of bivalves with fish cultures can improve growth and bivalve quality (e.g. condition index) (e.g. Peharda et al. 2007, Handå et al. 2012a, Appendix Table S2.4), while in areas, or during seasons, of high ambient food concentrations, no enhancement of growth or improved quality was observed (e.g. Peharda et al. 2007, Navarrete-Mier et al. 2010, Handå et al. 2012a, Appendix Table S2.4). In semi-open systems, positive effects on bivalve growth were observed when cultivated on phytoplankton grown on inorganic waste (i.e. fish-microalgae-bivalves), or on a mix of uneaten feed, faeces and phytoplankton (e.g. Shpigel and Blaylock 1991, Jara-Jara et al. 1997, Jones et al. 2002, Appendix Table S2.4) .

The balance method was mostly applied to closed systems, to calculate bivalve waste extraction efficiencies. Bivalves extracted up to 23% organic matter (OM), up to 33% organic N, up to 96% chlorophyll-a, up to 88% suspended solids and up to 88% bacteria biomass, when cultivated in effluents of fish or shrimp cultures. In a fish-microalgae-bivalve system, 100% of the microalgae were taken up by the bivalves, whereby the microalgae assimilated 67% of TAN-N and 47% of PO_4-

P released by the fish (Hussenot et al. 1998). It can be estimated that by feeding on these microalgae, bivalves retained 58% of TAN-N and 41% of PO_4-P excreted by the fish, if an AE of 87% is assumed for the bivalves (Appendix Table S2.3).

Tracer studies were applied in open-water IMTA systems. Results varied; while in some studies aquaculture waste was the main food source, in others food uptake was dominated by ambient plankton (Fig. 2.2). Cultivation area and seasons partially explain these differences (Mazzola & Sarà 2001, Deudero et al. 2011). For example, in the study of Mazzola and Sarà (2001), phytoplankton made up 5 to 100% of the total diet of *Mytilus galloprovinciales* in an open-water IMTA system, the percentage varying according to the season. This indicates that the role of bivalves in organic fish waste bioremediation may vary with the seasons. Most tracer studies showed that uneaten fish feed contributed more to the total diet than fish faeces (Fig. 2.2, Handå 2012). None of the studies in open-water IMTA reported if phytoplankton taken up by the bivalves grew on waste or ambient nutrients.

3.2.3 Sea cucumbers or polychaetes

Deposit feeders, like sea cucumbers and polychaetes, are included in IMTA to remove settled POM. Although often mentioned as candidate species in IMTA systems (e.g. Soto 2009, Chopin et al. 2012), sea cucumbers and polychaetes are not that frequently studied, compared to seaweeds and bivalves (Soto 2009). Data collected on the bioremediation potential of sea cucumbers and polychaetes in IMTA is summarised in Appendix Table S2.3 (removal rate) and Appendix Table S2.5 (retention and balance studies). These results show that responses of sea cucumbers and polychaetes to aquaculture waste vary between studies. Species, experimental set-up and waste composition contribute to these reported variations.

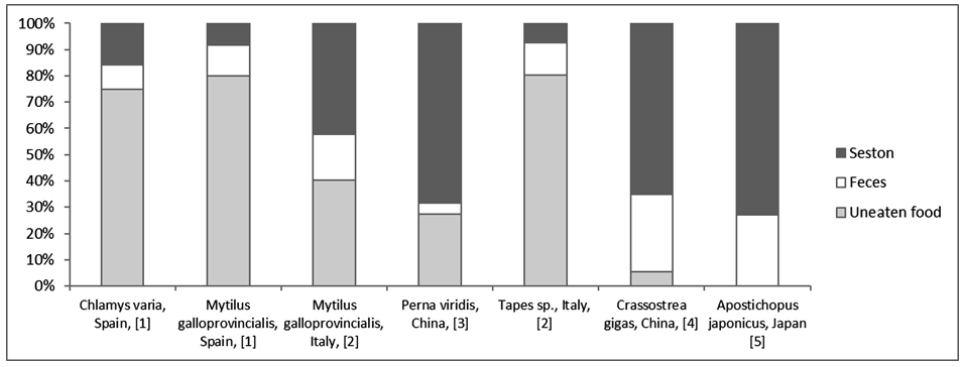

Figure 2.2
Contribution of food sources to the dietary consumption of bivalves and sea cucumbers in open water IMTA systems. Values are based on average stable isotope results from the corresponding papers.
1. Deudero et al. (2011); 2. Mazzola and Sarà (2001); 3. Gao et al. (2006); 4. Jiang et al. (2012); 5. Yokoyama (2013a).

Removal rate studies for sea cucumbers fed aquaculture waste reported an increase in consumption rate with decreasing substrate OM (Yuan et al. 2006, Zamora & Jeffs 2011), which reflects compensatory feeding, commonly observed in deposit feeders when (high quality) food is scarce (Lopez & Levinton 1987). Low substrate OM is further compensated by more active selection of OM particles (Paltzat et al. 2008, Yu et al. 2011, Yu et al. 2014a). Both compensatory feeding and active selection result in reworking of surface sediments, affecting sediment ecosystems by reallocation of resources and altering geochemical gradients and nutrient fluxes (MacTavish et al. 2012). This bioturbation by deposit feeders facilitates decomposition of OM in sediments, thereby increasing the net effect on bioremediation of organic waste (MacTavish et al. 2012). The assimilation efficiencies reported for sea cucumbers in integrated systems are highly variable and range from 14 to 88%.

Studies based on the retention method show contrasting results for sea cucumbers in open and semi-open integrated systems. Increased growth was observed for sea cucumbers integrated with fish or bivalves, while integration with shrimp was less successful. Less growth and higher mortality are reported in shrimp-sea cucumber cultures, compared to sea cucumber monocultures, likely due to the high TAN excreted by the shrimp (Purcell et al. 2006, Bell et al. 2007). Nevertheless, in a feeding trial, lowest growth was reported when *Stichopus monotuberculatus* was fed only waste from a shrimp farm, compared to a commercial sea cucumber diet, or a mixed diet of 50% waste from the shrimp farm and 50% sea mud (Chen et al. 2014).

Only a limited number of studies used the balance method to determine waste extraction efficiencies and they do show that sea cucumbers reduce aquaculture waste. Sea cucumbers can extract 0.1-20% OM, 3-10% organic C, 7-16% organic N and 21-25% organic P from the aquaculture waste fed directly, or from sediments enriched with aquaculture waste. No studies were found quantifying waste extraction efficiencies by sea cucumbers in open systems, but tracer studies indicate that also in open-water IMTA, sea cucumbers do assimilate aquaculture waste nutrients (Slater & Carton 2010, Yokoyama 2013a, b). Yokohama (2013a) estimated that when cultivated close to fish cages, the diet of *Apostichopus japonicus* consisted of 27% nutrients from aquaculture origin (Fig. 2.2).

In a similar way to sea cucumbers, AE reported for polychaetes in integrated systems is highly variable, ranging between 24 to 71%. The retention method was mainly used for polychaetes in closed integrated systems. These studies show that polychaetes survive and grow on aquaculture waste, but growth is lower compared to polychaetes fed a commercial worm diet, which contains more protein and energy. There are however indications that fish waste can improve fatty acid profiles of polychaetes, making them interesting marine resources (García-Alonso et al. 2008, Bischoff et al. 2009, Brown et al. 2011, Pajand et al. 2017, Marques et al. 2018, Nederlof et al. 2019).

Balance studies reported waste extraction efficiencies for polychaetes, which were higher compared to sea cucumbers; 20-85% OM, 40-91% organic C and 30-91% organic N of the aquaculture waste fed. Also for polychaetes, balance studies in open-water IMTA are scarce. Nevertheless, Tsutsumi et al. (2005) and Kinoshita et al. (2008) showed that mass cultivation of *Capitella* sp. significantly reduced OM levels in sediments underneath fish farms or in fish ponds.

3.3 Waste retention in IMTA; creating a balance

A key aspect of the bioremediation potential of IMTA is the balance between nutrient input and removal. The latter can be estimated by quantifying the nutrients retained in biomass gain of fed and extractive species. This was done for our conceptual four-species IMTA presented in *section 3.1* and *3.2*, assuming that only fed nutrients contribute to biomass gain of IMTA species and species perform at their reported optimum. Salmon was chosen as the fed species, and the starting point was the input of 1 ton of salmon feed (wet weight), for which the N, P and C waste production was calculated. The solid organic waste fraction was separated into 30% suspended solids consumed by bivalves and 70% settled solids consumed by deposit feeders (Wong & Piedrahita 2000). For the autotrophic species, kelp and microalgae were chosen and it was assumed that inorganic waste nutrients were incorporated in the tissue, according to the Atkinson ratio for seaweed (Atkinson & Smith 1983) or the Redfield ratio for microalgae (Redfield et al. 1963). An additional assumption made was that inorganic C was non-limiting, due to the exchange between the atmosphere and surface water. For the invertebrates, assimilated nutrients were calculated based on AE, with the highest values reported in literature for mussels and polychaetes. Bivalves were included, to directly remove feed-derived POM (i.e. faeces and feed fines), however as they are also capable of removing microalgae grown on feed-derived inorganic waste nutrients, this scenario (salmon-microalgae&mussel-mussel-polychaete IMTA; Scenario B, Fig. 2.3) was included as an alternative to the salmon-kelp-mussel-polychaete IMTA (Scenario A, Fig. 2.3). It was thereby assumed that all microalgae could be filtered by the bivalves and nutrient assimilation was calculated based on AE.

Data used in the mass balance calculations, with references, are reported in Table 2.1, while the resulting IMTA mass balances for nitrogen, phosphorous and carbon are shown in Fig. 2.3. Under these assumptions, the conceptual salmon–kelp–mussel–polychaete IMTA retains 94% N, 79% P and 94% C provided by the input of fish feed (Scenario A, Fig. 2.3). Scenario B, where seaweeds are replaced with a microalgae–bivalve combination, reduces the maximum retention efficiency to 78% N and 89% C and increases the P retention efficiency to 81% (Scenario B, Fig. 2.3).

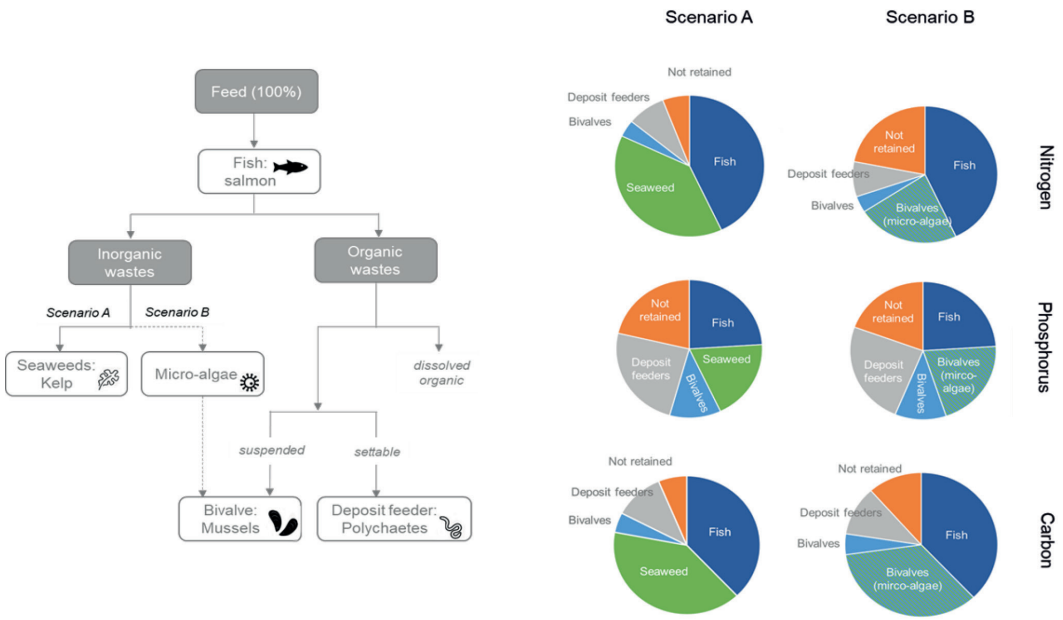

Figure 2.3
Nitrogen, phosphorus and carbon balance of a conceptual four-species IMTA system, indicating the theoretical retention potential (Fig. 2.1) Starting point for the balance was a commercial salmon farm, fed 1 ton of commercial feed. The following assumptions were made: (1) ambient nutrients are absent, (2) extractive species capture and ingest all of their target waste nutrients, (3) extractive species show optimal performance, i.e. highest assimilation efficiencies reported in literature. For *the inorganic waste stream*, two different conversion pathway scenarios were suggested; Scenario A) uptake by seaweeds resulting in a 'Salmon-Kelp-Mussel-Polychaete' IMTA and Scenario B) uptake by microalgae, which in turn can be assimilated by bivalves, resulting in a 'Salmon-Microalgae&Mussel-Mussel-Polychaete' IMTA. *The organic solid waste stream* was divided in two fractions; 30 % was assumed to be suspended and available for bivalves, while 70 % was assumed to settle on the sediment where they are available for deposit feeders. Pie charts are based on a mass-balance approach (calculations not shown). Data was collected from the literature and referred to in Table 2.1.

Table 2.1
Non-retained nutrients (i.e. waste) from salmon culture and maximum responses of extractive species to fish waste, as reported in literature. See supplementary material for full overview. DM, Dry Matter; AE, Assimilation Efficiency; NI, No Information.

		Nitrogen		Phosphorus		Carbon	
Parameter	Unit	Value	Ref.	Value	Ref.	Value	Ref.
Fed species (salmon)							
Input (feed)							
DM	%	98	1	98	1	98	1
Nutrient composition	% of DM	5.8	1	0.88	1	54	1
Retention (fish biomass)							
Nutrient retention	% of input	43	1	24	1	38	1
Output (waste)							
Inorganic	% of input	39	1	24	1	40	1
Organic_total	% of input	18	1	52	1	21	1
Organic_dissolved	% of input	3	1	8	1	3	1
Organic_solids	% of input	15	1	44	1	18	1
Organic_suspended solids	% of organic_solids	30	2	30	2	30	2
Organic_settled solids	% of organic_solids	70	2	70	2	70	2
Extractive species							
Seaweed							
DM content	%	12	3	12	3	12	3
Nutrient composition	% of DM	5	4	0.51	3	40	5
Bivalves							
Microalgae							
DM content	%	22	6	22	6	22	6
Nutrient composition	% of DM	9	7	1	8	36	9
Bivalves							
DM_mussels	%	25	10	25	10	25	10
DM_oysters	%	13	11	13	11	13	11
Nutrient composition_mussel	% of DM	11	12	1.2	13	37	14
Nutrient composition_oyster	% of DM	8	15	0.8	16	46	16
AE_mussel_phytoplankton	%	87	17	87	17	87	17
AE_oyster_phytoplankton	%	66	11	66	11	66	11
AE_mussel_faeces	%	86	17	86	17	86	17
AE_oyster_faeces	%	56	11	56	11	56	11
Deposit feeders							
DM_sea cucumber	%	8	18	8	18	8	18
DM_polychaete*	%	8	18	8	18	8	18
Nutrient composition_sea	% of DM	7	18	NI		31	19
Nutrient composition_polychaete	% of DM	10	20	NI		49	21
AE_sea cucumber_faeces	%	62	22	60	22	88	23
AE_polychaete_faeces	%	79	24	79$	25	79	25

*No info available for polychaetes, therefore the same data was used as for sea cucumbers.
$No data available for phosphorus assimilation efficiency, therefore the same data was used as for nitrogen and carbon efficiency.
1. Wang et al. (2013); 2. Wong and Piedrahita (2000); 3. Reid et al. (2013); 4. Handå et al. (2013); 5. Corey et al. (2014); 6. Amin (2009); 7. Schneider et al. (2005); 8. Brune et al. (2003); 9. Wang (2003); 10. Wang et al. (2012); 11. Lefebvre et al. (2000); 12. Haamer (1996); 13. Cantoni et al. (1977); 14. Smaal and Vonck (1997); 15. Ren et al. (2003); 16. Higgins et al. (2011); 17. Reid et al. (2010); 18. Dong et al. (2010); 19. Gao et al. (2011); 20. Brown et al. (2011); 21. Nederlof et al. (2019); 22. Hannah et al. (2013); 23. Maxwell et al. (2009); 24. Honda and Kikuchi (2002); 25. Fang et al. (2017).

4 Factors affecting nutrient retention efficiencies

The nutrient retention efficiencies calculated for the conceptual IMTA in *section 3.3* demonstrate the theoretical retention potential presented in Fig. 2.3 and is referred to as step 1 in the generic framework (Fig. 2.1). In practice, however, various factors limit retention efficiencies of extractive species and thereby influence the bioremediation potential of IMTA systems. Biological limiting factors are grouped under step 2 in the generic framework and reduce the theoretical retention potential to what can be realised in a 'closed IMTA system'. Environmental limiting factors are grouped under step 3 in the generic framework and reduce the retention potential further to what can be realised in an 'open-water IMTA' (Fig. 2.1). How biological and environmental limiting factors restrict the theoretical nutrient retention potential in IMTA is described in more detail below and summarised in Table 2.2.

4.1 Biological factors

Biological factors are independent of system openness, and are influenced by waste quality and the physiological responses of extractive species feeding on waste nutrients.

4.1.1 DOM

None of the species included in our conceptual IMTA extracts DOM, resulting in a small non-retained fraction of 3% N, 8% P and 3% C (Fig. 2.3, Table 2.2). Marine DOM represents the largest ocean reservoir of reduced carbon, and due to its key role in the global carbon cycle, the role of DOM in marine ecosystems has been studied extensively (Hansell et al. 2009, Lechtenfeld et al. 2015, Moran 2015). Although microbes play a role in the reduction of aquaculture derived DOM (Heilskov et al. 2006, Valdemarsen et al. 2012), an increase in microbial biomass may contribute to the microbialisation of marine ecosystems (Jackson et al. 2001, Haas et al. 2016). Only a few studies, mainly on sponges, looked into DOM removal and use efficiency (Fu et al. 2007, Osinga et al. 2010, Gökalp et al. 2020). Analysis of their role in the overall bioremediation potential of IMTA systems is still in its infancy, but all studies indicate that sponges can benefit from cultivation in an IMTA setting (Osinga et al. 2010, Gökalp et al. 2019).

Table 2.2
Potential limitations influencing the maximum nutrient retention efficiency of IMTA systems varying in openness (open, semi-open and closed systems). Values express non-retained nutrients as percentage of the total nutrient input (i.e. feed) to our conceptual four-species IMTA system described in Fig. 2.3. Percentages are calculated per factor (for a full description see section number given for each factor), per extractive species and for systems varying in openness. N, Nitrogen; P, Phosphorus; C, Carbon; DOM, Dissolved Organic Matter; PON, Particulate Organic Nitrogen; POC, Particulate Organic Carbon; DOC, Dissolved Organic Carbon; NA, Not Applicable; (*o*) non-retained nutrients contributing to the organic nutrient pool; (*i*) non-retained inorganic nutrients contributing to the inorganic nutrient pool.

Factor	Extractive species	Description	Nutrient	System			Reference
				Open	Semi-open	Closed	
Biological factors							
4.1.1 Non-retained DOM	None	No DON uptake* (*o*)	N	3	3	3	Wang et al. (2013)
		No DOP uptake* (*o*)	P	8	8	8	Wang et al. (2013)
		No DOC uptake* (*o*)	C	3	3	3	Wang et al. (2013)
4.1.2 Extractive species waste production	Seaweed	PON release (*o*)	N	16-21	16-21	16-21	Zhang et al. (2012)
			P	unknown	unknown	unknown	
		POC release (*o*)	C	0.4-24	0.4-24	0.4-24	Wada et al. (2007), Zhang et al. (2012)
		DOC release (*o*)		7-25	7-25	7-25	Wada et al. (2007)
	Bivalves_inorganic	Faeces production* (*o*)	N	NA	3-9	3-9	Lefebvre et al. (2000), Reid et al. (2010)
		Excretion (*i*)			6-8	6-8	Jansen (2012)
		Pseudofaeces production (*o*)			location dependent	NA	
		Faeces production* (*o*)	P	NA	3-8	3-8	Lefebvre et al. (2000), Reid et al. (2010)
		Excretion (*i*)			11-14	11-14	Jansen (2012)
		Pseudofaeces production (*o*)			location dependent	NA	
		Faeces production* (*o*)	C	NA	5-14	5-14	Lefebvre et al. (2000), Reid et al. (2010)
		Respiration (*i*)			21-27	21-27	Jansen (2012), Filgueira et al. (2019)
		Pseudofaeces production (*o*)			location dependent	NA	

Table 2.2 - *Continued*

Factor	Extractive Species	Description	Nutrient	System			Reference
				Open	Semi-open	Closed	
4.1.2 Extractive species waste production	Bivalves_organic	Faeces production* (*o*)	N	1-2	1-2	1-2	Lefebvre et al. (2000), Reid et al. (2010)
		Excretion (*i*)		1	1	1	Jansen (2012)
		Pseudofaeces production (*o*)		location dependent	location dependent	NA	
		Faeces production* (*o*)	P	2-6	2-6	2-6	Lefebvre et al. (2000), Reid et al. (2010)
		Excretion (*i*)		5-8	5-8	5-8	Jansen (2012)
		Pseudofaeces production (*o*)		location dependent	location dependent	NA	
		Faeces production* (*o*)	C	1-2	1-2	1-2	Lefebvre et al. (2000), Reid et al. (2010)
		Respiration (*i*)		2-4	2-4	2-4	Jansen (2012), Filgueira et al. (2019)
		Pseudofaeces production (*o*)		location dependent	location dependent	NA	
	Deposit feeder	Faeces production* (*o*)	N	2-4	2-4	2-4	Honda and Kikuchi (2002), Hannah et al. (2013)
		Excretion (*i*)		1-5	1-5	1-5	Honda and Kikuchi (2002), Yuan et al. (2013), Fang et al. (2016a)
		Faeces production* (*o*)	P	6-12	6-12	6-12	Hannah et al. (2013), Fang et al. (2016a)
		Excretion (*i*)		unknown	unknown	unknown	
		Faeces production* (*o*)	C	2-3	2-3	2-3	Maxwell et al. (2009), Fang et al. (2016a)
		Respiration (*i*)		6-9	6-9	6-9	Yuan et al. (2013), Fang et al. (2016a)

Table 2.2 - *Continued*

Factor	Extractive species	Description	Nutrient	System			Reference
				Open	Semi-open	Closed	
4.1.3 Waste quality	Seaweed	Stoichiometry[a] (*i*)	P	location dependent	location dependent	5	Atkinson and Smith (1983)
	Microalgae	Stoichiometry[b] (*i*)	N	NA	location dependent	13	Redfield et al. (1963)
	Bivalves_organic	Stoichiometry[c] (*i*)	P	location dependent	location dependent	10-11	Higgins et al. (2011), Jansen et al. (2012)
			C	location dependent	location dependent	3	Higgins et al. (2011), Jansen et al. (2012)
	Deposit feeder		N, P, C	location dependent	location dependent	unknown	
Environmental factors							
4.2.1 Capture efficiency	Bivalves_organic	Particle size range (*o*) Exposure time[d] (*o*)	N	unknown 1	0 NA	0 NA	Cripps (1995), Brinker and Rösch (2005) Cranford et al. (2013)
		Particle size range (*o*) Exposure time[d] (*o*)	P	unknown 3	0 NA	0 NA	Cripps (1995), Brinker and Rösch (2005) Cranford et al. (2013)
		Particle size range (*o*) Exposure time[d] (*o*)	C	unknown 1	0 NA	0 NA	Cripps (1995), Brinker and Rösch (2005) Cranford et al. (2013)
4.2.2 Ambient nutrients	Bivalves_organic	AE mixed diet[e] (*o*) Food preference (*o*)	N	2 unknown	2 unknown	NA NA	Reid et al. (Reid et al. 2010) Lefebvre et al. (2000), Handå (2012)
		AE mixed diet[e] (*o*) Food preference (*o*)	P	6 unknown	6 unknown	NA NA	Reid et al. (2010) Lefebvre et al. (2000), Handå (2012)
		AE mixed diet[e] (*o*) Food preference (*o*)	C	2 unknown	2 unknown	NA NA	Reid et al. (2010) Lefebvre et al. (2000), Handå (2012)
	Deposit feeder	AE mixed diet[e] (*o*) Food preference (*o*)	N	6 unknown	6 unknown	NA NA	Fang et al. (2017)
		AE mixed diet[e] (*o*) Food preference (*o*)	P	18 unknown	18 unknown	NA NA	Fang et al. (2017)
		AE mixed diet[e] (*o*) Food preference (*o*)	C	8 unknown	8 unknown	NA NA	Fang et al. (2017)

Table 2.2 - *Continued*

Factor	Extractive species	Description	Nutrient	System			Reference
				Open	Semi-open	Closed	
4.2.3 Temporal issues	Seaweed	Seasonal mismatch[f] (*i*)	N	18	18	NA	Broch et al. (2013)
		Seasonal mismatch[f] (*i*)	P	6	6	NA	Broch et al. (2013)
		Seasonal mismatch[f] (*i*)	C	19	19	NA	Broch et al. (2013)
	Deposit feeder	Seasonal mismatch[g] (*i*)	N	5	5	NA	Ren et al. (2012)
		Seasonal mismatch[g] (*i*)	P	15	15	NA	Ren et al. (2012)
		Seasonal mismatch[g] (*i*)	C	6	6	NA	Ren et al. (2012)
4.2.4 Spatial issues	Seaweed	Spatial arrangement[h] (*i*)	N	20	NA	NA	
		Spatial arrangement[h] (*i*)	P	7	NA	NA	
		Spatial arrangement[h] (*i*)	C	20	NA	NA	
	Bivalves_organic	Spatial arrangement[h] (*o*)	N	2	NA	NA	
		Spatial arrangement[h] (*o*)	P	7	NA	NA	
		Spatial arrangement[h] (*o*)	C	3	NA	NA	

[*]See also Fig. 2.3
[a]Based on the Atkinson ratio 550:30:1 (C:N:P, molar).
[b]Based on the Redfield ratio 106:16:1 (C:N:P, molar), for microalgae.
[c]Based on an average tissue content for mussels of 161:34:1 (C:N:P, molar) and an average tissue content for oysters of 140:21:1 (C:N:P, molar).
[d]Under the assumption of a current of 8 cm sec^{-1}, and 100000 mussels.
[e]Mixed diet; diet composed of fish waste nutrients and low quality ambient nutrients.
[f]Based on the integration of the kelp species *Saccharina latissima* with salmon in temperate region.
[g]Based on the integration of the sea cucumber *Apostichopus japonicus* with salmon.
[h]Based on an area where tidal currents dominate and extractive species are placed on one side of the fish cage.

4.1.2 Extractive species waste production

In our conceptual model, maximum AE data reported for invertebrates were applied to estimate nutrient assimilation. This shows that only a small nutrient fraction was not retained, due to the combined faeces production by bivalves and deposit feeders feeding on organic waste (3-6% N, 8-18% P and 3-5% C; Table 2.2). In addition, metabolic waste produced by the extractive species should be considered (Table 2.2). This was estimated as: 60-80% loss of assimilated C as inorganic C through respiration; 10-75% of assimilated N excreted as inorganic N; and 65% of assimilated P excreted as inorganic P (mussels, Jansen 2012 and Filgueira et al. 2019; polychaetes, Honda and Kikuchi 2002 and Fang et al. 2016a; sea cucumber, Yuan et al. 2013). No study reported a P budget for deposit feeders. The excreted and respired nutrients could serve as an additional nutrient source for autotrophs. In open-water systems it is expected that these inorganic nutrients dilute and disperse quickly or are taken up by autotrophs (Jansen et al. 2018), while in closed systems they will accumulate.

A specific characteristic of bivalves is their pre-ingestive selection of food particles, which occurs above a pseudofaeces threshold concentration of 3-5 mg SPM l^{-1} for the mussel *M. edulis* (Widdows et al. 1979) and 10 mg SPM l^{-1} for the oyster *Crassostrea virginica* (Epifanio & Ewart 1977). Oysters reject fish faeces as pseudofaeces when a mixed diet of faeces and microalgae is offered, indicating that faeces is not a preferable food source (Lefebvre et al. 2000). This has consequences for the bioremediation potential of bivalves in systems with ambient and waste nutrients present, i.e. open and semi-open systems (Table 2.2). In open systems it will depend on the location if threshold concentrations are reached; SPM concentrations in and around fish cages in Canada and the Mediterranean were occasionally above the threshold level (Sarà et al. 2009, Brager et al. 2016), while in a study in Norway threshold concentrations were not reached (Brager et al. 2016). For semi-open systems, concentrations above the threshold have been reported (e.g. Jones et al. 2001), but threshold concentrations can be avoided by adjusting the water flow.

In our conceptual IMTA we assumed that inorganic waste nutrients were retained in seaweed biomass, according to the Atkinson ratio. However, seaweeds do also release organic material as metabolic products (Campbell et al. 2019). It is estimated that 18-62% of their primary production is released as dissolved organic carbon (DOC) (Khailov & Burlakova 1969, Abdullah & Fredriksen 2004, Wada et al. 2007). Additionally, POM is released as a result of three processes: (1) fall-off, whereby a whole individual is lost; (2) break-off, whereby part of the thallus is lost; (3) distal erosion, whereby leaf tops erode (Zhang et al. 2012). Estimates on POM release vary; while Wada et al. (2007) reported that 1-13% of the primary production of the brown seaweed *Ecklonia cava* is released as particulate organic carbon (POC), Zhang et al. (2012) reported that kelp (*Saccharina japonica*) releases 45-61% and 41-54% of its primary production as POC and particulate organic nitrogen (PON), respectively. Release of DOC results in a non-retained fraction of 7-25% C, while release of POM results in a non-retained fraction of 16-21% N and 0.4-24% C (Table 2.2). It should be noted that seaweeds are inorganic

extractive species, but their non-retained fraction contributes to the organic nutrient pool in the (eco)system.

4.1.3 Waste quality

The majority of studies summarised in *section 3* focus on a single element, but organisms require nutrients in balanced amounts (i.e. stoichiometry) to sustain optimal growth and functioning (Elser et al. 2000). In our conceptual IMTA, the Atkinson ratio for seaweeds and the Redfield ratio for microalgae were compared to the C:N:P molar ratio of the inorganic waste fraction (264:24:1), indicating P limitation for microalgae and C and N limitation for seaweeds. Assuming a large enough surface area, C limitation is most likely prevented by carbon exchange between the atmosphere and surface water (carbon cycle) and instead N becomes most limiting for seaweeds. The imbalance of inorganic nutrients in fish waste results in an overall non-retained fraction of 5% P by seaweeds, or 13% N by microalgae, in closed systems. In open and semi-open systems the presence of ambient nutrients plays a role in the stoichiometry of the available nutrients, therefore the first limiting nutrient will be location dependent (Table 2.2). The molar ratio of the organic waste fraction in our conceptual IMTA is 65:5:1 (C:N:P), for both the suspended and settled solids. Jansen (2012) reported for the mussel *M. edulis* an average tissue C:N:P ratio of 173:35:1, while for oysters an average tissue C:N:P ratio of 140:21:1 was reported by Higgins et al. (2011). These ratios suggest that for bivalves N is the first limiting nutrient in organic fish waste, resulting in a non-retained fraction of 10-11% P and 3% C (Table 2.2). No information was found on tissue C:N:P ratios of sea cucumbers and polychaetes, and it remains unclear to what extent macronutrient composition of organic fish waste is balanced for deposit feeders. For both sea cucumbers and polychaetes, lower growth has been reported when feeding fish faeces, as compared to commercial diets (Honda & Kikuchi 2002, Brown et al. 2011, Chen et al. 2014), suggesting that waste quality is not sufficient to sustain optimal growth. For mussels it has also been suggested that fish faeces alone is insufficient; integration of *M. edulis* with Atlantic cod in a closed system resulted in nutritionally stressed mussels (Both et al. 2011). Presence of ambient nutrients in open and semi-open systems may overcome these limitations that are potentially faced in closed systems for bivalves and deposit feeders.

4.2 Environmental factors

Environmental factors influence the connectivity between waste nutrients and the extractive species. For closed systems it is assumed that this connectivity is optimal and these factors are therefore more relevant for open and semi-open systems.

4.2.1 Capture efficiency

The capture efficiency of bivalves depends on particle size and exposure time (Reid et al. 2011, Cranford et al. 2013). To be captured, waste particles should fall within a species-specific size range (Reid et al. 2011); the mussel *M. edulis* efficiently

filters particles between 3-1000 µm (Newell et al. 1998, Davenport et al. 2000); the oyster *C. gigas* efficiently filters particles between 5-541 µm (Dupuy et al. 2000). Little is known about the fraction of waste particles that fall within the bivalve filtering size ranges (Reid et al. 2011). Studies on waste particle sizes in land-based fish farms reported ranges between 8-269 µm (salmonid hatchery; Cripps 1995) and 8-512 µm (trout farm; Brinker & Rösch 2005) for the suspended solids. It is therefore suggested that in land-based systems all waste particles available for bivalves (i.e. suspended solids; 30% of POM, *section 3.3*) fall within the filtering size-ranges of both mussels and oysters, and results in 0% non-retained nutrient loss (Table 2.2). For open-water systems, information is lacking on the fraction of waste particles that fall within the filtering size range of bivalves, and it remains unknown to what extent this factor should be taken into account (Table 2.2).

Capture efficiency is also influenced by exposure time. While in land-based systems exposure time can be controlled, in open-water systems this depends on the current. Cranford et al. (2013) calculated the capture efficiencies for a cultivation unit of mussels for a range of current speeds and highlighted that exposure time (e.g. current) can seriously limit capture efficiency. In our conceptual model 5% of carbon supplied with fish feed is retained in mussel biomass (Fig. 2.3). This corresponds to a biomass of 100,000 mussels, with an assumed C content of 37% on a dry weight basis (Smaal & Vonck 1997, Table 2.1) and an average individual dry weight, without shell, of 0.7 g (Cranford et al. 2013). Based on Cranford et al. (2013), however, it was estimated that in areas with current speeds of 8 cm sec^{-1}, these mussels can only capture 80% of the suspended waste particles. In consequence, 20% of the suspended waste particles will be non-retained. Expressed as percentage of the total fish feed input to the system, this corresponds to a non-retained fraction of 1% N, 3% P and 1% C (Table 2.2).

4.2.2 Ambient nutrients

In open and semi-open systems water exchange imports ambient nutrients into the system, while waste nutrients are exported. Ambient nutrients are assumed to affect the bioremediation potential of open and semi-open IMTA systems, as they compete with waste nutrients in concentration and quality. For autotrophs it can be argued whether or not the presence of ambient nutrients influences their bioremediation capacity, since most likely inorganic N, P and C released by fish do not differ from their ambient counterparts. 'Direct uptake' of waste nutrients is therefore not of principal interest for inorganic extractive species, and instead a balance between nutrient inputs and outputs should be created (Reid et al. 2013). Quality differences between waste and ambient POM may influence the bioremediation potential of bivalves and deposit feeders, as low-quality ambient POM can reduce AE. Reid et al. (2010) observed that resuspension events and periodic fluxes of low-quality food resulted in a lower AE for mussels cultivated adjacent to salmon cages in the field (54%), compared to mussels fed salmon faeces in the laboratory (86%) (Appendix Table S2.3). Fang et al. (2017) showed

that polychaetes fed sediment collected underneath a fish farm had a lower AE (~40%), compared to polychaetes fed deposited material collected from sediment traps deployed in the centre of a fish farm (~60%) (Appendix Table S2.3). Deposited material consisted mainly of fresh faeces and feed spills, while sediment is a mix of fresh and decomposing faeces and microbial communities. The latter is more likely to represent a diet that can be expected in open and semi-open systems. In open and semi-open systems located in areas with (periodically) low-quality ambient nutrients, retention efficiencies of mussels and polychaetes are thus expected to decrease, due to a reduced AE from 86% to 54% for mussels and from 79% to 40% for polychaetes. Mixed diets composed of fish waste nutrients and low-quality ambient nutrients thus increase the total non-retained fraction (Table 2.2). High-quality ambient POM may also reduce bioremediation potential, as a result of food preferences. Bivalves prefer, for example, plankton over fish feed derived POM (Lefebvre et al. 2000, Handå 2012). In areas with high-quality ambient food sources it can therefore be expected that bioremediation potential of bivalves is lower than in our conceptual IMTA, however exact quantification will depend on local factors, like the ratio and quality difference between ambient and waste POM (Table 2.2). It should be noted that plankton taken up by bivalves can (partially) be grown on inorganic fish waste, contributing indirectly to the bioremediation capacity of bivalves in open and semi-open systems. For polychaetes and sea cucumbers no studies were found looking at potential preferences for either ambient food sources encountered at fish farms, or fish feed derived POM, and it remains unknown to what extent this should be taken into account (Table 2.2).

4.2.3 Temporal issues

For specific combinations of fed and extractive species, seasonal factors can result in a 'mismatch' between nutrient release and uptake. Broch et al. (2013) described such a mismatch between integration of the kelp species *Saccharina latissima,* with salmon in an open-water system located in a temperate region. Uptake rates of kelp peak during spring, while due to the start of distal erosion, kelp is harvested in early to mid-summer (Hurd et al. 2014). Waste production by salmon fluctuates seasonally, and highest release rates are at the end of the summer, when kelp is already harvested. Based on the model presented by Broch et al. (2013) it was estimated that 53% of the waste nutrients are released during the kelp growth cycle, suggesting that nearly half of the inorganic nutrients are non-retained. This results in a non-retained fraction of 18% N, 6% P and 19% C of the total amount fed to the system (Table 2.2). To survive high and low temperatures, various sea cucumber species undergo aestivation, hibernation or both (Ren et al. 2012, Chen et al. 2016). *Apostichopus japonicus* stops feeding during winter and summer (Ren et al. 2012), resulting in a mismatch between the highest waste release by the fed species during summer. Ren et al. (2012) indeed observed that in pond systems where *A. japonicus* was integrated with scallops (*Chlamys farreri*) both organic C and total N content of the sediment increased during hibernation and aestivation, but decreased during sea cucumber feeding seasons. Based on the salmon waste

production cycle presented in Broch et al. (2013), and the aestivation (July-September) and hibernation (October-December) period reported in Ren et al. (2012), it was estimated that only 50% of the waste nutrients are released during the feeding season of *A. japonicus* (Table 2.2). Both cases demonstrate that temporal issues can limit maximum waste retention in open and semi-open systems, but it should be noted that this is highly species and location specific.

4.2.4 Spatial design

Integration of extractive species to fish cultures has practical implications, such as a requirement for a greater farm area. Extractive species biomass and corresponding cultivation areas were calculated for our conceptual IMTA and are reported in Table 2.3. Upscaling to a commercial salmon farm (production of ~1800 tons over a 2 year-cycle, and an average FCR of 1.1) would in the best case scenario require (*i*) 47 ha seaweed, (*ii*) 12 ha bivalves and (*iii*) 237 ha deposit feeders, assuming that extractive species are harvested yearly. Studies modelling the seaweed and bivalve compartment in open water IMTA already highlighted that large areas are required (Sanderson et al. 2012, Broch et al. 2013, Reid et al. 2013, Holdt & Edwards 2014), which has major implications for the spatial design of IMTA systems. Addition of extractive species to land-based fish farms will increase pressure on space. While closed and semi-open systems can be designed to optimise connectivity between waste and the extractive species, in open systems the spatial arrangement determines connectivity. When tidal currents dominate, and seaweed and bivalve cultivation are placed on one side of the fish cages, they are only exposed to waste nutrients 50% of the time, increasing the non-retained fraction of the overall IMTA system (Table 2.2). In reality waste exposure time will probably be higher, as some suspended nutrients will oscillate around the farm. Deposit feeders should be cultivated underneath fish cages, within the farm scale, as their target waste flux settles relatively nearby, resulting in local impacts (Filgueira et al. 2017). In shallow areas (< 20 m depth) farm scale could mean within 30 m from the cages, as this is where most organic waste accumulation is observed (Findlay et al. 1995, Kempf et al. 2002), while for farms located in deeper areas, like fjords, this area is expanded and could reach up to 500 m from the farm (Bannister et al. 2016). With the trend of moving fish farms to deeper and more exposed locations, it is expected that the affected benthic area becomes larger (Valdemarsen et al. 2015).

Dispersal of organic waste particles in open water systems is dominated by a vertical flux, and only a small fraction ends up in the horizontal flux (Reid et al. 2011, Bannister et al. 2016, Filgueira et al. 2017). The latter is supported by field studies, indicating only minimal and temporal enhancement of suspended particles in the water column around fish farms (Brager et al. 2015, Brager et al. 2016). Given that mussels in open water systems are mostly cultivated in surface

Table 2.3
Scaling of a conceptual four-species IMTA system; biomass (tons wet weight) and area (m^2) required per extractive species for maximum retention of waste (salmon farm fed 1 ton of commercial feed). Biomass per extractive species is calculated based on assimilation efficiencies (AE) and tissue contents reported in literature. Data for these calculations can be found in table 2.1. Area per extractive species is calculated based on the following stocking densities: 95 tons ha^{-1} for seaweeds (Reid et al. 2013), 3 tons ha^{-1} for microalgae (Brune et al. 2003), 76 tons ha^{-1} for mussels (Hughes & Black 2016), 50 tons ha^{-1} for oysters (FAO 2005-2019), 10 tons ha^{-1} for sea cucumbers (FAO 2011-2019) and 3 tons ha^{-1} for polychaetes (Brown et al. 2011). ND, not determined.

Extractive compartment		Biomass (tons wet weight)	Area (m^2)
Seaweed	N	4 - 23	389 – 23091
	P	3 – 18	356 – 15680
	C	4 – 8	465 – 7571
Microalgae + bivalves			
Microalgae	N	1 – 10	4073 – 36653
	P	1	2852
	C	3 – 4	9722 – 14000
Mussels	N	0.7-0.9	96-357
	P	0.6-1	76-381
	C	2	737
Oysters	N	1.3 – 1.5	268 – 304
	P	1.3 – 1.5	263-300
	C	2 – 3	467 – 500
Mussels	N	0.08-0.11	11-41
	P	0.3-0.6	42-207
	C	0.3	98
Oysters	N	0.13-0.15	26-30
	P	0.6-0.7	123-140
	C	0.3	54
Sea cucumber	N	0.7-0.9	664-930
	P	ND	ND
	C	2 – 3	2366-2821
Polychaete	N	0.6-1	1842-3542
	P	ND	ND
	C	1 – 3	4225-9526

waters (up to 13 m), their exposure is only to a minor fraction of the organic waste (Cranford et al. 2013, Filgueira et al. 2017), suggesting that the 30% of POM available as suspended solids for the bivalves in our conceptual IMTA is an overestimation. In addition, due to biodeposition, mussels contribute to the already dominant vertical particle flux, increasing local benthic impact (Cranford et al. 2013). The bioremediation potential of mussels in open water IMTA has therefore gained critical attention. Cranford et al. (2013) estimated that, in open water systems, mussels contribute to a reduced impact of aquaculture on the benthic ecosystem, if their diets consist for a minimum of 15-30% of OM originating from fish faeces. Stable isotope analysis shows that contribution of fish faeces to the overall diet of bivalves is often lower (Fig. 2.2), limiting their role in open-water IMTA. Only in areas where seston concentration is low and organic content is high and if mussels can be cultivated close to cages, might it be possible to reach conditions whereby mussels can play a role in the bioremediation of aquaculture waste (Mazzola & Sarà 2001, Cranford et al. 2013, Filgueira et al. 2017).

In open-water fish cage systems, contrary to consensus, extractive species should not be cultured directly alongside the fed species. This is partly because they may hinder the optimal functioning of the system by, for example, making it difficult to access the cages by boat to feed the fish (Hughes & Black 2016). It is also, because inorganic and suspended waste is rapidly dispersed by currents. Therefore the extractive species in the water column, i.e. seaweeds and bivalves, simply need to be located in the area of nutrient dispersion (Sanderson et al. 2008, Brager et al. 2015, Brager et al. 2016, Jansen et al. 2018). Hence, in open-water IMTA, the bioremediation potential of extractive species could be evaluated at a more regional scale, creating a balance between nutrients excreted by the fed species and nutrients harvested via the extractive species. Such a 'balance approach' allows to evaluate 'connectivity' between the different functional groups at a larger scale than farm level (Sanz-Lazaro & Sanchez-Jerez 2020). Using this approach raises the question of where to establish the boundaries in evaluating IMTA performance. It has been shown that growth of seaweeds and bivalves is only significantly enhanced, compared to reference stations, when cultivated within tens to hundreds of meters from fish cages (Kerrigan & Suckling 2016, Fossberg et al. 2018). Using the balance approach for open-water IMTA designs, growth enhancement of extractive species (compared to monocultures of seaweeds and bivalves) should therefore not be expected.

5 Retention potential of IMTA systems

Based on the highest nutrient use efficiencies reported in literature, we demonstrated that a theoretical maximum nutrient retention potential of 94% for C and N and 79% for P administrated with the fish feed is possible in IMTA systems containing salmon as fed species and kelp, mussel and polychaete as extractive species (Fig. 2.4). These percentages, however, do solely consider the nutrients applied to produce fish, estimate use efficiency of extractive species based only on assimilation efficiencies and assume no ambient nutrients complement waste

nutrients from fed fish for extractive species. These percentages also take into account that a small fraction of fish waste is DOM, which extractive species included in our salmon-kelp-mussel-polychaete IMTA cannot use.
The theoretical maximum nutrient retention potential, however, does not account for the feeding metabolism of extractive species. When doing so, the retention efficiencies decrease to 65-75% for N, 65% for P and 45-75% for C with the fish feed still being the only nutrient input to the IMTA, which is the case for an IMTA operated as a closed system (Fig. 2.4).
In semi-open and open systems, limited control over environmental factors including exposure time to waste nutrients, presence of ambient nutrients influencing food preference and assimilation efficiencies of mixed diets by extractive species, seasonal mismatches between nutrient supply and food requirements, and sub-optimal spatial arrangements reducing nutrient access of extractive species, lessen the retention efficiencies that can be achieved to 50% for N, 40% for P and 40-50% for C, administrated with the feed to the IMTA system (Fig. 2.4).

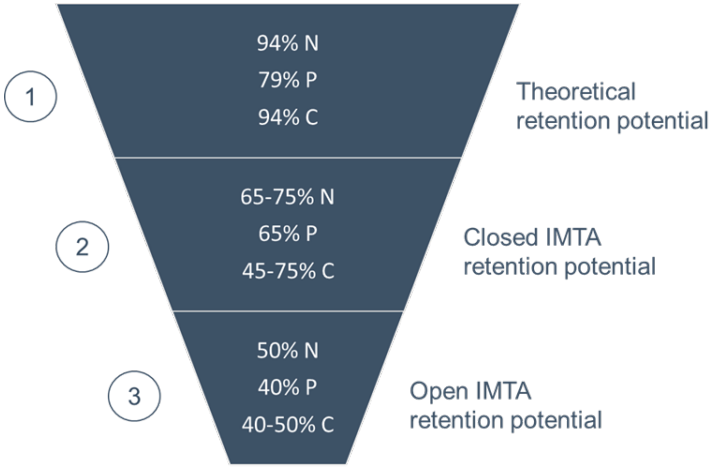

Figure 2.4
Nutrient retention potentials estimated for a conceptual four-species IMTA (salmon-kelp-mussel-polychaete) under different degrees of system openness. The numbers 1, 2 and 3 refer to the different steps of the generic framework, as presented in Fig. 2.1. In step 1 the maximum theoretical retention potential of a conceptual IMTA (fish – seaweed – bivalve – deposit feeder) is calculated. In Step 2 biological factors are considered, resulting in the retention potential of a closed IMTA system. Step 3 considers environmental factors, which besides the biological factors, have to be taken into account to calculate the retention potential of an open-water IMTA system.

Concluding, in this study we assumed that 43% N, 24% P and 38% C of the fed nutrients to salmon are retained in fish biomass gain. This means that in closed land-based IMTA system, an additional 22-32% N, 41% P and 7-37% C of the fed nutrients can be recycled by extractive species, while in an open IMTA system this is 7% for N, 16% for P and 2-12% for C. In most cases, for open IMTA systems the nutrient retention efficiencies are still overestimated as maximum retention efficiencies reported in literature were used. This makes it attractive to apply a 'mass balance approach' over a larger production area, aiming to extract the same amount of nutrients that were fed to fish cages with the nutrients contained in biomass gain of harvested fed and extractive species. An advantage is then that different species can be cultured independently, allowing to optimise production while minimising temporal and spatial mismatches. A disadvantage is that fed nutrients and nutrients retained by extractive species are not fully the same, and that local pollution or nutrient shortages may become an issue if the design and local conditions are not carefully investigated.

Acknowledgements

The authors would like to thank Miranda Maybank for her constructive comments on the manuscript. We also thank the 3 anonymous reviewers for their comments and suggested changes to earlier versions of this paper. This study was carried out as part of the PhD study of MAJ Nederlof (IPOP project TripleP@Sea KB-24- 001-019 at Wageningen University).

Supplementary material

Table S2.1

Fed species nitrogen, phosphorus and carbon budget, including nutrient content of the feed given in gram kg^{-1} dry matter (i.e. input), percentage of nutrients retained and released (i.e. output) by different marine fish species.

Retention and outputs are given as a percentage of the total nutrients provided with the feed. Nutrients released are divided into an inorganic and organic fraction, of which the latter is subdivided into the solid fraction (org_solids) and the dissolved fraction (org_dissolved, i.e. dissolution of solid waste). Numbers in *italic* are re-calculated from values reported in the corresponding paper.

Species	Nitrogen					Phosphorus					Carbon					Ref.
	Feed content	Retention	Output - inorganic	Output - org_solids	Output - org_dissolved	Feed content	Retention	Output - inorganic	Output - org_solids	Output - org_dissolved	Feed content	Retention	Output - inorganic	Output - org_solids	Output - org_dissolved	
Salmon	72	38	45	*15*	3	12	30	18	*44*	8	510	30	48	*19*	3	1
Salmon	55-63	43	39	15	---	6-10	24	24	44	---	519-555	38	40	19	---	2
Trout	---	---	---	---	---	---	---	---	---	---	500-520	*22*	49	23	6	3
Trout	---	---	---	---	---	11-16	*18-23*	25-30	47-54	2-4	---	---	---	---	---	4
Trout	66-94	*29-33*	48	20	1-3	---	---	---	---	---	---	---	---	---	---	5
Sea bream	*70*	*17*	*40*	*43*	---	---	*36*	*23*	*42*	---	---	*16*	*39*	*44*	---	6
Sea bream	*79*	22	61	10	7	*15*	29	19	44	8	---	---	---	---	---	7
Sea bass	*70*	*13*	*42*	*45*	---	---	*35*	*25*	*43*	---	---	*14*	*42*	*44*	---	6
Sea bass	---	32	63	5	---	---	---	---	---	---	---	33	52-70	*5*	1	8

1 Wang et al. (2012); 2 Wang et al. (2013); 3 Hall et al. (1990); 4 Holby and Hall (1991); 5 Hall et al. (1992); 6 Brigolin et al. (2014); 7 Lupatsch and Kissel (1998); 8 Norði et al. (2011)

Table S2.2

Seaweed production (growth and tissue content) and nutrient extraction parameters in integrated aquaculture systems varying in openness.

Country codes: CA, Canada; CL, Chile; TZ, Zanzibar (Tanzania); JP, Japan; NO, Norway; CN, China; IL, Israel; PT, Portugal; ES-CN, Canary Island; SA, Saudi Arabia; ES, Spain; AU, Australia; GB-SCT, Scotland (Great Britain).

Exchange rate refers to water exchange rate and is given in l day^{-1} for closed and semi-open systems, and in m sec^{-1} for open systems.

For the parameters (1) *ambient nutrients*, (2) *increase in integrated aquaculture*, (3) *load* and (4) *extraction efficiency,* time of the year when the parameter was measured is given within brackets: wi, winter; sp, spring; su, summer; au, autumn and year is averaged over a whole year.

Increase in integrated aquaculture indicates, for the parameters given, percentage benefit for the seaweed to be cultivated in integrated aquaculture systems, compared to a control group, reference station or monoculture and is calculated as: Increase in integrated aquaculture (%) = (value measured in IMTA – value measured in control)/value measured in control * 100.

Extraction efficiency is the percentage of nutrients removed from the inflow to the seaweed compartment. In literature, two methods are used to measure the extraction efficiency: (1) based on the tissue content of the seaweed (Tissue) and (2) based on the difference in nutrient concentration in the in- and outflow of the seaweed compartment (Water).

ND, Not Determined; SGR, Specific Growth Rate; RGR, Relative Growth Rate; Lab, measured in a laboratory set-up. Numbers in *italic* are calculated from values in the corresponding paper. Numbers in the column Ref. relate to the reference list.

System						Growth & tissue content of seaweed in integrated aquaculture		Nutrient extraction by seaweeds				Ref.
Country	System type	Exchange rate	Ambient nutrients (µM)		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg seaweed^{-1} day^{-1})	Extraction efficiency (%)	
CA	Closed	ND	N_{DIN} P_{PO4}	18 (wi/sp) 12 (su/au) 0.2 (au) 6 (wi)	Halibut with *Palmaria palmata*	SGR Tissue N Tissue C	*38* (sp) *22* (au) *0* (year)	Tissue	N_{DIN} C	*6* (wi/sp) *5* (su/au) *16* (wi/sp) *12* (su/au)	1 (wi/sp) 0.5 (su/au) 5 (wi/sp) 2 (su/au)	1
IL	Closed	2000		Negligible	Sea bream with *Ulva lactuca*	ND		Tissue	N_{DIN} P_{PO4}	*9* (su) *2* (su)	*53* (su) *30* (su)	2

Table S2.2 - *Continued*

System						Growth & tissue content of seaweed in integrated aquaculture		Nutrient extraction by seaweeds				Ref.
Country	System type	Exchange rate	Ambient nutrients (µM)		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg seaweed^{-1} day^{-1})	Extraction efficiency (%)	
PT	Closed	ND	ND		Sea bass & turbot with *Gracilaria bursa pastoris*	ND		Water	N_{TAN}	*0.3* (su/au)	84 (su/au)	3
CN	Closed (Lab)	0	N_{TAN} P_{PO4}	3 (au) 0.7 (au)	Scallops with *Gracilaria lemaneiformis*	ND		Water	ND		84 (au) 70 (au)	4
AU	Closed (Lab)	0	ND		Shrimp & oyster with *Gracilaria edulis*	ND		Water	N_{DIN} P_{PO4}	*0.04* *0.005*	*98* *95*	5
CL	Semi-open	*1200*	N_{DIN} P_{PO4}	*16* (au) *2* (su) 2 (au) 0.5 (su)	Grunt with *Gracilaria chilensis*	SGR	*150* (year)	Water	N_{DIN} P_{PO4}	*0.13* (au) *0.55* (su) *0.03* (au) *0.02* (su)	*77* (au) *100*(su) *37* (au) *86* (su)	6
TZ	Semi-open	*81864*	N_{TAN} P_{PO4}	4.8 (sp) 0.8 (sp)	Fish with *Ulva reticulata*	SGR	*60* (sp)	Water	N_{TAN} P_{PO4}	*11* (sp) *0.7* (sp)	65 (sp) 33 (sp)	7
IL	Semi-open	*96000*	ND		Sea bream with *Ulva lactuca*	ND		Tissue Water	N_{TAN}	*2* (au)	56 (au) 92 (au)	8
ES-CN	Semi-open	*1500*	ND		Sea bream with *Ulva rigida*	ND		Water	N_{DIN}	*1* (su) *0.8* (wi)	97 (su) 85 (wi)	9
IL	Semi-open	*2400*	ND		Sea bream with *Ulva lactuca*	ND		Water	N_{TAN}	*2* (wi) *8* (wi)	96 (wi) 39 (wi)	10
SA	Semi-open	*5400*	ND		Tilapia with *Ulva lactuca*	ND		Water	N_{TAN} P_{PO4}	*0.02-1* *5*	58-85 41	11

Table S2.2 - *Continued*

System						Growth & tissue content of seaweed in integrated aquaculture		Nutrient extraction by seaweeds				Ref.
Country	System type	Exchange rate	Ambient nutrients (µM)		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg seaweed^{-1} day^{-1})	Extraction efficiency (%)	
SA	Semi-open	*5400*	ND		Tilapia with *Gracilaria arcuata*	ND		Water	N_{TAN} P_{PO4}	*0.02-1* *5*	66-87 41	11
ES	Semi-open	*2100*	N_{DIN} P_{PO4}	9 (sp) 1 (sp)	Sea bream with *Ulva rotundata*	ND		Water	N_{DIN} P_{PO4}	*2-8* (sp) *1-3* (sp)	54 (sp) 9 (sp)	12
ES	Semi-open	*2100*	N_{DIN} P_{PO4}	9 (sp) 1 (sp)	Sea bream with *Gracilaria longissima*	ND		Water	N_{DIN} P_{PO4}	*2-8* (sp) *1-3* (sp)	17 (sp) 3 (sp)	12
JP	Open	*0.1*	N_{DIN}	*0.6* (sp) *2* (su)	Seabream & yellowtail with *Ulva ohnoi*	SGR Tissue N	*574* (sp) *10* (su) *140* (sp) *0* (su)	ND	ND	ND	ND	13
NO	Open	*0.1*	N_{DIN}	116 (sp) 70 (wi)	Salmon with *Saccharina latissima*	SGR Tissue N Tissue C	*74* (sp) *26* (sp) *0* (sp)	ND	ND	ND	ND	14
CN	Open	ND	N_{DIN} P_{PO4}	*7* (wi) *4* (sp) 0.5 (wi) 0.4 (sp)	Snapper & Grouper & Cobia with *Sargassum hemiphyllum*	SGR Tissue N Tissue C Tissue P	*50* (year) *23* (sp) *2* (sp) *-18* (sp)	ND	ND	ND	ND	15
CN	Open	ND	N_{DIN} P_{PO4}	*7* (wi) *4* (sp) 0.5 (wi) 0.4 (sp)	Snapper & Grouper & Cobia with *Sargassum henslowianum*	SGR Tissue N Tissue C Tissue P	*14* (year) *-5* (sp) *-2* (sp) *-20* (sp)	ND	ND	ND	ND	15

Table S2.2 - *Continued*

System						Growth & tissue content of seaweed in integrated aquaculture		Nutrient extraction by seaweeds				Ref.
Country	System type	Exchange rate	Ambient nutrients (μM)		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg seaweed^{-1} day^{-1})	Extraction efficiency (%)	
NO	Open	ND	N_{DIN}	0.1 (sp) 8 (su)	Salmon with *Saccharina latissima*	SGR Tissue N Tissue C	*25* (au) *-50* (wi) *0* (sp) *67* (su) *-2* (year)	ND	ND	ND	ND	16
CL	Open	*0.05*	N_{DIN}	5 (su) 24 (au)	Salmon with *Gracilaria chilensis*	RGR Tissue N	*14* (su) *33* (au) *21* (au)	ND	ND	ND	ND	17
CL	Open	ND	ND		Salmon & Trout with *Gracilaria chilensis*	SGR	*10* (su)	ND	ND	ND	ND	18
CL	Open	*0.075*	ND		Salmon with *Gracilaria chilensis*	SGR Agar	*87* (su) *30* (au) *100* (su) *31* (au)	Tissue	N_{DIN} P_{PO4}	*0.17*(year) *0.01* year)	6.5 (year) 27 (year)	19
NO	Open	*0.05*	ND		Salmon with *Saccharina latissima*	ND		Tissue	N_{TAN}	*2* (year)	0.34 (year)	20
GB-SCT	Open	ND	ND		Salmon with *Palmaria palmata*	Tissue N RGR	*100* (su) *36-48* (wi)	Tissue	N_{DIN}	*0.18* (year)	12 (year)	21
GB-SCT	Open	ND	ND		Salmon with *Saccharina latissima*	Tissue N	*54* (su)	Tissue	N_{DIN}	*0.14* (year)	5 (year)	21

1 Corey et al. (2014); 2 Krom et al. (1995); 3 Matos et al. (2006); 4 Mao et al. (2009); 5 Jones et al. (2002); 6 Chow et al. (2001); 7 Msuya et al. (2006); 8 Neori et al. (2003); 9 Jiménez del Río et al. (1996); 10 Cohen and Neori (1991); 11 Al-Hafedh et al. (2012); 12 Hernández et al. (2005); 13 Yokoyama and Ishihi (2010); 14 Wang et al. (2014); 15 Yu et al. (2014b); 16 Handå et al. (2013); 17 Abreu et al. (2009); 18 Halling et al. (2005); 19 Troell et al. (1997); 20 Broch et al. (2013); 21 Sanderson et al. (2012)

Table S2.3
Effect of food source (waste, ambient or a mix between waste and ambient) on assimilation efficiency (AE), clearance rate (CR) and consumption rate of bivalves, sea cucumbers and polychaetes (the latter two are referred to as deposit feeders).

Country codes: US, United States of America; JP, Japan; FR, France; CA, Canada; MX, Mexico; NO, Norway; CN, China; GB, Great Britain; NZ, New Zealand; ES, Spain; . Numbers in the column Ref. relate to the reference list.

Country	System type	Species (*extractive species in italics*)	Extractive species	AE (%)			CR (l ind^{-1} h^{-1})			Consumption rate (mg gram deposit $feeder^{-1}$ day^{-1})			Ref
				Fish Waste	Mix	Natural	Fish Waste	Mix	Natural	Fish Waste	Mix	Natural	
US	Closed	Halibut with *Nereis virens*	Polychaete	24-48 *Protein*									1
JP	Closed	Japanese flounder with *Perinereis nuntia vallata*	Polychaete	46						12-63			2
FR	Semi-open	Sea bass with *Crassostrea gigas*	Bivalves	56	70	66	2.2	1.7	2.7				3
CA	Semi-open	Salmon with *Mytilus edulis* and *Mytilus trossulus*	Bivalves	86-90		81-87							4
MX	Semi-open	Shrimp with *Crassostrea corteziensis*	Bivalves		94		0.4						5
MX	Semi-open	Shrimp with *Anadara tuberculosa*	Bivalves		96		0.3						5
CA	Semi-open	Salmon food with *Mytilus edulis*	Bivalves	25-39		38	2		2				6
NO	Semi-open	Salmon with *Mytilus edulis*	Bivalves				2.4-2.8		2.4				7
CN	Semi-open	Scallops with *Stichopus japonicus*	Sea cucumber							11 *OM* 0.6 *N* 0.04 *P*			8
GB	Semi-open	Sea bass with *Holothuria forskali*	Sea cucumber							17			9
NZ	Semi-open	Mussel with *Australostichopus mollis*	Sea cucumber		45						106		10
NZ	Semi-open	Mussel with *Australostichopus mollis*	Sea cucumber							58			11
NZ	Semi-open	Abalone with *Australostichopus mollis*	Sea cucumber	83-88						31-59			12

Table S2.3 - *Continued*

Country	System type	Species (*extractive species in italics*)	Extractive species	AE (%)			CR (l ind^{-1} h^{-1})			Consumption rate (mg gram deposit feeder^{-1} day^{-1})			Ref
				Fish Waste	Mix	Natural	Fish Waste	Mix	Natural	Fish Waste	Mix	Natural	
CN	Semi-open	Flounder with *Perinereis aibuhitensis*	Polychaete	61-71						1-7 *OM* 0.02-0.06 *N* 0.5-2 *P*			13
CN	Semi-open	Fish with *Perinereis aibuhitensis*	Polychaete	55	32					0.9-3 *C* 0.17-0.34 *N*			13
CA	Open	Salmon with *Mytilus edulis* and *Mytilus trossulus*	Bivalves		54								4
ES	Open	Sea bream with *Mytilus galloprovincialis*	Bivalves		35-95								14
CN	Open	Sea bass with *Crassostrea gigas*	Bivalves	10-44									15
CN	Open	Snapper, Cobia and Grouper with *Apostichopus japonicus*	Sea cucumber		14	15							16
CN	Open	Snapper, Cobia and Grouper with *Holothuria leucospilota*	Sea cucumber		15								17
CA	Open	Sablefish with *Parastichopus californicus*	Sea cucumber		60 *C* 62 *N*								18
CA	Open	Oyster with *Parastichopus californicus*	Sea cucumber		39-42 *OM* 37-41 *C* 42-47 *N*								19

1 Brown et al. (2011); 2 Honda and Kikuchi (2002); 3 Lefebvre et al. (2000); 4 Reid et al. (2010); 5 Peña-Messina et al. (2009); 6 MacDonald et al. (2011); 7 Handå (2012); 8 Zhou et al. (2006); 9 MacDonald et al. (2013); 10 Zamora and Jeffs (2011); 11 Slater et al. (2009); 12 Maxwell et al. (2009); 13 Fang et al. (2017); 14 Irisarri et al. (2013); 15 Jiang et al. (2012); 16 Yu et al. (2014a); 17 Yu et al. (2011); 18 Hannah et al. (2013); 19 Paltzat et al. (2008)

Table S2.4
Bivalve production (growth and tissue content) and nutrient extraction parameters in integrated aquaculture systems varying in openness.

Country codes: CA, Canada; NO, Norway; CN, China; IL, Israel; ES, Spain; AU, Australia; GB-SCT, Scotland (Great Britain); FR, France; HR, Croatia; IT, Italy.
Exchange rate refers to water exchange rate and is given in l day^{-1} for closed and semi-open system, and in m sec^{-1} for open systems.
For the parameters (1) *ambient nutrients*, (2) *increase in integrated aquaculture*, (3) *load* and (4) *extraction efficiency,* time of the year when the parameters were measured are given within brackets: wi, winter; sp, spring; su, summer; au, autumn, and year is averaged over a whole year.
Increase in integrated aquaculture indicates, for the parameters given, percentage benefit for the bivalves to be cultivated in integrated aquaculture systems, compared to a control group, reference station or monoculture and is calculated as: Increase in integrated aquaculture (%) = (value measured in IMTA – value measured in control)/value measured in control * 100).
Extraction efficiency is the percentage of nutrients removed from the inflow to the bivalve compartment (calculated as the difference between nutrient concentrations measured in the in- and outflow to the bivalve compartment).
* range shown from 3 different locations, # 2 different study locations, Loch Etive (E) and Loch Leven (L). ND, not determined, Chl-a, chlorophyll-a; TSS, total suspended solids; OM, organic matter; TN, total nitrogen; TP, total phosphorus; CI, condition index; DW, dry weight; AFDW, ash free dry weight. Numbers in *italic* are calculated from values in the corresponding paper. Numbers in the column Ref. relate to the reference list.

System					Growth & tissue content of bivalves in integrated aquaculture		Nutrient extraction by bivalves			Ref
Country	System type	Exchange rate	Ambient nutrients (mg L^{-1})	Species *(extractive species in italics)*	Parameter	Increase in integrated aquaculture (%)	Parameter	Load (g kg bivalve^{-1} day^{-1})	Extraction efficiency (%)	
AU	Closed	0	ND	Shrimp with *Saccostrea commercialis*	ND		Chl-a TSS OM TN TP	*0.002* *2.7* *0.8* *0.04* *0.005*	*92* *88* *23* *33* *37*	1
AU	Closed	0	ND	Shrimp with *Mytilus edulis*	ND		TSS Bacteria TN TP		*51* *61* *5* *-47*	2

Table S2.4 - *Continued*

System						Growth & tissue content of bivalves in integrated aquaculture		Nutrient extraction by bivalves			Ref
Country	System type	Exchange rate	Ambient nutrients (mg L^{-1})		Species (*extractive species in italics)*	Parameter	Increase in integrated aquaculture (%)	Parameter	Load (g kg bivalve^{-1} day^{-1})	Extraction efficiency (%)	
FR	Closed	0	ND		Sea bass with (1) microalgae and (2) *Crassostrea gigas*	ND		N_{TAN} P_{PO4} Si Microalgae	 *0.57*	67 (algae) 47(algae) 50 (algae) 92 – 100 (oyster)	3
AU	Closed	ND	ND		Shrimp with *Saccostrea commercialis*	ND		Bacteria Chl-a	ND	55-88 88-96	4
IL	Semi-open	*3600*	ND		Sea bream with *Crassostrea gigas*	SGR FR	*200* (su) *133* (wi) *2760* (su) *2711* (wi)				5
AU	Semi-open	ND	ND		Shrimp with *Saccostrea commercialis*	Growth	*83*	Bacteria Chl-a	ND	61 70	4
ES	Semi-open	*26880*	Chl-a	0.002 (au) 0.005 (su)	Turbot with *Ruditapes decussatus*	Shell length DW-meat CI Carbohydrates Lipids Proteins OM	*35* (wi) *204* (wi) *33* (wi) *220* (wi) *362* (wi) *224* (wi) *222* (wi)				6
CA	Semi-open	*2736*	ND		Atlantic cod with *Mytilus edulis*	Lipid FA	*-33* *-40*	ND	ND	ND	7
CA	Open	ND	Chl-a Seston	3 (au) 0 (su) 16 (au) 2 (su)	Salmon with *Mytilus edulis*	CI Carbohydrate Crude protein	*20* (au) *0* (wi) *59* (au) *0* (wi)				8

Table S2.4 - *Continued*

System						Growth & tissue content of bivalves in integrated aquaculture		Nutrient extraction by bivalves			Ref
Country	System type	Exchange rate	Ambient nutrients (mg L^{-1})		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Parameter	Load (g kg bivalve^{-1} day^{-1})	Extraction efficiency (%)	
NO	Open	0.2-0.5	SPM POC Chl-a	6 (wi) 10 (au) 0.07 (wi) 0.35 (su) $6*10^{-5}$ (wi) 0.007 (su)	Salmon with *Mytilus edulis*	Growth	*-40* (year) *-34* (su) *25* (wi)				9
CA*	Open	ND	TPM POM Chl-a Energy content seston	1.8-4 (su) 0.4-4 (su) 0.003-0.009 (su) 11-589(su)	Salmon with *Mytilus edulis*	Feeding response	*4-35* (su)				10
CN	Open	*0.48*	ND		Sea bass with *Crassostrea gigas*	Growth	*30* (year)				11
AU	Open	*0.034*	Chl-a TPM POM	ND (wi) 0.006 (au) 4 (su) 9 (au) 1 (su) 5 (au)	Salmon with *Mytilus planulatus*	Shell length AFDW-meat CI	*2.5* (year) *7* (year) *14* (year)				12
CA	Open	ND	ND		Salmon with *Mytilus edulis*	Shell length WW – meat CI	*20* (year) *112* (year) *67* (year)				13
HR	Open	ND	ND		Sea bream & Sea bass with *Mytilus galloprovincialis*	Shell length CI	*22* (year) *36* (wi) *-17* (su)				14

Table S2.4 - *Continued*

System						Growth & tissue content of bivalves in integrated aquaculture		Nutrient extraction by bivalves			Ref
Country	System type	Exchange rate	Ambient nutrients (mg L^{-1})		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Parameter	Load (g kg bivalve^{-1} day^{-1})	Extraction efficiency (%)	
IT	Open	ND	TSM TSM_{OM} Chl-a POM	0.0081 0.0012 $1.1*10^{-6}$ $2.4*10^{-4}$	Sea bream & Sea bass with *Mytilus galloprovincialis*	AFDW-meat	*30* (year)				15
GB-SCT	Open#	ND	POM_E POM_L Chl-a_E Chl-a_L	0.0028(wi) 0.0072(sp) 4 (wi) 4.8 (sp) $2.5*10^{-7}$ $4.8*10^{-6}$ $2.5*10^{-7}$ $3.8*10^{-6}$	Salmon with *Mytilus edulis*	Shell length_E Shell length_L AFDW-E AFDW-L	*5* *13* *5* *53*				16
ES	Open	*0.11*	ND		Sea bass & Sea bream with *Mytilus galloprovincialis*	Shell length DW-meat	*0* (sp) *0* (sp)				17
ES	Open	*0.11*	ND		Sea bass & Sea bream with *Ostrea edulis*	Shell length DW-meat	*-36* (sp) *0* (sp)				17

1 Jones et al. (2001); 2 Van Khoi and Fotedar (2012); 3 Hussenot et al. (1998); 4 Jones et al. (2002); 5 Shpigel and Blaylock (1991); 6 Jara-Jara et al. (1997); 7 Both et al. (2011); 8 Taylor et al. (1992); 9 Handå et al. (2012a); 10 MacDonald et al. (2011); 11 Jiang et al. (2012); 12 Cheshuk et al. (2003); 13 Lander et al. (2012); 14 Peharda et al. (2007); 15 Sarà et al. (2009); 16 Stirling and Okumuş (1995); 17 Navarrete-Mier et al. (2010)

Table S2.5
Deposit feeder production (growth and tissue content) and nutrient extraction parameters in integrated aquaculture systems varying in openness.

Country codes: GB, Great Britain; JP, Japan; US, United States of America; CL, Chile; IR, Iran; PT, Portugal; ZA, South Africa; NC, New Caledonia; CN, China; NZ, New Zealand; CA, Canada; TR, Turkey.
Exchange rate refers to water exchange rate and is given in l day^{-1} for closed and semi-open systems, and in m sec^{-1} for open systems.
Food quality is given in percentage of food dry weight for crude protein (CP) and crude fat (CF), in mg l^{-1} for total suspended solids (TSS) and in kJ gram $food^{-1}$ for energy.
Increase in integrated aquaculture indicates, for the parameters given, percentage benefit for the deposit feeder to be cultivated in integrated aquaculture systems, compared to a control group, reference station or monoculture and is calculated as: Increase in integrated aquaculture (%) = (value measured in IMTA – value measured in control)/value measured in control * 100).
Extraction efficiency is the percentage of nutrients removed from the sediment of the deposit feeder compartment.
* Has been changed to *Hediste diversicolor*. ND, not determined; FA, fatty acid; CP, crude protein; CF, crude fat; TSS, total suspended solids; OM, organic matter; TOC, total organic carbon; TN, total nitrogen; TP, total phosphorus. Numbers in *italic* are calculated from values in the corresponding paper. Numbers in the column Ref. relate to the reference list.

System						Growth & tissue content of deposit feeders in integrated aquaculture		Nutrient extraction by deposit feeders				Ref.
Country	System type	Exchange rate	Food quality		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg deposit $feeder^{-1}$ day^{-1})	Extraction efficiency (%)	
GB	Closed	0	ND		Eel with *Nereis diversicolor*	FA	58	ND				1
JP	Closed	0	CP_{waste} $CP_{polychaete\ diet}$	21 49	Flounder with *Perinereis nuntia vallata*	Growth N content	*-55* *-53*	Feces	TN	*0.6-1.5*	31-54	2
US	Closed	0	CP_{waste} $CP_{pol\ diet}$ CF_{waste} $CF_{pol\ diet}$ $Energy_{waste}$ $Energy_{pol\ diet}$	50 39 22 11 4 19	Halibut with *Nereis virens*	Growth Protein content Fat content	*-6* *-8* *-3*	ND				3

Table S2.5 - *Continued*

System						Growth & tissue content of deposit feeders in integrated aquaculture		Nutrient extraction by deposit feeders				Ref.
Country	System type	Exchange rate	Food quality		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg deposit feeder^{-1} day^{-1})	Extraction efficiency (%)	
CL	Closed	0	TOM C N	82 29 4	Yellowtail kingfish with *Abarenicola pusilla*	ND		Sludge	TOM C N	*1741-5935* *623-2123* *87-296*	32-85 91 91	4
IR	Semi-open	*2160-2592*	ND		Sturgeon with *Nereis diversicolor*	ND		Water	TN TP	ND	56 53	5
PT	Semi-open	*4320*	ND		Sole with *Hediste diversicolor*	ND		Sediment	OM	ND	70	6
AU	Semi-open	1500	ND		Tiger shrimp with *Perinereis nuntia* and *Perinereis helleri*	ND		Sediment	OM	ND	50	7
ZA	Semi-open	*4493*	TSS_{waste} $TSS_{seawater}$ CP_{waste}	42 6 34	Silver kob with *Arenicola loveni loveni*	Growth	*69*	Water	TSS	*220*	*94*	8
NC	Semi-open	ND	ND		Shrimp with *Holothuria scabra*	Growth Survival	*-33* *-21*	ND				9
NC	Semi-open	*50*	ND		Shrimp with *Holothuria scabra*	Growth	*-38*	Sediment	OM	ND	*19*	10
CN	Semi-open	ND	TSS_{waste}	22	Scallops with *Apostichopus japonicus*	Growth Survival	*59* *13*	Sediment	OM TOC TN TP	ND *53-20* *2-7* *0.5-1*	*20* *7* *7* *25*	11
CN	Semi-open	*10000*	ND		Scallops with *Stichopus japonicus*	ND		Sediment	OM TOC TN TP	ND	*8* *8* *16* *21*	12
GB	Semi-open	*2880*	ND		Sea bass with *Holothuria forskali*	ND		Sediment	TOC TN	7 1	*10* *11*	13

Table S2.5 - *Continued*

System						Growth & tissue content of deposit feeders in integrated aquaculture		Nutrient extraction by deposit feeders				Ref.
Country	System type	Exchange rate	Food quality		Species (*extractive species in italics*)	Parameter	Increase in integrated aquaculture (%)	Method	Parameter	Load (g kg deposit feeder^{-1} day^{-1})	Extraction efficiency (%)	
NZ	Semi-open	*720*	OM TOC PON	7 1 0.2	Mussels with *Australostichopus mollis*	ND		Sediment	TSS OM TOC TN	*638*	 *0.1* *3* *0*	14
CN	Semi-open	*0.01*	OM_{waste} TOC_{waste} TON_{waste}	11 3 0.3	Flounder with *Perinereis aibuhitensis*	ND		Sediment	OM TOC TN	ND	20-35 40-50 30-40	15
JP	Open	ND	ND		Sea bream with *Apostichopus japonicus*	Growth	*58*	ND				16
JP	Open	ND	ND		Oysters with *Apostichopus japonicus*	Growth	*25*	ND				17
CN	Open	0.1	OM_{farm} OM_{ref} TOC_{farm} TOC_{ref} TN_{farm} TN_{ref}	15 12 4 2 0.5 0.3	Snapper, Cobia and Grouper with *Apostichopus japonicus*	Growth	*100*	ND				18
CA	Open	ND	ND		Sablefish with *Parastichopus californicus*	Growth	*164*	ND				19
TR	Open	ND	OC_{farm} OC_{ref}	4/5 0.1/0.2	Sea bass and sea bream with *Holothuria tubulosa*	Growth	*220*	ND				20

1 García-Alonso et al. (2008); 2 Honda and Kikuchi (2002); 3 Brown et al. (2011); 4 Gómez et al. (2019); 5 Pajand et al. (2017); 6 Marques et al. (2017); 7 Palmer (2010); 8 Yearsley et al. (2011); 9 Bell et al. (2007); 10 Purcell et al. (2006); 11 Ren et al. (2012); 12 Zhou et al. (2006); 13 MacDonald et al. (2013); 14 Slater and Carton (2009); 15 Fang et al. (2017); 16 Yokoyama (2013a); 17 Yokoyama (2013b); 18 Yu et al. (2014a); 19 Hannah et al. (2013); 20 Tolon et al. (2017)



Chapter 3

Ulva spp. performance and biomitigation potential under high nutrient concentrations in recirculating IMTA systems

Marit A.J. Nederlof, Amir Neori, Marc C.J. Verdegem, Aad C. Smaal, Henrice M. Jansen

Submitted to Journal of Applied Phycology

Abstract

The growth, tissue content and nutrient removal rates of *Ulva* spp. when exposed to moderate to high nitrogen (0.5-5 mM) and phosphorus (0.01-0.9 mM) concentrations, were examined in simulated recirculating IMTA (Integrated Multi-Trophic Aquaculture) systems of fish and seaweed. It was hypothesized that fish waste effluents might lead to, for seaweed, unfavourable nutrient stoichiometry, and/or toxic conditions resulting in reduced *Ulva* spp. performance. Results of these experiments showed that: (I) the unfavourable N:P stoichiometry (N:P<< Atkinson atomic ratio of 30:1) did not restrict *Ulva* spp. growth nor tissue content, indicating that nutrients were supplied in concentrations exceeding the minimum requirements; (II) a high orthophosphate concentration (0.9 mM) was toxic to *Ulva* spp., whereas (III) high nitrate concentrations (5 mM) did not inhibit phosphorus uptake; (IV) *Ulva*'s growth was not enhanced when nitrate was exchanged for similarly high ammonium concentrations. However, tissue nitrogen content was 1.4 times higher when exposed to ammonium compared with nitrate, suggesting that the former N-form was stored faster in the seaweed's tissue and that other factors limited growth with the high concentrations of ammonium. This study also highlights the importance of relatively long acclimatization periods (one week) when maintenance uptake (Vm) is evaluated, as surge uptake (Vs) may result in considerably different, and more variable, rates. These results contribute to a better understanding of the application of *Ulva* spp. as the extractive component in closed IMTA systems, and thus to the development of sustainable and circular production techniques.

1 Introduction

Green seaweeds belonging to the genus *Ulva* (Chlorophyta) are well known for their high nutrient uptake capacity and high biomass productivity (Bruhn et al. 2011, Kang et al. 2011, Luo et al. 2012). Furthermore, they can be cultivated in artificial media, as well as in wastewater effluents (Guist Jr & Humm 1976, Cohen & Neori 1991, Luo et al. 2012, Kumari et al. 2013). These characteristics make them ideal model species for small scale laboratory experiments on eco-physiological processes, like nutrient uptake kinetics (Lubsch & Timmermans 2018), but also for land-based integrated aquaculture systems, for biomitigation of the inorganic waste fraction resulting from fish cultures (Krom et al. 1995, Shpigel & Neori 1996, Neori et al. 2003, Schuenhoff et al. 2003, Msuya et al. 2006). Fish excrete inorganic phosphorus as orthophosphate (PO_4) and inorganic nitrogen in the form of ammonia (NH_3-N + NH_4-N, combined referred to as total ammonia nitrogen, TAN), which by bacterial nitrification processes convert to nitrate (NO_3). In recirculation aquaculture systems (RAS), water renewal is minimized and non-retained nutrients may accumulate, resulting in relatively high nutrient concentrations in the culture water. The bacterial biofilter in RAS transforms practically all TAN into nitrate, to create conditions that are less toxic for fish. In near-zero water exchange bacterially-biofiltered RAS systems, nitrate levels of up to 6 mM and orthophosphate levels of up to 1 mM can be reached in seawater systems (Tal et al. 2009, van Bussel et al. 2013, Yogev et al. 2017), but may even reach levels as high as 20 mM nitrate and 3 mM orthophosphate for marine zero-exchange RAS systems (Neori et al. 2007). Integrated recirculating fish-seaweed systems can be designed in two ways; either with or without bacterial biofilters, both characterised by high nutrient concentrations but varying in the form of nitrogen supplied to the seaweeds. Nutrient dynamics in integrated aquaculture based on closed systems (i.e. RAS) are also different compared to open-water IMTA systems, where fish wastes are quickly mixed with ambient nutrients (Jansen et al. 2018). Several studies using seaweed as extractive species in integrated aquaculture systems have focussed on biomitigation under low to moderate nutrient conditions (Krom et al. 1995, Al-Hafedh et al. 2012, Kang et al. 2021). These conditions are not always representative of the high nutrient concentrations in RAS effluents.

Molar N:P ratios of inorganic fish waste range between 5–35, depending on different factors like fish species and diet type (Wang et al. 2012, Hadley et al. 2015). For seaweeds in general the Atkinson atomic ratio (N:P 30:1) is considered optimal (Atkinson & Smith 1983). However, tissue ratios between 16:1 and 24:1 (N:P) have been reported to sustain maximum growth in *Ulva* spp. (Björnsäter & Wheeler 1990, Tremblay-Gratton et al. 2018). Indeed, suboptimal N:P ratios have been shown to reduce the growth rates of seaweed (Björnsäter & Wheeler 1990), indicating that one of the nutrients was limiting. The N:P ratios in fish wastes can be well below the Atkinson ratio, considering that phosphorus may accumulate in an integrated RAS system and suggesting that phosphorus uptake, and thereby growth of seaweeds, might be limited under such conditions. However, at the same

time, the high nutrient concentrations in RAS effluents may be well above minimum values required for maximum growth (0.8 µM P and 6.7 µM N; Pedersen & Borum 1997, Pedersen et al. 2010), suggesting that N:P ratios in the RAS effluent may not limit the uptake of either nutrient and will thus not result in reduced growth.

Apart from potentially limited growth as a result of unbalanced stoichiometry in fish effluents, high nutrient concentrations may create toxic conditions for seaweeds. As highlighted above, particularly phosphorus may accumulate in integrated RAS systems. Some studies suggest that a high (0.8-3.2 mM) orthophosphate concentration may lead to reduced seaweed growth (Friedlander & Ben-Amotz 1991, Navarro-Angulo & Robledo 1999), but it is unclear whether that is caused by the high orthophosphate concentration itself or by the low N:P ratios. As far as we know, it is largely unknown at what concentrations phosphorus becomes toxic and inhibits growth in *Ulva* spp. There are, however, indications that exposure of *Ulva lactuca* to high nitrate concentrations may inhibit phosphorus uptake and therefore growth (Lundberg et al. 1989). The opposite has been reported for *Fucus vesiculosus* (Perini & Bracken 2014). This highlights the limited understanding of the interactions between nitrate and phosphorus uptake kinetics in seaweed. As nitrate concentrations are high in RAS effluents, these processes are particularly relevant for such systems and may result in an even higher phosphorus accumulation in the system, but also reduced seaweed performance.

Fish excreta consist among others of TAN, which by the bacterial biofilters in RAS systems is transformed into nitrate (Krom et al. 1995, Neori et al. 2007). Although *Ulva* spp. can assimilate both nitrogen sources, uptake of nitrate is generally much slower compared to TAN (Neori 1996, Hadley et al. 2015), which is also reflected in a lower growth (Ale et al. 2011). The differential nutrient uptake and growth response of *Ulva* spp. to these nitrogen sources are related to differences in uptake and assimilation pathways, which affects the energy requirements (Shahar et al. 2020): uptake and assimilation of nitrate uses much metabolic energy (active) while assimilation of TAN is passive and requires less energy (Taylor et al. 2006). This suggests that seaweed growth in bacterial driven RAS IMTA systems might be sub-optimal since nitrogen is mostly available as nitrate-N. Replacing the bacterial biofilter with a seaweed filter, may result in good water quality conditions for fish (low TAN concentrations) and at the same time benefit the seaweed production, potentially resulting in higher growth and better biomitigation potential. However, care should be taken as TAN is not only more toxic to fish but also to seaweed in comparison to nitrate (Harrison & Hurd 2001, Moustafa et al. 2014).

In this study, the growth and nutrient assimilation were measured in *Ulva* spp., when exposed to relatively high and moderate nitrogen and phosphorus concentrations. Such conditions have been insufficiently addressed in the literature (Lundberg et al. 1989, Demetropoulos & Langdon 2004, Tremblay-Gratton et al. 2018), and would give a better understanding of biomitigation potential and seaweed performance under high nutrient concentrations. The following four

hypotheses were examined: (I) *Ulva* spp. performance is not influenced by N:P stoichiometry in fish waste effluents under high nutrient concentrations, (II) High orthophosphate concentrations typical in RAS effluents are not toxic to *Ulva* spp*.,* (III) High nitrate concentrations typical in RAS effluents can limit the phosphorus uptake, (IV) High TAN concentrations improve the performance of *Ulva* spp. in comparison to comparably high nitrate concentrations.

2 Materials & Methods

2.1 Experimental design

This study consisted of two experiments evaluating maintenance (V_m; i.e. internal nutrient pools are filled) and surge (V_s; i.e. filling of internal nutrient pools) uptake kinetics (Lubsch & Timmermans 2018) in *Ulva* spp. exposed to different nutrient regimes. Maintenance uptake (V_m) kinetics were assessed in an experiment where separate batches of *Ulva* spp. were continuously exposed to six different nutrient regimes for a period of two weeks. These nutrient regimes reflected conditions as can be expected from fish effluents in a RAS facility, and were chosen in such a way that all four hypotheses could be tested. In an additional experiment, hypothesis III was also tested for surge uptake (V_s). In this experiment phosphorus uptake was studied when exposing starved *Ulva* spp*.* to moderate and high nitrate concentrations for five hours.

2.2 Holding conditions

The experiments were conducted in a temperature- and light- controlled room at the Aquatic Research Facility of the Wageningen University (ARF - Carus, Wageningen, The Netherlands). *Ulva* spp. was obtained from the greenhouse facility of the Wageningen University and Research Centre (Nergena, Wageningen, The Netherlands), where it has been cultivated since 2012. It was assumed that the culture stock consisted of *Ulva lactuca*, but recent analysis from the location where initial samples were collected indicates the presence of additional *Ulva* species (Fort et al. 2021). We therefore refer to '*Ulva* spp.' for the species investigated in this study. Prior to the experiments, seaweeds were maintained in a stock tank (1 m^3) filled with artificial seawater (Reef Crystals™, Aquarium Systems, Inc., Mentor, Ohio, USA), to which once a week plant fertilizer (Pokon© Groene Planten Voeding, Veenendaal, The Netherlands) was added. Light tubes (T5 TL 24 watt – AquaBlue Special - ATI) were placed above the tank (~400 µmol m^{-2} sec^{-1}), temperature was maintained at 17.5 ± 0.5 °C and aeration was added at the bottom of the tank, to maintain vertical water movement (Msuya & Neori 2008).

2.3 Maintenance uptake (Vm) and growth experiments

Six treatments were formulated varying in nutrient concentrations between 0.7-5.0 mM nitrogen (either nitrate or ammonium) and 0.013-0.9 mM orthophosphate, using dilution steps with a factor 10, and resulting in stoichiometries that were either high (N:P 60-70) or low (N:P 9-10) (Table 3.1). Four experimental systems (header tank plus three associated seaweed cultivation tanks) were available and

treatments were therefore divided over two consecutive runs, using new seaweed stocks in each run. The first run included all nitrate based treatments, while the second run included all ammonium based treatments. One nitrate based treatment (A) was included in both runs, to elucidate a potential effect of 'run'. Treatment concentrations were formulated by daily addition of a mix of artificial seawater (29.7 ± 1.8 ‰, Reef Crystals™, Aquarium Systems, Inc., Mentor, Ohio, USA), wastewater from a RAS system with sea bass (2 l) and stock solutions, to header tanks with a total volume of 110 l. The stock solutions were prepared with NaH_2PO_4 and $NaNO_3$ for the nitrate treatments and NaH_2PO_4 and NH_4Cl for the ammonium treatments. Water samples were collected daily in the header tanks. Water samples were directly stored in the freezer (-20 °C) until nutrient analyses.

Seaweed tanks consisted of 15 l white round tanks (0.07 m^2 surface area) with an overflow PVC pipe in the centre. Each overflow pipe was covered with an additional perforated PVC pipe topped with a perforated cap, preventing seaweed pieces from floating out of the tank. Aeration was added via the bottom of each tank, approximately 5 cm from the centre, creating sufficient turbulence to suspend the seaweed pieces and moving them through the water column. A slow, internal recirculation flow through a UV-light removed potential microalgal and bacterial contaminants. A double perforated bottom allowed the water to pass the UV light, without damaging the seaweed. Light tubes (T5 TL 24 watt – AquaBlue Special - ATI) were placed above the seaweed tanks, resulting in irradiance of 396 ± 46 µmol m^{-2} sec^{-1} (measured by LI-COR LI193R PAR meter, just underneath the water surface). A 12 hour light, 12 hour dark light regime was maintained. All seaweed tanks were connected to a flow-through set-up with a continuous supply of nutrients. Nutrients from one header tank were pumped to three replicate seaweed tanks with a flow rate of 1.6 ± 0.09 l h^{-1}.

Table 3.1
Treatment overview including dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentrations and N:P ratio (molar) in the culture media. Planned values refer to the targeted concentrations prior to the experiment and realized values are actual daily average concentrations measured in the header tanks. Values are given as mean ± SD.

		Planned			Realized			
Treatment	Run	DIN	DIP (mM)	N:P	NO_3/TAN (mM)	DIN (mM)	DIP (mM)	N:P
A1	1	5.0 NO_3	0.160	30	4.9 ± 0.9 NO_3	5.0 ± 0.9	0.13 ± 0.02	38 ± 3
B	1	0.5 NO_3	0.016	30	0.8 ± 0.1 NO_3	0.8 ± 0.1	0.013 ± 0.003	62 ± 10
C	1	0.5 NO_3	0.080	6	0.7 ± 0.1 NO_3	0.7 ± 0.1	0.07 ± 0.01	10 ± 1
D	1	5.0 NO_3	0.800	6	5.5 ± 0.4 NO_3	5.5 ± 0.4	0.86 ± 0.17	7 ± 1
A2	2	5.0 NO_3	0.160	30	4.9 ± 0.6 NO_3	4.9 ± 0.6	0.14 ± 0.01	34 ± 4
E	2	0.5 TAN	0.016	30	0.6 ± 0.1 TAN	1.0 ± 0.2	0.015 ± 0.003	68 ± 13
F	2	0.5 TAN	0.080	6	0.5 ± 0.1 TAN	0.8 ± 0.1	0.09 ± 0.01	9 ± 1

Each experimental run consisted of an acclimation week and an experimental week. This acclimatization period stabilized the internal nutrient pools (Lubsch & Timmermans 2018). Nutrient removal rates measured in the second week were thus assumed to reflect metabolic controlled uptake rates (V_m). At the start of the acclimation period each seaweed tank was stocked with 60.3 ± 0.4 gram fresh *Ulva* spp. material (spin dried, using a lettuce hand-centrifuge). Start samples were collected to determine initial tissue content (n = 3 samples for each run). After the acclimation week, all seaweed material was harvested, spin dried and weighted to determine fresh weight. Then, 30.5 ± 0.3 gram of the harvested *Ulva* spp. material from each tanks was returned to the tank, while the remaining material was collected for analysis. At the end of the second week, all seaweed material was collected. Seaweed samples were rinsed with deionized water, spin dried and thereafter stored in the freezer (-20 °C) until analyses. Specific growth rate (SGR, % day^{-1}) was calculated based on dry weight (DW) for week 1 and week 2 separately, using the following formula:

$$SGR = ((\ln(W_f) - \ln(W_i))/T) \times 100$$

where W_f is the final biomass in gram dry weight, W_i the initial biomass in gram dry weight and T the number of experimental days.

All seaweed samples were analysed for dry matter, ash, N and P tissue content (see *section 2.5 biochemical analyses*). Nitrogen and phosphorus removal rates were calculated for week 1 and week 2 separately, using the following equation after Kim et al. (2007):

$$\text{Removal rate } (\mu mol\ gram^{-1}\ DW\ day^{-1}) = ((W_f * TC_f) - (W_i * TC_i))/DW/T$$

where W_f is the final biomass in gram dry weight, W_i the initial biomass in gram dry weight, TC_f is the final N or P tissue content (in µmol), TC_i is the initial N or P tissue content (in µmol), DW is the mean biomass in gram dry weight of either week 1 or week 2 and T the number of experimental days.

2.4 Surge uptake (Vs) experiment

For the measurement of the surge uptake related to hypothesis III, *Ulva* spp. was exposed to either high (5 mM) or moderate (0.5 mM) nitrate concentrations, both with a fixed orthophosphate concentration (0.026 ± 0.005 mM). The solutions were prepared by artificial seawater (29.7 ± 1.8 ‰, Reef Crystals™, Aquarium Systems, Inc., Mentor, Ohio, USA) and NaH_2PO_4 and $NaNO_3$. Surge uptake measurements were based on the method described by Hurd and Dring (1990). Prior to the experiment, *Ulva* spp. were nutrient-starved for 3 days. At the start of the experiment, pieces of *Ulva* spp. of comparable weights (1.43 ± 0.03 gram fresh weight) were placed in 500 ml glass jars, filled with 400 ml of one of the above-described solutions, in four replicates per treatment. For each treatment, two control jars were added, containing nutrient solution without seaweed. The jars were placed in a water bath shaker, creating a constant water movement for mixing and reducing the diffusion boundary layer between seaweed and the medium. Light tubes (T5 TL 24 watt – AquaBlue Special - ATI) were placed above

the seaweed tanks, resulting in irradiance of 826 ± 14 µmol m^{-2} sec^{-1} (measured by LI-COR LI193R PAR meter, just underneath the water surface). Water samples (in duplicate; 2 x 1 ml) were taken at t = 0, 10, 20, 30 and 300 minutes, and instantly analysed as described below for nitrate and orthophosphate concentrations. Phosphorus uptake rate was calculated as:

Uptake rate (µmol $gram^{-1}$ FW h^{-1}) = ((WC_f * V) – (WC_i * V))/FW/T

where WC_f is the nutrient concentration in the water (in µmol) at Tx, WC_i the initial nutrient concentration in the water (in µmol), V is the volume in litres, FW is the fresh weight of the *Ulva* spp. (in gram) and T is the time in hours.

2.5 Biochemical analyses

Water samples of the growth experiment (maintenance uptake) were analysed for TAN, nitrate-N and orthophosphate-P using an auto-analyzer (SANplusSYSTEM, Skalar). Water samples of the surge uptake experiment were analysed for nitrate-N and orthophosphate-P using a SmartChem 200 Discrete Analyzer. Seaweed samples were freeze dried, and then ground with a centrifugal grinding mill (Retsch/Brinkmann ZM 100/w 1 mm sieve, Verder NV, The Netherlands). Dry matter and ash were determined according to ISO-6496 (1983) and ISO-5984 (1978), respectively. Phosphorus content in the seaweed was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007). Carbon and nitrogen content of the seaweed were analysed by combusting the samples with an element analyzer (Flash 2000, Therm Fisher) at 1020 °C in the presence of oxygen, converting carbon and nitrogen to CO_2 and NO_x respectively. Thereafter, NO_x was reduced to N_2 in a reduction column.

2.6 Statistical analyses

Statistical analyses were performed in R studio 3.4.0. Prior to the analysis, residuals of the data were checked for homogeneity of variance and normality using respectively the Shapiro-Wilk and Levene test. A two-way analyses of variance (2-way ANOVA) was used to check for a potential interaction effect between N:P ratio and N-source (treatments B, C, E, F). Since no interaction effect (p > 0.05) was observed for uptake and growth performance parameters, comparative treatments related to hypotheses I and IV were tested independently. For each hypothesis, differences in uptake and performance of *Ulva* (i.e. growth, tissue content, V_m nutrient removal rates) in the corresponding treatments (Table 3.2), were tested by a one-way analyses of variance (1-way ANOVA). In addition to hypothesis III, a 1-way ANOVA was used to detect potential differences in phosphorus surge uptake rates (Vs) by *Ulva* spp. exposed to either a high or moderate nitrate concentration. All statistics for the V_m experiments were based on data collected in the second week only. To verify whether acclimation is indeed relevant, Paired-Samples T-tests were used to test the difference in growth and tissue content between week 1 and week 2, for each treatment separately.

Table 3.2
Overview of hypotheses and associated treatment comparisons.

	Hypothesis	Treatment comparison
I	*Ulva* spp. performance is not influenced by stoichiometry in fish waste effluents under high nutrient concentrations	Compare high N:P ratios with low N:P ratios (B versus C; E versus F)
II	Orthophosphate concentrations in RAS effluents are not toxic for *Ulva* spp.	Compare between different orthophosphate concentrations (low=B, moderate=C, high=D)
III	High nitrate concentrations in RAS effluents will limit the phosphorus uptake	Compare between high nitrate (A) and moderate nitrate (B) concentrations
IV	High TAN concentrations will result in better performance of *Ulva* spp. in comparison to comparably high nitrate concentrations	Compare between nitrate and ammonium conditions (B versus E; C versus F)

3 Results

Actual realized nutrient concentrations were not always in range with the planned formulated culture media. Especially treatment B and E showed variations and the measured DIN concentrations were almost double the planned ones, resulting in deviations to the N:P ratios (Table 3.1). Culture media formulated based on NH_4Cl resulted not only in TAN-nitrogen but also additional NOx-nitrogen concentrations were observed, leading to slightly higher DIN concentrations than expected. The origin of these additional NOx concentrations remains unknown, but seems to fall within the variation observed for the other treatments and may relate to initial NOx concentrations present in the artificial seawater. This was however not analysed. Despite these variations all hypothesises could still be evaluated.

3.1 Hypothesis I: *Ulva* spp. performance under contrasting N:P ratios

The two contrasting N:P ratios (9-10 vs 60-70) did not impact growth (Fig. 3.1) nor tissue content of the seaweed (Fig. 3.2) (1-way ANOVA; $p > 0.05$ B vs C; $p > 0.05$ E vs F) during the maintenance uptake experiment. As a result nutrient removal rates were comparable for the high and low N:P treatments (Fig. 3.3) (1-way ANOVA; $p > 0.05$ B vs C; $p > 0.05$ E vs F). Tissue N:P ratios varied between 27-28 for the nitrate based treatments (B and C) and 39-42 for the ammonium based treatments (E and F), irrespective of the N:P ratio provided in the culture medium.

3.2 Hypothesis II: Toxicity of high orthophosphate concentrations

Ulva spp. cultivated under the highest orthophosphate concentration (treatment D; 0.9 mM P) showed a different, unhealthy, tissue structure compared to the other treatments; tissue was hard, easy to break and felt brittle, suggesting degradation (visual observation). Most of the material of this treatment was lost during the sampling procedure (spin drying), and we were therefore unable to derive valid measurements. Treatment D was therefore excluded from the statistical analyses (Fig. 3.1, 3.2 and 3.3). As described for hypothesis I (*section*

3.1), no significant differences were observed for the *Ulva* spp. in the remaining low (treatment B) and medium (treatment C) orthophosphate concentrations.

3.3 Hypothesis III: Inhibiting effects of high nitrate concentration

Phosphorus removal during maintenance (Vm) was approximately 60% higher (1-way ANOVA; $p < 0.05$) for *Ulva* spp. cultivated under high nitrate (treatment A) compared to moderate nitrate concentrations (treatment B) (Fig. 3.3). This difference was not the result of growth, since SGR did not differ between the treatments (Fig. 3.1; 1-way ANOVA; $p > 0.05$). More likely is that the difference in phosphorus removal was driven by differences in tissue content, as phosphorus content in *Ulva* spp. of the high nitrate treatment was higher by approximately 25% (1-way ANOVA; $p < 0.0001$) compared to the moderate nitrate treatment (Fig. 3.2). This also resulted in a nearly 25% lower tissue N:P ratio for *Ulva* spp. in the high nitrate treatment, compared to *Ulva* spp. cultivated in the moderate nitrate treatment (Fig. 3.2; 1-way ANOVA; $p = 0.0002$). No differences were observed in tissue nitrogen content, tissue C:N ratio and nitrogen removal rates (Fig. 3.2 & 3.3; 1-way ANOVA; $p > 0.05$). Similarly, surge uptake (Vs) of phosphorus did not vary between the moderate (0.60 ± 0.09 µmol gram^{-1} FW day^{-1}) and high (0.51 ± 0.04 µmol gram^{-1} FW day^{-1}) nitrate conditions (Fig. 3.4; 1-way ANOVA, $p > 0.05$).

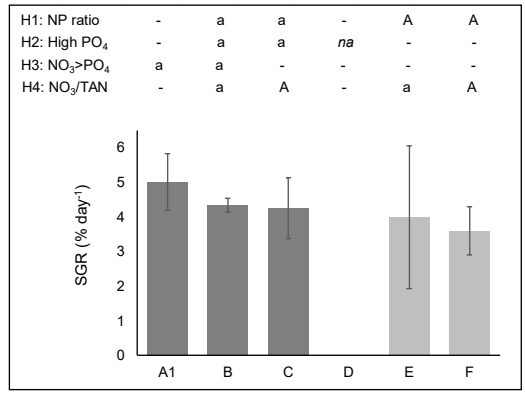

Figure 3.1
Growth performance (Specific Growth Rate, % day^{-1}) of *Ulva* spp cultivated in the six experimental treatments. For treatment description see Table 3.1, and for a description of the hypotheses tested see Table 3.2. Dark grey bars represent nitrate based treatments and light grey bars represent ammonium treatments. Bars represent mean values (n = 3 tanks treatment^{-1}), and error bars represent standard deviations. Within a hypothesis, treatments lacking a common letter differ significantly ($p < 0.05$); - indicates that that treatment is not included to evaluate the hypotheses. If within a hypothesis treatments are compared separately (i.e. H1 & H4), the compared treatments are indicated with either lower case letters or capital case letters.

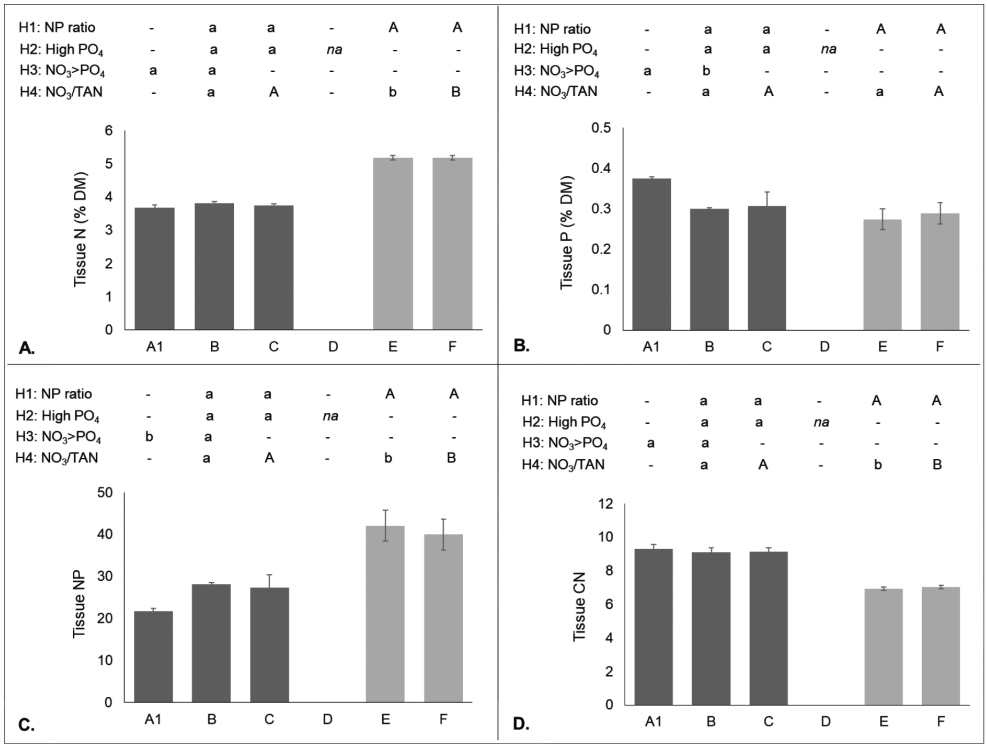

Figure 3.2
Tissue content (tissue nitrogen and phosphorus, % of dry matter and tissue N:P and C:N molar ratio) of *Ulva* spp cultivated in the six experimental treatments. Treatment descriptions are given in Table 3.1, and description of the hypotheses tested in Table 3.2. Dark grey bars represent nitrate based treatments and light grey bars represent ammonium treatments. Bars represent mean values (n = 3 tanks treatment^{-1}), and error bars represent standard deviations. Within a hypothesis, treatments lacking a common letter differ significantly ($p < 0.05$); - indicates that that treatment is not included to evaluate the hypotheses. If within a hypothesis treatments are compared separately (i.e. H1 & H4), the compared treatments are indicated with either lower case letters or capital case letters.

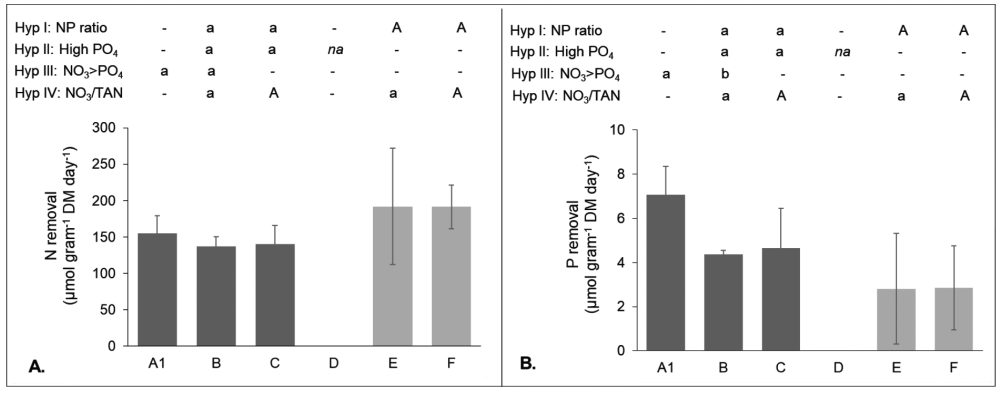

Figure 3.3
Nitrogen (A) and Phosphorus (B) removal rate (µmol gram^{-1} dry matter day^{-1}) of *Ulva* spp cultivated in the six experimental treatments. Treatment descriptions are given in Table 3.1, and description of the hypotheses tested in Table 3.2. Dark grey bars represent nitrate based treatments and light grey bars represent ammonium treatments. Bars represent mean values (n = 3 tanks treatment^{-1}), and error bars represent standard deviations. Within a hypothesis, treatments lacking a common letter differ significantly ($p < 0.05$); - indicates that that treatment is not included to evaluate the hypotheses. If within a hypothesis treatments are compared separately (i.e. H1 & H4), the compared treatments are indicated with either lower case letters or capital case letters.

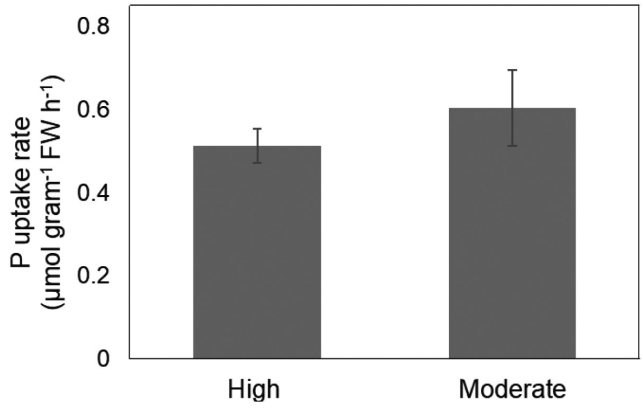

Figure 3.4
Phosphorus surge uptake (µmol gram^{-1} fresh weight hour^{-1}) for *Ulva* spp exposed to either high (5 mM) or moderate (0.5 mM) nitrate concentration. Bars represent mean values (n = 4 tanks treatment^{-1}) and error bars standard deviations. Treatments did not differ significantly ($p < 0.05$).

3.4 Hypothesis IV: Effect of N-source (nitrate or ammonium)

To elucidate any potential effect of 'run', treatment A (high nitrate concentration) was included in both runs (Table 3.1; A1 and A2). As no significant difference in growth was observed (data not shown; 1-way ANOVA; $p = 0.07598$), it seems valid to compare nitrate treatments tested in run 1 with ammonium treatments tested in run 2. While growth and tissue phosphorus content were not affected by the type of N-source (Fig. 3.1 & 3.2; 1-way ANOVA; $p > 0.05$ B vs E; $p > 0.05$ C vs F), tissue nitrogen content was (Fig. 3.2) higher for *Ulva* spp. in the ammonium treatments (5.2% DM) compared to the nitrate treatments (3.8% DM) (1-way ANOVA; $p < 0.0001$ B vs E; $p < 0.0001$ C vs F). This was also reflected in a significant higher tissue N:P ratio, but lower tissue C:N ratio for *Ulva* spp. provided with ammonium-N (Fig. 3.2). Interestingly, nitrogen removal did not differ between nitrate and ammonium treatments (Fig. 3.3; 1-way ANOVA; $p > 0.05$). Numerically, highest nitrogen removal rates were obtained in the ammonium based treatments (Fig. 3.3).

3.5 The relevance of acclimatization

With the exception of treatment B, a significant increase in tissue nitrogen content was observed between the first and second weeks in all treatments, while tissue phosphorus content remained similar over time (Appendix Table S3.1; Paired-Samples T-tests; $p > 0.05$). However, the increased nitrogen content did not result in a difference in tissue N:P ratio between the two weeks. The C:N ratio decreased significantly in the second week only for the *Ulva* spp. that was cultivated in treatment A1 and treatment F (Appendix Table S3.1; Paired-Samples T-test; $p < 0.01$). Interestingly, growth seemed to increase over time when nitrogen was provided in the form of ammonium (treatment E & F), while growth decreased in most cases when nitrate was provided (treatment B & C) (Appendix Table S3.1). Nevertheless, a significant time effect for growth was only observed for treatment C (Paired-Samples T-test; $p < 0.05$).

4 Discussion

Our data suggests that *Ulva* spp. growth is not influenced by (unfavourable) stoichiometry under high nutrient concentrations, and high nitrate concentrations do not limit phosphorus uptake. This is promising for closed IMTA systems, where marine fish in RAS are integrated with seaweed. Nevertheless, our data also suggests that high nutrient concentrations in (simulated) fish waste effluents may, in specific cases, lead to reduced *Ulva* spp. performance. Care should therefore be taken to avoid such conditions in the design of integrated seaweed-fish aquaculture systems.

4.1 Limiting nutrients

Under nutrient limiting conditions, the ratio between macro-elements (N:P) regulates growth and nutrient uptake in seaweeds (Björnsäter & Wheeler 1990, Fan et al. 2014, Perini & Bracken 2014), highlighting the importance of studying nutrient interactions, rather than a single nutrient at a time. Under the high

nutrient concentrations in the current study, nutrient removal and growth rates were not different for the two contrasting N:P ratios (9-10 versus 60-70; B vs E; C vs F). This suggests that neither of the nutrients was limiting at any time. Both nitrogen and phosphorus tissue content were above the critical tissue values required to sustain maximum growth reported for *Ulva* spp. (0.20% P of DW and 2.17% N of DW; Pedersen & Borum 1997, Pedersen et al. 2010), supporting non-limiting concentrations for both nitrogen and phosphorus in the current study. Steffensen (1976) also reported for *U. lactuca* that maximum growth can be achieved under a wide variety of N:P ratios in the medium (N:P ratios of 1:48 – 1:2), demonstrating that variations from the Atkinson ratio do not always result in reduced growth, but when absolute values are above the saturation threshold other factors are limiting seaweed growth. Phosphorus tissue content measured in the current study was on the lower end of the in literature reported highest phosphorus tissue content for *Ulva* spp. (0.4–1.5% P of DW; Runcie et al. 2004, Pedersen et al. 2010, Lubsch & Timmermans 2018). Tremblay-Gratton et al. (2018) also reported relatively low tissue N and P content under moderate to high nutrient conditions and they suggested that low tissue reserves might have been the result of a deficiency of trace elements in the media. Trace elements are required to stimulate the phosphate transport system of algae (Lobban & Harrison 1994). In the current study seaweed was continuously supplied with new culture media, consisting of Artificial Sea Water (ASW), water from the sea bass RAS facility, supplemented with nitrogen (either as nitrate or as ammonium) and orthophosphate. Both ASW and RAS water contains trace elements, but their absolute concentrations and potential limiting effects were not analysed. It can therefore not be ruled out that trace elements were limiting phosphorus uptake in our study.

The results of the treatments of hypotheses I indicate that nutrients are supplied in concentrations exceeding the minimum requirements for growth and unfavourable stoichiometry in fish wastes (N:P << Atkinson ratio) is not limiting seaweed growth. Whether these high nutrient concentrations might have an overall negative effect on seaweed performance is discussed below.

4.2 Toxicity of high orthophosphate concentrations

The highest orthophosphate concentration (0.9 mM; treatment D) in this study resulted in sub-optimal conditions, which led the *Ulva* to degenerate. Orthophosphate was added to the culture medium in the form of NaH_2PO_4, a natural salt. Salinity did not vary between treatments and could thus not explain such results. Other studies reported inhibited growth for *Gracilaria cornea* (824 µM P; Navarro-Angulo & Robledo 1999), *Gracilaria conferta* (3.2 mM P; Friedlander & Ben-Amotz 1991), *Palmaria mollis* (83.3 µM P; Demetropoulos & Langdon 2004) and *Porphyra columbina* (120 µM P; Frazer & Brown 1995). It remains unclear whether inhibited growth in these studies is caused by high orthophosphate concentrations or low N:P ratios. To the best of our knowledge it is unknown at what concentration phosphorus becomes toxic for *Ulva* spp. However, Tremblay-Gratton et al. (2018) showed high growth of *U. lactuca* at orthophosphate

concentrations up to 291 µM, which is approximately 2.5 times higher than the moderate orthophosphate concentration used in the current study. The exact levels of phosphorus toxicity for *Ulva* spp. therefore need to be further elucidated but will likely fall within the range of 0.3-0.9 mM.

4.3 Inhibiting effects of high nitrate concentration

The maintenance (V_m) uptake of phosphorus was 1.6 times higher under high nitrate concentrations in comparison to moderate nitrate concentrations, and surge uptake (Vs) was similar for both nitrate concentrations. Although growth did not differ between the treatments, the higher phosphorus removal rate and tissue content in the high nitrate treatment was associated with higher orthophosphate concentrations in the culture media. Despite of the lower orthophosphate concentration supplied in the moderate nitrate treatment, saturating orthophosphate concentrations (0.8 µM; Pedersen et al. 2010, Lubsch & Timmermans 2018) were assumed for both treatments.

Unlike in the studies of Lundberg et al. (1989) and Kumari et al. (2013) the present results show no inhibiting effects of high nitrate levels on phosphorus uptake by *Ulva* spp. Similar results were reported by Lubsch & Timmermans (2018) who studied phosphorus uptake kinetics of *U. lactuca* under saturating nitrate concentrations (5 mM NO_3). The higher than expected DIN concentration, and subsequent high N:P ratio, in treatment B, is not expected to have influenced the results as nitrogen concentrations, as well as N:P ratios, were still contrasting. It remains unclear why in some studies high nitrate level seem to have an inhibiting effect on phosphorus uptake, while in other studies this is not observed.

4.4 Effect of N-source (nitrate or ammonium) under high nitrogen concentrations

Seaweed provided with either ammonium or nitrate as a nitrogen source grew in similar rates. This is opposite to Ale et al. (2011), who showed a favourable growth response to ammonium when exposed to concentrations of 0.05 mM nitrogen. As in these experiments culture medium was not replaced it is likely that these results resemble surge uptake (Vs) rather than maintenance (Vm). In that respect they seem opposite to the results obtained during our acclimatization week, which could be regarded as Vs, and where lower growth was observed for the ammonium based treatments. Both Neori (1996) and Shahar et al. (2020) found better growth of *U. lactuca* with ammonium than with nitrate for non-starved *Ulva*, representing Vm, and attributed this difference to the different uptake and assimilation pathways that are involved for the two N-sources. Interestingly, even though growth rates were comparable between nitrate and ammonium treatments in our study, higher tissue nitrogen contents were achieved in the ammonium based treatments, suggesting an accumulation of nitrogen which is not used for growth. Due to the high tissue nitrogen content, 1.4 times higher nitrogen removal rates are estimated for the ammonium based treatments.

It is largely unknown what levels of nitrogen are toxic to *Ulva* spp. Waite & Michell (1972) suggest that TAN is toxic to *U. lactuca* at concentrations >65 uM, whereas

other studies (e.g. Fujita 1985, Neori et al. 1991, Ji et al. 2019, Shahar et al. 2020) applied higher concentrations and did not report reduced growth or degenerating seaweed. It seems however unlikely that the high nitrogen concentrations (ammonium nor nitrate) in our study were toxic, since no mortality or debilitation was observed, as seen for the *Ulva* cultured under the high orthophosphate concentration.

4.5 General patterns on the biomitigation potential and seaweed performance in recirculating IMTA systems

One of the aims of integrated cultures is to remove excess nutrients from the water. For intensive fish cultivation systems, such as RAS, this is not only important for the discharged waste water but also to keep good water quality that can be re-used in the fish units. Nutrient removal rates observed in the current study were in line with or lower compared to other studies on different *Ulva* species (Table 3.3). This shows the general pattern observed in literature of highly variable nutrient uptake/removal rates. Besides differences in species, or even strains (Jansen, unpublished data), a potential explanation for the variation might be the method used. Studies either define nutrient removal as a function of biomass increase and nutrient tissue content, while others determine uptake rates based on depletion of nutrients in the medium. Results from both methods may vary, and it was shown that the nutrient depletion method results in a 2–4.5 times higher nutrient uptake (Tremblay-Gratton et al. 2018). The nutritional state of the seaweeds may also play a role in the observed variation in literature, since nutrient uptake by seaweeds is among others a function of their internal nutrient storage (Lobban & Harrison 1994, Hadley et al. 2015). Our study specifically addressed nutrient uptake for maintenance (Vm), but it is not always clear from other studies whether Vm or Vs (surge uptake) is addressed. This may change results considerably as nutrient uptake during surge is much higher (Neori et al. 2003).

Besides the capacity to remove excess nutrients from the system, growth performance and quality (i.e. protein content) also determines the success of extractive species in integrated systems. Growth rates in the current study ranged between 3.6 and 5.0% day^{-1} under continuous high nutrient concentrations. As for nutrient uptake rates, highly variable growth rates are reported in literature for *Ulva* species (Table 3.3), but growth rates measured in the current study were in line with maximum growth rates measured under natural conditions for an *Ulva* strain (up to 6.2% day^{-1}) collected from the same location as the *Ulva* used in the current study (Jansen, unpublished data). As the high nutrient concentrations resulted in conditions where neither nitrogen nor phosphorus was limiting for maximum growth (see *section 4.1*), the role of trace elements or other limiting factors should be considered in future studies, in particular for integrated RAS where the main nutrient supply to the extractive species results from non-retained nutrients by the fed species.

Table 3.3
A literature overview of *Ulva* performance (tissue content and growth) and nutrient removal rates.
DM, dry matter; accl., acclimatization period; exp., experimental period; biomass & tissue content, nutrient removal rate determined based on biomass growth and initial and final tissue content; nutrient depletion, nutrient removal rate determined based on nutrient depletion in the media.

Species	Tissue content (% DM)		Removal rate (µmol gram^{-1} DM day^{-1})		Growth (% day^{-1})	Concentration culture medium (mM)		Experimental design		Ref.
	N	P	DIN	DIP		DIN	DIP	Removal rate	Performance	
Ulva spp	3.67-3.82 5.18	0.30-0.37 0.27-0.29	140-155 191	4-7 3	4.5-5.0 3.6-4.0	0.7-5 NO_3 0.5-0.6 TAN	0.07-0.9 0.01-0.1	Flow-through, 1 wk accl., 1 wk exp, biomass & tissue content	Flow-through, 1 wk accl., 1 wk exp,	This study
Ulva sp.	1.30-3.52				0.8-6.2				Flow-through, 5 months, frequently sampled	Jansen et al. unpublished
Ulva fasciata	5.8 7.1	0.17 0.39	528 2736	260 82	7.5 14.3	0.7 NO_3 0.8 TAN	0.06–0.07 0.06-0.07	Flow-through, 3 wk accl., 4 d exp., nutrient depletion	Flow-through, 3 wk accl., 4 d exp.	Shahar et al. (2020)
Ulva lactuca		0.87	896	27	0.6-4.4	5 NO_3	0.001–0.05	Daily media replacement, 10 d exp., nutrient depletion	Daily media replacement, 10 d exp.	Lubsch & Timmermans (2018)
Ulva lactuca	4.41	0.26	126 49-67	10 2	1.5-2.8	2.9-4.3 NO_3	0.195–0.291	No media exchange, 6 d exp., nutrient depletion; biomass & tissue content	No media exchange, 6 d exp.	Tremblay-Gratton et al. (2018)
Ulva lactuca		0.14–0.39		100		0.001–0.032 NH_4NO_3	0.0005-0.01 0.00006–0.002	No media exchange, 4 h exp., nutrient depletion	Flow-through, 2 yrs, frequently sampled	Pedersen et al. (2010)
Ulva lactuca		0.35–1.53		52 - 112 600 1588		0.5 NH_4NO_3 0.5 NH_4NO_3 0.04 NH_4NO_3	0.02 <0.0005 0.05 0.001–0.03	No medium exchange, no accl., 18 min exp., nut. depl. Regular media exchange, 17-18 d accl., 12 min exp, nutrient depletion	No media exchange, 13-15 d	Runcie et al. (2004)

Table 3.3 - *Continued*

Species	Tissue content (% DM)		Removal rate (µmol gram^{-1} DM day^{-1})		Growth (% day^{-1})	Concentration culture medium (mM)		Experimental design		Ref.
	N	P	DIN	DIP		DIN	DIP	Removal rate	Performance	
Ulva rotundata	1.57-2.62 1.35-2.32	0.07-0.10 0.05-0.09	2136 50-90 50-150	67 1.5-5 0-1		0.03-0.06 TAN 0.02-0.06 TAN 0.02-0.06 TAN	Up to 0.005 Up to 0.005 Up to 0.005	No media exchange, 7 h exp., nutrient depletion Flow-through, 6 d accl. (starved), 1 wk exp., nut. depl Flow-through, 6 d accl. (non-starved), 1 wk exp., nut. depl		Martínez-Aragón et al. (2002); Hernández et al. (2002)
Ulva lactuca			1728 480			0.0035-0.085 TAN 0.0035-0.045 NO_3		No media exchange, 4–6h exp., nutrient depletion		Pedersen et al. (1997)
Ulva lactuca					7-11 8-17 15 - 18	0.010-0.014 TAN 0.027-0.048 TAN 0.071–0.078 TAN			Flow-through, 3 wk exp.	Neori et al. (1991)
Ulva lactuca	1.15 3.59		3312 3312		5.3 9.3	0.04 TAN 0.2 TAN		No medium exchange, 1-2h exp., nutrient depletion	Medium exchange every other day, 19d exp.	Fujita (1985)

5 Conclusion

Integrated aquaculture systems, where marine fish in RAS systems are combined with seaweed production are characterised by high nutrient concentrations, which is different to most studies that have previously looked at nutrient uptake kinetics of *Ulva* species. Exposing *Ulva* spp. to high nutrient concentrations allowed us to test the following hypotheses:

Hypothesis (I) - Ulva spp. performance is not influenced by stoichiometry in fish waste effluents under high nutrient concentrations: it was indeed confirmed that nutrient concentrations in the culture medium and tissue content were above the critical threshold for maximum growth, suggesting that other factors than nitrogen or phosphorus level were limiting seaweed growth. The unfavourable stoichiometry in fish wastes (N:P<< Atkinson ratio) typical for RAS systems is therefore not limiting seaweed growth.

Hypothesis (II) - Orthophosphate concentrations in RAS effluents are not toxic for Ulva spp.: Highest orthophosphate concentrations of 0.9 mM did result in degenerating *Ulva* spp*.,* suggesting toxic conditions. The exact levels of phosphorus toxicity need to be further elucidated but results suggest they will most likely fall within the range 0.3-0.9 mM. As orthophosphate accumulates within RAS systems, and may reach high concentrations, this requires further care for the development of integrated RAS systems.

Hypothesis (III) - High nitrate concentrations in RAS effluents will limit the phosphorus uptake: Because the TAN excreted by fish is transformed into nitrate by the bacterial biofilter in RAS systems, nitrate may reach high levels. In contrast to suggestions in literature, phosphorus uptake was not reduced in response to high nitrate concentrations in this study, not for surge nor for maintenance uptake, indicating that phosphorus removal of *Ulva* spp. is not limited by high nitrate concentrations (5 mM).

Hypothesis (IV) - High ammonium concentrations will result in better performance of Ulva spp. in comparison to comparably high nitrate concentrations: *Ulva* growth was not affected when high nitrate-N concentrations were changed to high ammonium-N concentrations, but the nitrogen content in tissue increased significantly. This suggests an accumulation of nitrogen in the ammonium-based treatments, which was not used for growth.

Growth and nitrogen removal rates did not differ between high and moderate nitrate concentrations, nor for high versus low N:P ratios, suggesting that under these conditions maximum growth and nitrogen removal rates were achieved. These results contribute to a better understanding of the application of *Ulva* spp. as extractive component in closed integrated aquaculture systems, where a continuous supply of moderate to high nutrient concentrations can be expected.

Acknowledgment

The authors would like to thank Menno ter Veld and the staff of ARF-CARUS for their technical support in preparation and running the experiment. In addition, we would like to thank Tino Leffering and Ronald Booms for their support during the chemical analyses. At last, Willem Brandenburg and Willem de Visser are acknowledged for their support to obtain the seaweed material. This research was carried out as part of the PhD study of MAJ Nederlof (IPOP project TripleP@Sea KB-24-001-019 at Wageningen University).

Supplementary material

Table S3.1

Growth performance (specific growth rate, % day^{-1}) and tissue content (tissue nitrogen and phosphorus, % of dry matter and tissue N:P and C:N molar ratio) of *Ulva* spp. for the first and second weeks. Data are shown as mean ± standard deviation. Week effect indicates the *p*-values of the Paired-Samples T-test, where significant differences are highlighted in bold.

		Treatment						
	Week	A1	A2	B	C	D	E	F
SGR	1	4.61 ± 1.04	2.27 ± 1.24	5.24 ± 0.55	6.12 ± 0.77	ND	3.06 ± 0.79	1.77 ± 1.38
	2	5.00 ± 0.82	2.79 ± 1.28	4.34 ± 0.20	4.25 ± 0.87	ND	3.99 ± 2.05	3.60 ± 0.70
	Week effect	0.7449	0.3626	0.167	**0.03519**		0.6137	0.1264
Tissue N	1	3.38 ± 0.10	3.54 ± 0.08	3.59 ± 0.10	3.43 ± 0.02	ND	4.66 ± 0.04	4.52 ± 0.10
	2	3.67 ± 0.09	3.94 ± 0.07	3.82 ± 0.05	3.75 ± 0.04	ND	5.18 ± 0.07	5.18 ± 0.07
	Week effect	**0.006896**	**0.01145**	0.1063	**0.002413**		**0.01185**	**0.004587**
Tissue P	1	0.35 ± 0.02	0.33 ± 0.02	0.30 ± 0.006	0.30 ± 0.004	ND	0.29 ± 0.01	0.30 ± 0.007
	2	0.37 ± 0.004	0.35 ± 0.07	0.30 ± 0.002	0.31 ± 0.035	ND	0.27 ± 0.03	0.29 ± 0.03
	Week effect	0.1911	0.6853	0.2585	0.5742		0.302	0.5749
Tissue NP	1	21.7 ± 2.06	23.6 ± 2.7	26.8 ± 0.86	25.8 ± 0.3	ND	35.5 ± 1.3	33.3 ± 0.30
	2	21.7 ± 0.71	26.0 ± 6.3	28.1 ± 0.5	27.3 ± 3.1	ND	42.1 ± 3.7	40.0 ± 3.6
	Week effect	0.9967	0.4857	0.2491	0.4477		0.0596	0.08223
Tissue CN	1	9.8 ± 0.4	9.3 ± 0.1	9.3 ± 0.3	9.6 ± 0.05	ND	7.3 ± 0.07	7.5 ± 0.03
	2	9.3 ± 0.2	8.8 ± 0.03	9.1 ± 0.3	9.1 ± 0.2	ND	6.9 ± 0.1	7.0 ± 0.1
	Week effect	0.06852	**0.01113**	0.4161	0.1089		0.06111	**0.009787**



Chapter 4

Application of polychaetes in (de)coupled integrated aquaculture: an approach for fish waste bioremediation

Marit A.J. Nederlof, Jinghui Fang,
Thomas G. Dahlgren, Samuel P.S. Rastrick,
Aad C. Smaal, Øivind Strand, Harald Sveier,
Marc C.J. Verdegem, Henrice M. Jansen

Aquaculture Environment Interactions (2020), 12: 385-399

Abstract

Development of benthic components within integrated multi-trophic aquaculture (IMTA) systems warrants more attention, and the development of polychaetes as an extractive component in IMTA systems is ongoing. This study estimates the bioremediation potential of *Capitella* sp. and *Ophryotrocha craigsmithi* for coupled and decoupled salmon-driven IMTA. In coupled IMTA, polychaetes receive fresh faeces, while in decoupled IMTA, preservation of faeces is applied. Respiration and ammonia excretion rates were measured for polychaetes fed fresh, oven-dried or acidified salmon faeces, and combined with nutrients incorporated into tissue growth, to estimate nutrient requirements. Nutrient requirements were subsequently used to evaluate bioremediation potential. Metabolic rates were highest for *O. craigsmithi* and contributed notably to their overall nutrient requirement (20−30%). For the 2 polychaete species, nutrient requirements ranged from 5 to 26 mg C and from 2 to 6 mg N g^{-1} AFDW d^{-1}. These requirements were comparable with or higher than other polychaete species, highlighting the potential for fish waste bioremediation by *Capitella* sp. and *O. craigsmithi*. Preserved diets reduced bioremediation potential 1.5 and 3−5 times for, respectively, *Capitella* sp. and *O. craigsmithi*. Assuming that polychaetes are efficient fish-faeces convertors, the bioremediation potential indicates that benthic cultivation units containing 65 000−95 000 ind. m^{-2} of *Capitella* sp. or 36 000−194 000 ind. m^{-2} of *O. craigsmithi* can convert the daily organic waste flux deposited below an average salmon farm. These densities were within ranges reported for wild populations, indicating that, based on the bioremediation potential, development of benthic IMTA with these 2 polychaete species seems realistic and efficient for waste conversion.

1 Introduction

Sustainability issues in aquaculture have fuelled interest in integrated multi-trophic aquaculture (IMTA) systems. In IMTA systems, fed cultures (e.g. fish) are coupled with extractive cultures in such a way that waste resulting from the fed cultures (i.e. uneaten feed, faeces and metabolic excreta) serve as a nutrient source for algae or invertebrates. Due to the potential to reduce aquaculture waste while generating a valuable crop, IMTA is considered to be sustainable (Chopin 2015, Hughes & Black 2016). The concept of IMTA has developed over the years (Chopin 2013b, Hughes & Black 2016), and alternative concepts have been presented, including coupled and decoupled integrated systems (Goddek et al. 2016, Jansen et al. 2019). While coupled IMTA refers to the more conventional approach whereby extractive species are integrated in proximity of the fed species, in decoupled IMTA, the different compartments (i.e. fed species and extractive species) are integrated as separate functional units. Spatial connection between the cultivation units is not required in decoupled integrated aquaculture (Goddek et al. 2016).

One of the ecological concerns of finfish aquaculture is the impact of solid wastes on the benthic ecosystem (Taranger et al. 2015, Quiñones et al. 2019). Nonetheless, research on benthic species in IMTA has received less attention than species in the water column (Granada et al. 2016, Filgueira et al. 2017, Jansen et al. 2019, Strand et al. 2019). Deposit-feeding polychaetes have been suggested as potential candidates for benthic IMTA (e.g. Bischoff et al. 2009, Bergström et al. 2015, Gómez et al. 2019, Jansen et al. 2019) and several studies have quantified their bioremediation potential, reporting removal efficiencies ranging from 20 to 85% for organic matter (OM), 65 to 91% for organic C, and 31 to 91% for organic N (Honda & Kikuchi 2002, Palmer 2010, Fang et al. 2017, Marques et al. 2017, Pajand et al. 2017, Gómez et al. 2019). In the field, it has been demonstrated that the opportunistic polychaete species of the genus *Capitella* Blainville, 1828, which thrives under eutrophic conditions, is able to significantly reduce OM in sediments underneath cage farms and in pond systems (Tsutsumi et al. 2002, Kinoshita et al. 2008). These studies highlight the potential of polychaetes as extractive species in IMTA systems.

Underneath salmon farms, large densities of opportunistic polychaete species are naturally present (Kutti et al. 2007b, Wiklund et al. 2009b, Salvo et al. 2015b). Among these opportunistic species, *Capitella* sp. and *Ophryotrocha craigsmithi* Wiklund, Glover & Dahlgren, 2009 (see Wiklund et al. 2009a) have been identified (Kutti et al. 2007b, Bannister et al. 2014, Valdemarsen et al. 2015). In a recent study, Nederlof et al. (2019) showed that these species grow well on salmon faeces in laboratory trials, and highlighted that based on their nutritive quality, both species seem interesting as a high-quality marine ingredient for aquatic diets. Their ability to convert salmon faeces into valuable biomass shows that *Capitella* sp. and *O. craigsmithi* have potential as extractive species for cultivation in connection with Atlantic salmon (*Salmo salar*). In addition, development of benthic

cultivation methods for these polychaete species is ongoing (Jansen et al. 2019). Apart from increasing the productivity of a production system, the IMTA approach also strives to reduce wastes produced by fed species via extractive species (Chopin 2013b, Hughes & Black 2016). To fully understand the potential of *Capitella* sp. and *O. craigsmithi* as an extractive component in IMTA systems, their bioremediation potential should thus be studied.

Since the grow-out phase of salmon aquaculture is predominantly open-water cage culture, environmental concerns motivate an exploration of alternative production systems, such as enclosed or semi-enclosed sea cages where waste collection could be possible (Lekang et al. 2016). While deposit feeders cultivated underneath open-water cages (i.e. coupled integrated aquaculture) can directly feed on the fresh solid waste, (semi-)enclosed systems make it possible to collect and upgrade solid wastes before use (e.g. decoupled integrated aquaculture). In decoupled IMTA systems, preservation of the collected organic wastes is recommended, as fish waste degrades quickly (Beristain 2005). In the current study, 2 preservation techniques were chosen, drying and acidification. These methods are relatively fast and easy to apply, which makes commercial application more feasible. Both preservation methods may inactivate microbial activity and thereby reduce decomposition rates (Luckstadt 2008, Betoret et al. 2016). However, microbes may also contribute to the diets of *O. craigsmithi* and *Capitella* sp. (Fauchald & Jumars 1979, Findlay & Tenore 1982, Salvo et al. 2015a). Further, compositional changes of solid waste due to preservation potentially affects the nutritional value for the polychaetes, which is most likely to occur during preservation by acidification (Hardy et al. 1984, Özyurt et al. 2016).

The main objective of the present study was to estimate the bioremediation potential of *Capitella* sp. and *O. craigsmithi* fed fresh and preserved salmon faeces in order to evaluate their application in coupled and decoupled IMTA systems. Respiration and ammonia excretion rates measured in this study, combined with nutrients incorporated in tissue growth (based on data from Nederlof et al. 2019), were used as a proxy to estimate the nutrient requirements (i.e. absorbed nutrients) of the polychaetes. Based on high assimilation efficiencies (AE) reported in previous studies for polychaetes fed aquaculture waste (Honda & Kikuchi 2002, Fang et al. 2016a, Fang et al. 2017), it can be assumed that the proxies used in this study serve as a valid indication for the nutrient requirements of the polychaetes. Nutrient requirements were subsequently related to deposition of fish waste as a proxy for the bioremediation potential of the 2 polychaete species studied. It should be noted that in this study, bioremediation potential includes recycling of organic waste nutrients by polychaete mineralization (i.e. metabolic processes) and nutrient incorporation in polychaete biomass, but excludes information on consumption and AE, and thus on the amount of waste nutrients that remain in the system.

In addition to the main objective, whether metabolic rates differed when measured either on population or on individual scale was also investigated for both

polychaetes. As metabolic rates can be affected by several drivers, the metabolic response of individuals may differ from the response of populations, as a population can be more than the sum of its parts (Hassall 1983). Measurements on a population scale provide information on what can be expected in the field (e.g. cultivation underneath salmon cages), whereas measurements on an individual scale facilitate an understanding of the underlying physiology. *Ophrytorocha* species 'cluster' naturally in dense colonies on organically enriched substrates forming polychaete–mucus complexes (Salvo et al. 2014). *Capitella* species are known to form dense patches in organic enriched sediments (Tsutsumi et al. 2002). It was hypothesized that formation of these polychaete–mucus complexes and dense polychaete patches may influence the maintenance of physiological processes, resulting in differences between measurements on population or individual scale.

2 Materials & Methods

2.1 Species collection

Animals were collected underneath 2 different commercial Atlantic salmon (*Salmo salar*) farms located in the coastal area of western Norway. One farm was characterized by a soft bottom (125 m depth), where individuals of the genus *Capitella* were collected using a Van Veen grab. In this study, the name '*Capitella* sp.' was used for the species investigated, since the species belonging to the genus *Capitella* occurring in Norwegian waters are morphologically similar (cryptic) and include currently undescribed species. A taxonomic revision of annelids in nutrient-rich habitats (e.g. underneath fish farms) in Norway, including *Capitella* species, is ongoing (T. G. Dahlgren unpubl. data). The substrate below the second salmon farm, where *Ophryotrocha craigsmithi* was collected, was characterized by a hard bottom (140 m depth). For this polychaete, which belongs to a genus of morphologically similar species (Wiklund et al. 2009a), species determination was confirmed using cytochrome c oxidase subunit I (COI) barcode data (Hebert et al. 2003). For the collection of *O. craigsmithi*, 3 iron trays (1.2 × 1.2 × 0.1 m, with a perforated base to allow water to pass through), covered with different plastic substrates, were placed underneath the fish farm. The trays were deployed at depths varying between 50 and 150 m, and were left submerged for 3 wk. After 3 wk, the trays were brought to the surface and polychaetes were collected. Both the Van Veen grab and benthic tray samples were carefully washed and polychaetes were collected. Collected polychaetes were immediately placed in an aerated tank containing seawater from 200 m depth, ensuring comparable salinity and water quality to their natural conditions. Then the polychaetes were transported to the experimental facilities of Austevoll Research Station (Institute of Marine Research, Norway), where the experiments were conducted.

2.2 C and N mass balance

Nutrient requirements (C and N) were estimated using a mass balance approach based on metabolic processes (measured as respiration and excretion) and nutrients incorporated into tissue growth. Mass balances were calculated for polychaetes fed fresh and preserved salmon faeces. Metabolic processes were measured and the experimental procedure is described below. Nutrients incorporated into tissue growth were calculated based on growth and tissue content data published in Nederlof et al. (2019), which includes a detailed description of how growth and tissue content data were obtained. It should be noted that the experiment described in the present study and the experiment described in Nederlof et al. (2019) were run in parallel, under comparable conditions and with the same dietary treatments, namely fresh, acidified and dried salmon faeces fed to the 2 polychaete species.

2.2.1 Diet preparation and treatments

Capitella sp. and *O. craigsmithi* were fed 3 different diets: fresh, acid-preserved and oven-dried faeces. To prepare the diets, faeces were collected twice a week by stripping salmon (individual weight: ca. 2−4 kg) kept at the sea cage facility of Austevoll Research Station. The collected faeces were directly centrifuged (6300 × *g* for 3 min; Eppendorf 5810R), and liquid was carefully removed. This process was repeated twice. The remaining solid fraction was homogenized and used to formulate the experimental diets. For the fresh diet treatment, polychaetes were directly fed with fresh centrifuged faeces. For the acid diet treatment, centrifuged faeces were preserved by the addition of formic acid (80%), creating a pH < 4 (pH: 3.4 ± 0.1), and left for 24h at room temperature. Before feeding, excessive liquid was carefully removed and the faeces were washed twice with seawater to reduce acidity. For the washing procedure, seawater was added to the acidified faeces, which was then centrifuged (6300 × *g* for 3 min; Eppendorf 5810R), and liquid was removed. For the oven-dried diet treatment, centrifuged faeces were preserved by oven-drying for 48h at 100°C, which is assumed to kill the majority of bacteria present in the faeces. Treatments were started on 3 successive days, as preservation of the diets needed different time spans, i.e. Day 1: fresh diet treatment (direct use), Day 2: acid diet treatment (24 h), and Day 3: oven-dried diet treatment (48 h). In total, polychaetes were fed the experimental diets for 2.5 wk.

Prior to the respiration and excretion measurements, animals were kept in 1 l flow-through holding tanks (flow rate: 28 ± 2 ml min^{-1}), placed in the dark (ca. 50−70 ind. $tank^{-1}$). These tanks received filtered (1 µm) seawater (salinity: 34.8 ± 0.1, temperature: 8.7 ± 0.2°C) pumped from 200 m depth. These conditions were comparable to conditions measured underneath the salmon farms where the polychaetes were collected (conductivity, temperature and depth scans, data not shown). For *Capitella* sp., glass marbles (5 mm diameter, VWR Norway) were added to the tanks (~1 cm of the bottom was covered), to mimic natural substrates while providing the opportunity to observe animals during daily check-ups. For *O. craigsmithi*, a pre-combusted (overnight, 550°C) stone (~5 cm width and length)

was added to the tanks. The rough surface of the stone was assumed to mimic natural substrates for mucus attachment. Animals were fed the experimental diets for 2.5 wk before respiration and ammonia excretion measurements were started by feeding them, in excess (~1.5 g chamber^{-1} feeding^{-1}), fresh or preserved salmon faeces twice a week. Leftover feed was always observed, confirming that feed was provided in excess. During each feeding, feed samples were collected and stored in the freezer (-20°C) before analyses.

2.2.2 Respiration and ammonia excretion measurements

Respiration and excretion were measured after animals were placed in clean tanks receiving filtered (1 µm) seawater and were left for 2 d in order to defecate and empty their guts. The next day animals were transferred to the respiration chambers and measurements on metabolic rates started. Directly after the respiration and ammonia excretion measurements, polychaetes were sampled to determine their average weight (on an ash-free dry weight [AFDW] basis). For polychaetes fed fresh salmon faeces, respiration and excretion were measured at both individual and population scales. For polychaetes fed preserved diets, measurements were only done on a population scale. Respiration chambers consisted of closed chambers filled with filtered (1 µm) seawater (salinity: 34.8 ± 0.1, pH: 7.9 ± 0.1). For *O. craigsmithi*, respiration chambers with a volume of 1 and 17 ml for, respectively, measurements on individual scale (n = 1 ind. chamber^{-1}) or what was defined as population scale (n = 10 ind. chamber^{-1}) were used. Chamber volume for individuals of *Capitella* sp. was doubled (2 ml), as these chambers included marbles to mimic natural substrates (1 ml of the respiration chamber was filled with marbles). Marbles were also added to the population-scale respiration chamber used for *Capitella* sp. (total volume of 17 ml, 4 ml was filled with marbles; n = 13 ind. chamber^{-1}). For individual measurements, average weight was 2.1 ± 1.0 and 1.2 ± 0.6 mg AFDW for *Capitella* sp. and *O. craigsmithi* respectively, and for population measurements, average individual weight was 1.8 ± 0.7 and 1.3 ± 0.3 mg AFDW for *Capitella* sp. and *O. craigsmithi* respectively. Population measurements were made with 8 repetitions per treatment (i.e. diet) per polychaete species, while there were 18 repetitions per species for the individual measurements. However, 3 individuals of *O. craigsmithi* died during the respiration and excretion measurements done on individual scale and were therefore excluded, resulting in a sample size of 15 for this species. For a summary of the experimental set-up, see Table 4.1.

Oxygen consumption rates were determined using an optical system as described by Rastrick & Whiteley (2011). A PreSens® 10-channel non-invasive oxygen-analysing system (PreSens® OXY-10) was used for the measurements. As preservation of the diets needed different time spans, treatments (i.e. fresh, acid and dried) were run independently per polychaete species. Each population run consisted of 8 experimental (i.e. replicates) and 1 control chamber (i.e. without polychaetes). Individual measurements were performed on the same day, with 2 runs of 9 experimental and 1 control chamber per polychaete species. Oxygen concentrations were logged every 15 s.

Table 4.1
Summary of the experimental set-up.

	Capitella sp.	*Ophryotrocha craigsmithi*
Experimental conditions		
Salinity	35	35
Temperature (°C)	9-10	9-10
pH	8	8
Metabolic rates		
Population scale		
Start weight (mg AFDW*)	1.8 ± 0.7	1.3 ± 0.3
No. individuals	13	10
Chamber volume (ml)	17$	17
Replicates	8	8
Individual scale		
Start weight (mg AFDW)	2.1 ± 1.0	1.2 ± 0.6
No. individuals	1	1
Chamber volume (ml)	2$	1
Replicates	18	15$$
Nutrients incorporated in tissue growth$$$		
Start weight (mg AFDW)	1.7 ± 0.1	1.9 ± 0.1
No. individuals	66	50
Chamber volume (ml)	1000$	1000
Replicates	4	4

* AFDW, Ash Free Dry Weight.
$ Marbles were added to mimic natural substrates.
$$ 3 individuals died during the measurements and were excluded from the experiment.
$$$ Published in Nederlof et al. (2019)

Incubations were terminated when O_2 concentrations in all chambers had decreased at least 20%. All chambers showed a linear decrease in pO_2 throughout the incubation period (R^2 = 0.6095−0.9963, min−max), showing that oxygen uptake was not affected by handling stress or by changes in oxygen saturation during the measurement period. Oxygen consumption rates were calculated as the difference in the rate of pO_2 change between the experimental chamber and the control chamber, multiplied by the volume of the chambers and the solubility coefficient for oxygen in seawater (adjusted for salinity of 35 and temperature of 9−10°C; Benson & Krause 1980, 1984). To convert oxygen consumption rates to carbon loss, a respiratory quotient of 0.9 was used (Cammen 1985).

Following the oxygen consumption incubations, water from each respiration chamber was sampled, including the control chamber (approximately 1 ml for individual measurements and 10 ml for population measurements). These samples were stored at -20°C, for the determination of ammonia excretion rates. Ammonia excretion rates were calculated as ammonia concentrations (see *Section 2.3*) measured in the experimental chambers minus ammonia concentrations measured in the control chamber, multiplied by chamber volume. Finally, dry matter and ash of the polychaetes were determined, and oxygen consumption and ammonia excretion rates were standardized to 1 g body weight (AFDW) and per hour. Based on the respiration and excretion measurements, O:N ratios were calculated (mol:mol).

2.2.3 Nutrients incorporated in tissue growth

Growth and tissue content (C and N) data reported in Nederlof et al. (2019) for *Capitella* sp. and *O. craigsmithi* fed fresh and preserved salmon faeces were used to calculate the amount of nutrients incorporated in tissue growth. Increase in tissue C and N were first calculated per individual polychaete by the difference in body content (mg C or N ind.$^{-1}$) between the start and end of the experiment. Subsequently these results were standardized for time (per day) and polychaete weight (1 g AFDW). For the latter, geometric mean body weight (mg AFDW) of the polychaetes during the growth period was calculated using the following formula:

$$\text{Geometric mean body weight} = \sqrt{(W_f \times W_i)} \quad (1)$$

where W_f is the average individual final weight (mg AFDW) and W_i is the average individual initial weight (mg AFDW).

2.3 Analytical analyses

Diet and polychaete samples were freeze-dried. Diet samples were then ground using a bullet mill. Diet and polychaetes samples were analysed for dry matter (freeze-dried) and ash (550°C, 6 h). Diet samples were also analysed for C and N content. This was done by combusting the samples with an elemental analyser (Flash 2000, Thermo Fisher) at 1020°C, in the present of oxygen, to convert C and N to CO_2 and NOx, respectively. Thereafter, NOx was reduced to N_2 in a reduction column. Nitrogen content was multiplied by 6.25 to estimate crude protein content

in the diets. Sampled material was not enough for fat analyses. Nevertheless, diet samples were collected in parallel with samples collected by Nederlof et al. (2019), and therefore diets are assumed to be comparable between the 2 studies. Nederlof et al. (2019) reported fatty acid contents of the diets.

Water samples taken to determine ammonia excretion rates were thawed to room temperature and analysed using the phenol blue method described by Solórzano (1969). Briefly, 8 µl of a phenol alcohol solution (10 g phenol in 100 ml 95% ethyl alcohol), 8 µl of a 0.5% sodium nitroferricyanide solution (1 g sodium nitroferricyanide in 200 ml deionized water) and 20 µl oxidizing solution (100 g trisodium citrate and 5 g sodium hydroxide in 500 ml deionized water; on the day of the analysis, 100 ml of this solution was mixed with 25 ml sodium hypochlorite) were added to 200 µl of the water sample in a well plate (volume: 300 µl). This was left for 1 h at room temperature, after which absorbance was measured using a spectrophotometer (640 nm, SpectraMax M5 with SoftMax Pro software, Molecular Devices LLC). Standard curves were made using ammonium sulphate (1.5 mg ammonium sulphate in 1 l deionized water).

2.4. Statistical analyses

Statistical analyses were performed using the R statistical program (version 3.4.0.; R Development Core Team 2017).

Prior to statistical analysis, residuals of the data were checked for homogeneity of variance and normality using Q–Q plots and Shapiro-Wilk and Levene tests. Student's *t*-test was performed to assess potential differences in respiration and excretion rates between measurements on individual and population scale. One-way ANOVA was used to test differences between diet composition (AFDW, C, N and crude protein). As described by Garland & Adolph (1994), statistical comparison of species should be done with care. Since the aim of the present study was to estimate the bioremediation potential of each polychaete species independently, 1-way ANOVA was used to test, within each polychaete species, the effect of diet on respiration, excretion and O:N ratios. Where assumptions of homogeneity of variance or normality were violated, data were transformed. If after transformation, assumptions of homogeneity of variance and normality were still violated, a non-parametric Kruskal-Wallis test was used. When the ANOVA tests were significant ($p < 0.05$), treatments were compared using Tukey's HSD post hoc multiple comparison tests. Significant results found with the non-parametric test were followed by Mann-Whitney *U*-test with Bonferroni correction.

3 Results

3.1 Diets

Composition of diets fed to the polychaetes can be found in Table 4.2. Preservation by acidification affected diet composition, as this resulted in a significantly lower AFDW (Tukey HSD; $p < 0.001$), N (Mann-Whitney U; $p < 0.01$) and crude protein (Mann-Whitney U; $p < 0.01$) content compared to the other 2 diets, which did not differ. C content was significantly higher in the dried diet compared to the acidified diet (Tukey HSD; $p < 0.05$). Both preserved diets did not significantly differ in C content compared with the fresh salmon faeces (Tukey HSD; $p > 0.05$).

3.2 Polychaete respiration and excretion rates

Excretion rate of *Ophryotrocha craigsmithi* was affected by diet, and polychaetes fed the acid-preserved diet had a significantly higher ammonia excretion rate compared to polychaetes fed the fresh diet (Table 4.3; Tukey HSD; $p < 0.05$). Excretion rate of *O. craigsmithi* fed the oven-dried diet did not significantly differ from *O. craigsmithi* fed the 2 other diets (Table 4.3; Tukey HSD; $p > 0.05$). A significant diet effect was also found for the O:N ratios measured for *O. craigsmithi* (Table 4.3; 1-way ANOVA; $p < 0.01$); *O. craigsmithi* fed the acid-preserved diet had a significantly lower O:N ratio compared to *O. craigsmithi* fed the other 2 diets (Table 4.3; Tukey HSD; $p < 0.05$). The latter did not significantly differ from each other (Table 4.3; Tukey HSD; $p > 0.05$). Respiration rates of *O. craigsmithi* were not affected by diets (Table 4.3; 1-way ANOVA; $p > 0.05$). For *Capitella* sp., no diet effect was observed for respiration rates, excretion rates and O:N ratio (Table 4.3; 1-way ANOVA; $p > 0.05$).

Both polychaete species did not show significant differences when respiration and excretion rates were measured either at population or individual scale (Fig. 4.1; Student's t-test; $p > 0.05$). Overall, for *O. craigsmithi*, higher respiration and excretion rates were observed in comparison to rates measured for *Capitella* sp., while *Capitella* sp. showed higher O:N ratios.

Table 4.2
Composition of the experimental diets fed to the polychaetes in the weeks prior to respiration and excretion measurements, i.e. fresh salmon faeces and salmon faeces preserved by acidification (formic acid, pH <4) or by oven-drying (100 °C).

	Fresh	Acid	Dried	Significance
AFDW	790 ± 37^{a}	700 ± 39^{b}	782 ± 21^{a}	***
C	437 ± 12^{a,b}	435 ± 11^{b}	450 ± 4^{a}	*
N	24 ± 3^{a}	14 ± 2^{b}	25 ± 3^{a}	**
Crude protein	149 ± 21^{a}	89 ± 12^{b}	157 ± 21^{a}	**

Values are mean ± SD (n = 6 samples treatment^{-1}, except for AFDW of the Fresh treatment where n = 16 samples).
AFDW: ash-free dry weight (g kg^{-1} dry matter); C, N and crude protein (N x 6.25) are in g kg^{-1} AFDW.
AFDW data were log transformed before statistical analyses, non-parametric test was used for potential differences in N and crude protein content of the diets.
*** = P-value < 0.001, ** = P-value < 0.01, * = P-value < 0.05, ns = not significant.
Means within a row lacking a common subscript letter (a, b, ..) differ significantly (P < 0.05).

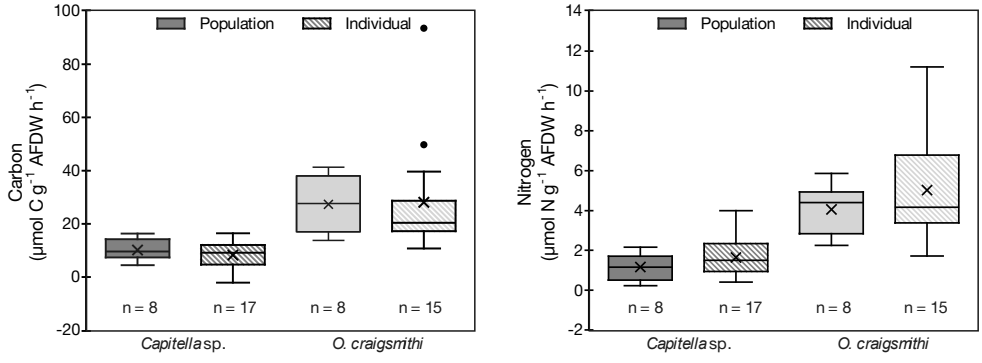

Figure 4.1
Carbon and Nitrogen required for metabolic processes (i.e. respiration and ammonia excretion), measured at population and individual scale for *Capitella* sp. and *Ophryotrocha craigsmithi* fed fresh salmon faeces. Carbon requirement based on respiration measurements; Nitrogen requirement based on ammonia excretion measurements. Box: interval between lower and upper quartiles of the distributions; cross: mean value; whiskers: minimum and maximum. Data of *O. craigsmithi* log transformed before statistical analyses. Within each species, no significant differences (p > 0.05) were found between measurements at population or individual scale for both Carbon and Nitrogen requirement.

Table 4.3
Carbon and nitrogen required for metabolic processes based on respiration (carbon, mg g^{-1} AFDW day^{-1}) and excretion (nitrogen, mg g^{-1} AFDW day^{-1}) and subsequent O:N ratios (mol:mol) of *Capitella* sp. and *Ophryotrocha craigsmithi* fed 3 different diets (fresh, acid-preserved and oven-dried salmon faeces).

	Capitella sp.				*O. craigsmithi*			
	Fresh	Acid	Dried	Sig.	Fresh	Acid	Dried	Sig.
Respiration	2.94 ± 1.16	2.51 ± 0.73	4.92 ± 3.85	ns	7.88 ± 3.01	6.70 ± 2.82	9.36 ± 1.82	ns
Excretion	0.39 ± 0.22	0.26 ± 0.10	0.59 ± 0.42	ns	1.37 ± 0.42^{b}	2.15 ± 0.41^{a}	2.04 ± 1.09^{a,b}	*
O:N ratio	14 ± 14	15 ± 10	12 ± 4	ns	8 ± 4^{a}	4 ± 2^{b}	7 ± 2^{a}	**

Values are given as mean ± SD (n = 8 tanks treatment^{-1}).
AFDW, Ash Free Dry Weight; Sig., significance.
All data of *Capitella* sp. and excretion data and O:N ratios of *O. craigsmithi* were log transformed before statistical analyses.
** = P-value < 0.01, * = P-value < 0.05, ns = not significant.
Means within a row lacking a common subscript letter (a, b, ...) differ significantly (P < 0.05).

3.3 C and N mass balances

Fig. 4.2 shows nutrient requirements of *Capitella* sp. and *O. craigsmithi*, including C and N required for, respectively, respiration and excretion combined with nutrients incorporated in body tissue. For *Capitella* sp., C and N, incorporation rates ranged between 8 and 13 mg C g^{-1} AFDW d^{-1} and between 2 and 3 mg N g^{-1} AFDW d^{-1}, with the highest values found for animals fed the fresh diet. Feeding *O. craigsmithi* the acid-preserved diet resulted in negative values for C and N incorporation, due to negative growth rates as reported by Nederlof et al. (2019), while for O*. craigsmithi* fed fresh salmon faeces, the highest C and N incorporation rates were observed (18 and 5 mg g^{-1} AFDW d^{-1} for C and N respectively).

With the exception of *O. craigsmithi* fed the acid diet, for both polychaete species it was observed that growth makes up a significant fraction in the overall C and N requirement (up to 82% for C for *Capitella* sp. on the fresh diet, and up to 87% for N for *Capitella* sp. on the fresh and acid diets). Overall, C requirement ranged between 11 and 16 mg C g^{-1} AFDW d^{-1} for *Capitella* sp. and between 5 and 26 mg C g^{-1} AFDW d^{-1} for *O. craigsmithi*. N requirement ranged between 2 and 3 mg N g^{-1} AFDW d^{-1} for *Capitella* sp. and between 2 and 6 mg N g^{-1} AFDW d^{-1} for *O. craigsmithi.*

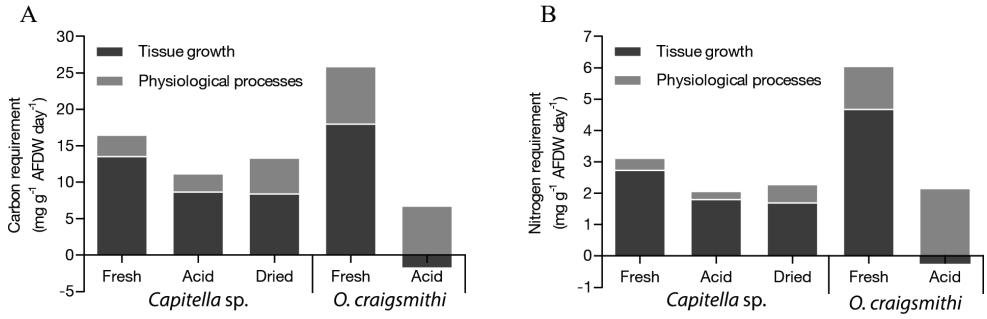

Figure 4.2

(A) Carbon and (B) Nitrogen requirement estimated for *Capitella* sp. and *Ophryotrocha craigsmithi* fed fresh or preserved (acidified or oven-dried) salmon faeces. For nutrients incorporated into tissue growth, growth and tissue content data from Nederlof et al. (2019) were used (see also our *Section 2.2.3* for calculation methods). Nutrients required for physiological processes were based on respiration (C) and excretion (N) measurements from the present study (see *Section 2.2.2* and Table 4.3).

4 Discussion

This study estimated the bioremediation potential of *Capitella* sp. and *Ophryotrocha craigsmithi* for a salmon−polychaete integrated system. Metabolic rates and the amount of nutrients incorporated in biomass differed between the diets fed, which has consequences for the bioremediation potential in coupled and decoupled IMTA systems.

4.1 Polychaete metabolism

4.1.1 Metabolic activity of Capitella sp. and O. craigsmithi

A 6 times lower respiration rate was measured in this study for *O. craigsmithi* (26−36 µmol O_2 g^{-1} AFDW h^{-1}) compared to rates reported for *O. labronica* (Rodríguez-Romero et al. 2016). Although factors such as differences in species and experimental set-up should not be excluded, the higher respiration rate observed by Rodríguez-Romero et al. (2016) may be attributed to the higher incubation temperature (Q10 rule; e.g. Hochachka 1991), i.e. 20°C in Rodríguez-Romero et al. (2016) versus 9−10°C in the current study. Both respiration and ammonia excretion rates (4−6 µmol N g^{-1} AFDW h^{-1}) of *O. craigsmithi* were in line with rates measured during a pilot study for the same species and under comparable environmental conditions (48 µmol O_2 g^{-1} AFDW h^{-1} and 7 µmol N g^{-1} AFDW h^{-1}) (Brennan 2018), while a twice as high respiration rate was observed in an earlier pilot study for *Ophryotrocha* spp. (Eikje 2013).

Even though, based on incubation temperatures, higher rates were expected, both Chareonpanich et al. (1994) (20°C) and Gillam (2016) (15−20°C) reported, for *Capitella* sp. and *C. teleta* respectively, respiration rates which were in line with the rates measured in our study. Other studies reported higher respiration rates (Brennan 2018, 11°C; Gamenick et al. 1998, 18°C; Linke-Gamenick et al. 2000, 18°C), but lower ammonia excretion rates (Brennan 2018, 11°C; Gardner et al. 1993, 20−25°C) for *Capitella* spp. These results may suggest that metabolic rates are highly plastic, as was shown for other ectotherms collected from sites different in thermal histories (e.g. latitudes) (Sokolova & Pörtner 2003, Whiteley et al. 2011). Other factors could also play a role in the differences in metabolic rates reported — size differences for example, since animals in both Brennan (2018) and Gardner et al. (1993) had a higher average individual weight than animals used in our study, suggesting that in those studies, the animals were larger. Furthermore, in the different studies, animals might have differed in their physiological status. Metabolic rates are highly influenced by the physiological status of an individual, and changes in O:N ratios can for example provide information on the reproductive status of an animal (Barber & Blake 1985). O:N ratios were measured, but not over time, and conclusions on the reproductive status based on a single time moment can be risky (Jansen et al. 2012). Respiration and excretion measurements were done with individuals from the same batch as used in the experiment in Nederlof et al. (2019), where reproduction in the tanks with *Capitella* sp. was observed, and it is therefore likely that in the current study, metabolic rates of *Capitella* sp. were influenced by their

reproductive status. In both our study species, the O:N ratios fall within the ranges indicative for a protein-driven metabolism (3–16) (Mayzaud 1973), but the lower values indicate that the metabolism of *O. craigsmithi* may rely more on proteins compared to *Capitella* sp.

While in the holding tanks, individuals of *O. craigsmithi* clustered together in polychaete–mucus complexes, clustering in dense polychaete patches was not observed for *Capitella* sp. (M. A. J. Nederlof pers. obs.). It was hypothesized that the natural formation of mucus–polychaete complexes observed for *O. craigsmithi* (Salvo et al. 2014) and the formation of dense polychaete patches observed for *Capitella* sp. (Tsutsumi et al. 2002) would affect metabolic rates. No population specific effects on respiration and excretion rates were observed for both polychaete species, suggesting that measurements obtained on an individual level can directly be scaled to the population level. It should be noted though that the clustering behaviour of *O. craigsmithi* observed in the holding tanks was not seen in the respiration chambers, and it can therefore not be excluded that population-specific effects do play a role.

4.1.2 Effect of diet preservation

Diets used in this study differed in nutrient composition; the oven-dried faeces contained a higher C, N and crude protein content (on an AFDW basis) compared to faeces preserved by acidification, but it was not different from the fresh salmon faeces. In Nederlof et al. (2019), similar diets were used, and a significantly higher total fatty acid content was reported for the fresh and acid diet compared to the oven-dried diet (100°C). In aerobic metabolic processes, macronutrients differ in their degree of oxidation, and therefore respiration is influenced by diet composition (Richardson 1929). Lipid metabolism requires relatively lower oxygen consumption (Richardson 1929), and lower respiration rates for polychaetes fed the fresh and acid-preserved diets can thus be expected compared to the oven-dried diet. Although values were indeed lower, no significant diet effect was found.

No difference in excretion rates and O:N ratios were observed for *Capitella* sp. fed the 3 different diets, while for *O. craigsmithi*, both parameters were affected by diet. The increased ammonia excretion rate of *O. craigsmithi* fed the acid diet compared to the fresh diet, and as a result the reduced O:N ratio, suggests that feeding *O. craigsmithi* with acid-preserved salmon faeces results in a higher protein turnover. This was surprising, since N and crude protein content of the acid diet was significantly lower compared to the other 2 diets, and it remains unclear why *O. craigsmithi* increased ammonia excretion when fed the acid diet. Interestingly, from a bioremediation perspective, the respiration results do suggest that for decoupled systems, preservation of waste by oven-drying would result in a higher C removal, while the excretion results indicate that for N removal, preservation by acidification is preferred.

4.2 Bioremediation potential

4.2.1 C and N mass balance

Bioremediation potential can be defined as the relationship between nutrient intake, growth (and reproduction), respiration/excretion and egestion. Several approaches are used to determine this potential, each with their own advantages and disadvantages (Table 4.4). Which approach is selected is often driven by practical or methodological constraints. In the present study, the initial aim was to define all processes described as approaches II−IV in Table 4.4. Pilot tests showed, however, that quantification of food intake and assimilation were not accurate due to mucus excreted by the polychaetes; feed and faeces were trapped in this mucus layer, from where it no longer could be removed. In particular, it was observed (visually) that *O. craigsmithi* 'actively' stored food and faeces in mucus strings. Besides splitting food and faeces, mucus also interfered with OM collection, since filters were easily clogged. Furthermore, the small size of the animals resulted in small faeces fragments, making it difficult to separate polychaete faeces from food leftovers, as is done for larger polychaetes to define assimilation (e.g. Fang et al. 2017). Instead, bioremediation potential was estimated based on nutrient requirements for growth and metabolic processes. This method excludes information on consumption and AE, overestimating waste turnover capacity. Nevertheless, relatively high AE values have been reported for polychaetes fed aquaculture waste (65−80% for both N and C) (Honda & Kikuchi 2002, Fang et al. 2016a, Fang et al. 2017). Hence it is assumed that polychaetes are efficient convertors of fish faeces, and that nutrient requirements for growth and metabolic processes provide a good estimate of their bioremediation potential.

Nutrient requirements determined in this study resulted in rates of 16 mg C g^{-1} AFDW d^{-1} and 3 mg N g^{-1} AFDW d^{-1} for *Capitella* sp. and 26 mg C g^{-1} AFDW d^{-1} and 6 mg N g^{-1} AFDW d^{-1} for *O. craigsmithi* fed fresh faeces. To compare with the literature, these rates were converted to g wet weight (WW), resulting in 3.5 mg C g^{-1} WW d^{-1} and 0.7 mg N g^{-1} WW d^{-1} for *Capitella* sp. and 4 mg C g^{-1} WW d^{-1} and 1 mg N g^{-1} WW d^{-1} for *O. craigsmithi*. Fang et al. (2016a) reported a C and N budget for *Perinereis aibuhitensis* fed defrosted fish faeces. Combining their data on growth and metabolism resulted in requirements of 2 mg C g^{-1} WW d^{-1} and 0.24 mg N g^{-1} WW d^{-1}, which is lower than the values obtained in the present study. Honda & Kikuchi (2002) reported a N budget for *P. nuntia vallata* fed flounder faeces, and combining their data on growth and metabolism resulted in a requirement of 0.82 mg N g^{-1} WW d^{-1}, which is in line with our study. Interestingly, the C budget for *P. aibuhitensis* presented by Fang et al. (2016a) was dominated by nutrients required for metabolic processes (56−82% of consumed carbon), while in the current study, C incorporated in tissue growth dominated the mass balance. In the N budget of *P. aibuhitensis*, relatively more N was allocated for growth (60−64% of consumed N), compared to excretory N (8−15% of consumed N) (Fang et al. 2016a), which was similar to the current study. Still, especially for *O. craigsmithi* fed fresh faeces, it was observed that nutrients required for metabolic processes made up a notable

Table 4.4
Overview of approaches applied in IMTA studies to define bioremediation potential of extractive species.

Approach		Advantage	Disadvantage	References
i	Bioremediation is defined as the amount of nutrients incorporated in cultivated or harvested biomass, determined by growth and nutrient content of the extractive species .	1) When extractive species are harvested, this presents the actual amount of nutrients removed from the system. 2) Relatively easy method that can be applied in both field and laboratory studies. 3) No need to determine the origin (i.e. waste or ambient) of nutrients taken up by the extractive species.	1) Overestimates organic extractive species maximum cultivation biomass, since waste consumption is not taken into account. 2) Underestimates benthic bioremediation potential since respiration/excretion and bioturbation processes are not taken into account.	e.g. Sanderson et al. (2012), Krom et al. (1995) and Wang et al. (2012)
ii	Bioremediation is defined as the amount of nutrients removed due to consumption, determined by food intake rates of the extractive species.	1) Relatively easy method when applied in laboratory studies, with non-mucus producing extractive species. 2) A good method to determine maximum cultivation biomass for organic extractive species.	1) Overestimates the bioremediation potential, since egestion is not taken into account. 2) A less suitable method for mucus producing extractive species, when feed, mucus and faeces are hard to separate.	e.g. Brown et al. (2011) and Honda and Kikuchi, (2002)
iii	Bioremediation is defined as the difference between food intake and egestion, i.e. assimilation, determined by among others the Conover method.	1) No need for quantitative recovery of extractive species' faeces, and therefore a relatively easy method for non-mucus producing extractive species. 2) Can be applied in both field and laboratory studies.	1) Does not take into account the amount of waste ingested by the extractive species. If food consumption is unknown this method can only provide qualitative bioremediation, and not quantitative bioremediation. 2) A less suitable method for mucus producing extractive species, when feed, mucus and faeces are hard to separate.	e.g. Fang et al. (2017) and Paltzat et al. (2008)
iv	Bioremediation is defined by respiration/excretion as a proxy for maintenance of physiological processes.	1) Can be applied to small individuals and mucus producing extractive species. 2) Can be applied in both field and laboratory studies.	1) Underestimates the bioremediation potential, since consumption and assimilation are not taken into account. 2) Can only be applied when benthic bioremediation is studied.	e.g. Honda and Kikuchi, (2002)
v	Bioremediation is defined by all processes described in approach *ii – iv*, determined by Scope for Growth.	1) Provides the most insights in the bioremediation capacity of extractive species.	1) Labour intensive. 2) A less suitable method for mucus producing extractive species, when feed, mucus and faeces are hard to separate. 3) Less suitable for field studies.	e.g. Jansen et al. (2012), Irisarri et al. (2015) and Fang et al. (2016a)

fraction of the total requirement (20–30%), highlighting the importance of metabolic processes in organic waste reduction by extractive species. Overall, compared to other studies, both *Capitella* sp. and *O. craigsmithi* show a high potential for waste bioremediation.

4.2.2 Farm-scale bioremediation

Organic waste production measured during peak moments of salmon cultivation in a commercial farm in Norway can reach up to 2500 mg organic C m^{-2} d^{-1} (Kutti et al. 2007a), which can negatively impact benthic ecosystems (Kutti et al. 2007a, Kutti et al. 2007b, Kutti et al. 2008). Based on C requirements determined in the present study, it can be estimated that for 100% removal of the daily organic C waste flux, approximately 65 000 ind. m^{-2} (152 g AFDW m^{-2}) of *Capitella* sp. or 37 000 ind. m^{-2} (97 g AFDW m^{-2}) of *O. craigsmithi* are required in coupled systems where the polychaetes are provided with fresh faeces (Table 4.5). These numbers need to increase in decoupled systems, where fish waste is preserved before being fed to the polychaetes (Table 4.5). It should be noted that the numbers provided in Table 4.5 are an overestimation, since consumption was not measured. Nevertheless, as mentioned earlier (*Section 4.2.1*), polychaetes are assumed to be efficient waste convertors (Fang et al. 2016a).

Underneath fish farms, opportunistic polychaete species such as *Capitella* spp. and *Ophryotrocha* spp. can reach high densities (Tsutsumi et al. 2005, Kutti et al. 2007b, Salvo et al. 2015b, Jansen et al. 2019). Tsutsumi et al. (2005) cultivated *Capitella* sp. underneath a fish farm (*Pagrus major* and *Seriola quinqueradiata*) in the Kuusura Bay in Japan and reported population densities of approximately 130 000 ind. m^{-2}. Paxton and Davey (2010) estimated that the density of *O. shieldsi* underneath sea cages (*Salmo salar*) at Macquarie Harbour in Tasmania, Australia, reached up to 100 000 ind. m^{-2}. Quantitative data on polychaete abundances underneath salmon farms in Norway is limited, as the depth of fjord systems makes it a challenge to measure these abundances (Jansen et al. 2019). However, in the current study, the estimated densities required for 100% waste removal (Table 4.5) fall within the ranges reported in the literature.

It should be noted that 100% waste removal does not equal 100% waste reduction to the benthic environment, as polychaetes expel faeces as well. Hughes (2016) indicated that there is a need to define IMTA in terms of environmental performance, raising the question of what percentage of waste should be removed for an effective and sustainable system. In benthic IMTA, the role of microbes in waste mineralization should not be underestimated (Heilskov et al. 2006, Valdemarsen et al. 2012). These microbes can in turn serve as a food source for the polychaetes. The lower bioremediation potential observed for both polychaete species fed the preserved salmon faeces suggests the importance of microbes in their diets, in particular for *O. craigsmithi*. Underneath fish farms, *Ophryotrocha* species commonly form a complex matrix of polychaetes, mucus and chemo-autotrophic bacteria (Salvo et al. 2015b), but the role of these matrixes in

Table 4.5
Capitella sp. and *Ophryotrocha craigsmithi* biomass (gram Ash Free Dry Weight m^{-2}) and number of individuals (ind. m^{-2}) required to remove 100% or 20% of the particulate organic carbon (POC) flux to the benthic system of a commercial salmon farm in Norway. Polychaete requirement is calculated for coupled (i.e. fresh salmon waste) and decoupled systems where waste preservation is recommended (acid-preservation and oven-drying). Deposition rate is derived from Kutti et al. (2007a). Carbon requirements are the sum of respiration rates (this study) and tissue growth rates (based on Nederlof et al. 2019). To calculate the number of individuals required, an individual geometric mean body weight of 2.35 mg AFDW for *Capitella* sp. and 2.64 mg AFDW for *O. craigsmithi* was used, which was based on the growth experiment reported in Nederlof et al. (2019).

	Capitella sp.			*O. craigsmithi*		
	Fresh	Acid	Dried	Fresh	Acid	
Deposition rate	2500	2500	2500	2500	2500	mg POC m^{-2} day^{-1}
Carbon requirement	16	11	13	26	5	mg C g^{-1} AFDW day^{-1}
Polychaetes required						
100%	152	224	188	97	511	g AFDW m^{-2}
	64643	95353	79935	36664	193632	ind. m^{-2}
20%	30	45	38	19	102	g AFDW m^{-2}
	12929	19071	15987	7333	38726	ind. m^{-2}

the bioremediation of fish waste is not yet understood. Next steps in understanding the full bioremediation capacity of benthic IMTA with *Capitella* sp. or *O. craigsmithi* should focus on the interactions that could be encountered at the farm level, like bacteria−polychaete interactions and the aggregation in mucus complexes. It should also be taken into account that opportunistic polychaete species are often characterized by a boom−bust population dynamics, whereby relatively small changes in organic loadings can have large impacts on the population (Ramskov & Forbes 2008). Polychaete cultivation densities aiming for 100% waste removal might therefore be risky. Based on the role of microbes and the risk of population collapses, polychaete cultivation densities required for 20% waste removal are therefore assumed to be more realistic and are also included in Table 4.5.

While in Nederlof et al. (2019), production potential (i.e. growth and survival) and body composition (i.e. fatty acid and amino acid profiles) of *Capitella* sp. and *O. craigsmithi* fed salmon faeces were evaluated, the present study shows their bioremediation potential for salmon−polychaete integrated systems. Combined, these studies highlight that both *Capitella* sp. and *O. craigsmithi* are interesting species to include in IMTA systems. The next steps should focus on cultivation and harvesting techniques, adjusted to the polychaete species selected. For decoupled integrated systems, this would mean that polychaete cultivation units should be developed, but other waste preservation methods should also be explored, as the bioremediation potential was reduced when polychaetes were fed either acidified or dried salmon faeces. Also for coupled integrated systems, the development of polychaete cultivation methods is still in its infancy, but some steps have been taken. Kinoshita et al. (2008) described, for example, a method whereby

individuals of *Capitella* sp. cultivated in a hatchery were introduced under a fish farm. Instead of introducing the polychaetes in coupled integrated systems, there are also opportunities to enhance native polychaete species, as shown by Jansen et al. (2019) for *Ophryotrocha* spp. and other species. This method can be compared with suspended mussel cultivation (Smaal 2002), in which an artificial substrate is provided (ropes in case of mussels, benthic trays in case of polychaetes) on which juveniles can settle and grow and which are eventually harvested. The fast colonization of benthic production systems shown by Jansen et al. (2019) in combination with the high bioremediation potential estimated in this study highlights the opportunity to boost native *Ophryotrocha* species as an extractive component in open water IMTA systems.

5 Conclusion

This study demonstrates that C requirements range between 11 and 16 mg C g AFDW^{-1} d^{-1} and between 5 and 26 mg C g AFDW^{-1} d^{-1} for *Capitella* sp. and *O. craigsmithi* respectively. N requirements range between 2 and 3 mg N g AFDW^{-1} d^{-1} and between 2 and 6 mg N g AFDW^{-1} d^{-1} for *Capitella* sp. and *O. craigsmithi* respectively. These values were in line with or higher than values reported for other polychaete species, highlighting the potential for fish-waste bioremediation by both *Capitella* sp. and *O. craigsmithi*. The highest values were observed for *O. craigsmithi* fed fresh salmon faeces. Preservation of salmon waste (either by oven-drying or acidification) reduced the bioremediation potential of both species, which could primarily be ascribed to reduced growth. This suggests the importance of polychaete–microbe interactions, and shows that in decoupled integrated systems, higher biomass is required to mitigate fish waste, compared to systems where the polychaetes can be cultivated directly underneath the cages (i.e. coupled IMTA). The polychaete densities required for bioremediation of fish waste lay within the ranges reported for wild populations, and development of benthic cultivation systems boosting these numbers of polychaetes above the natural numbers seems therefore a feasible option.

Acknowledgements

We thank Lerøy Vest AS and Marine Harvest AS for access to the farms and help with collection of the polychaetes. We also thank the technical staff of Austevoll Research Station for help with the experimental set-up and laboratory assistance. We thank the editor and 3 anonymous reviewers for their comments and suggested changes to earlier versions of this paper. This research was supported by Ocean Forest AS (Research and Development licence FD0036-1608763/Institute of Marine Research), the Norwegian species initiative and carried out as part of the PhD study of MAJ Nederlof (IPOP project TripleP@Sea KB-24-001-019 at Wageningen University).



Chapter 5

Application of polychaetes in (de)coupled integrated aquaculture: production of a high-quality marine resource

Marit A.J. Nederlof, Henrice M. Jansen,
Thomas G. Dahlgren, Jinghui Fang, Sonnich Meier,
Øivind Strand, Harald Sveier,
Marc C.J. Verdegem, Aad C. Smaal

Aquaculture Environment Interactions (2019), 11: 221-237

Abstract

Capitella sp. and *Ophryotrocha craigsmithi* received a diet of salmon faeces to evaluate their potential to convert fish waste into valuable marine products, e.g. ingredients for fish feed formulation. Production rate and body composition (focusing on fatty acid [FA] profiles) were determined for polychaetes fed fresh, acid-preserved or oven-dried salmon faeces to evaluate their application in (de)coupled integrated multi-trophic aquaculture (IMTA) systems. Coupled production refers to direct integration of fish and polychaetes within the same (eco)system, while in decoupled production, units can be spatially or functionally separated. For decoupled production, preservation of fish waste is recommended. Although diets contained relatively low polyunsaturated FA (PUFA) levels (5−9% of total FAs), both species were rich in PUFAs (> 30% of total FAs) and contained the essential FAs for fish. Feeding *Capitella* sp. the acid-preserved diet enriched its FA profile. Accumulation of PUFAs, *de novo* synthesis and/or transfer via bacterial biomass could have played a role in the upregulation of PUFA content. Amino acid profiles indicated that these polychaetes contained the amino acids essential for fish. Highest growth for both species was observed when fed fresh faeces, whereas preserved diets resulted in negative growth rates for *O. craigsmithi*, suggesting an important role of microbes in polychaete diets. Our results indicate that both species are potential valuable marine products. Given growth rates with different diets, *O. craigsmithi* seems more suitable for integration in coupled systems, while *Capitella* sp. is interesting for both coupled and decoupled integrated systems.

1 Introduction

Facing the challenge of feeding the growing world population, an important role for aquaculture is foreseen (Duarte et al. 2009, Kobayashi et al. 2015). However, the predicted expansion of the aquaculture sector generates several environmental concerns (Ottinger et al. 2016). For example, discharges of metabolic wastes, uneaten feed and faeces in cage aquaculture raise concerns about increasing nutrient loads to coastal ecosystems (Mente et al. 2006, Bostock et al. 2010, Holmer 2010). Expansion of the aquaculture sector also contributes to the growing need for high-quality feed (Tacon & Metian 2015). Ingredients traditionally used in fish feed, like fishmeal and oil, have become costly and limited, especially fish-oil (Gatlin et al. 2007, Froehlich et al. 2018). These traditional fish feed ingredients are rich in the essential n-3 long-chain polyunsaturated fatty acids (PUFAs), which need to be provided in fish diets (Izquierdo 2005, Tocher 2015). Although terrestrial-based plant materials are often suggested as alternative feed ingredients, they lack essential n-3 PUFAs (Miller et al. 2008). Furthermore, amino acid profiles are of importance for alternative resources for fish diets; in particular, the essential amino acids should be well balanced. Terrestrial plant-based alternatives are often deficient in the essential amino acids methionine and lysine, which must be supplemented in aquatic diets (Van Der Meer & Verdegem 1996, Gatlin et al. 2007). This highlights the need for sustainable and high-quality alternatives for fishmeal and oil for aquatic diets.

Integrated multi-trophic aquaculture (IMTA) systems are a promising approach to tackle bottlenecks of high nutrient loading and the need for high-quality ingredients. IMTA combines fed culture with extractive culture in such a way that waste produced by the fed culture becomes input for the extractive cultures (Troell et al. 2003, Neori et al. 2004, Chopin 2013a). By smart selection of extractive species, IMTA provides opportunities to re-use nutrients in waste products and to simultaneously produce ingredients for fish feed formulation, increasing productivity of the system (Chopin 2013a, Hughes & Black 2016). It should be noted that, in Europe, the produced ingredients (i.e. extractive species) cannot be re-used in diets for the same species as the fed species in the IMTA system, due to EU regulations (European Commission 2001).

To date, most research has focused on seaweeds and bivalves as extractive species (Fang et al. 2016b, Hughes & Black 2016, Buck et al. 2018). Seaweeds extract inorganic waste nutrients, and filter feeding bivalves are able to extract fine solid waste particles from the water column (Troell et al. 2003, Handå et al. 2012a, Wang et al. 2014). However, major ecological concerns are directed to the benthic ecosystem below fish farms, due to the deposition of relatively high quantities of organic waste such as feed and faeces (Sarà et al. 2006, Bannister et al. 2016). Extractive species that convert the solid waste fraction into valuable biomass are less studied.

Polychaetes have been suggested as extractive species in IMTA systems (Tsutsumi et al. 2005, Kinoshita et al. 2008, Brown et al. 2011, Fang et al. 2017), but their

potential has not been fully explored. The high level of PUFAs measured in several polychaete species (Marsh et al. 1989, Bischoff et al. 2009, Brown et al. 2011, Salvo et al. 2015a) make it interesting to explore the feasibility of using polychaetes to convert waste from fish farms into alternative resources for fish diets. Various opportunistic polychaete species are found in large densities underneath cage cultures in Norwegian fjords and coastal zones, showing their preference for organic-rich conditions (Kutti et al. 2007b, Eikje 2013, Bannister et al. 2014). *Capitella* sp. and *Ophryotrocha craigsmithi* were the most abundant polychaetes found under the fish farms visited in the current study, and were therefore selected for the laboratory trials. Both species occur at high densities beneath fish farms (Kutti et al. 2007b, Eikje 2013).

Species belonging to the genus *Capitella* are opportunistic, soft-sediment dwellers, classified as non-specialized subsurface deposit feeders, feeding on detritus and the associated microbes on this detritus (Fauchald & Jumars 1979, Tenore 1981, Findlay & Tenore 1982). The pioneering work of Tsutsumi et al. (2005) and Kinoshita et al. (2008) showed that cultivation of *Capitella* sp. underneath net pens in Kusuura Bay (Japan) resulted in rapid growth of the polychaete population and enhanced decomposition of organic matter in the enriched sediments, highlighting the potential of *Capitella* spp. as extractive species for IMTA systems.
Species belonging to the genus *Ophryotrocha* can be found in organically enriched soft sediments (Dahlgren et al. 2001), but the highest densities have been found in the deep sea on whale-falls (Dahlgren et al. 2006, Wiklund et al. 2009a,b, Wiklund et al. 2012, Salvo et al. 2014) and hard substrates underneath fish farms in Norway and Canada (Murray et al. 2012, Eikje 2013, Salvo et al. 2014). *Ophryotrocha* spp. are often found in communities that form a complex matrix of polychaetes, their mucus, organic waste and chemoautotrophic bacteria (Salvo et al. 2015a). Fatty acid (FA) profiles revealed that both organic wastes and bacteria contributed to the diet of *O. cyclops* collected underneath finfish aquaculture sites in Canada (Salvo et al. 2015a).

System- and site-specific characteristics determine the production success of extractive species in IMTA. In this study, polychaete species were selected with potential for cultivation in connection with Atlantic salmon (*Salmo salar*) aquaculture in Norway. Salmon aquaculture is dominated by open-water cage systems where wastes are discharged into the water column and eventually settle on the bottom (Bergheim 2012, Lekang et al. 2016, Clarke & Bostock 2017). Recently, efforts have been made to develop (semi-)enclosed sea farm systems where waste can be collected (Lekang et al. 2016, Klebert et al. 2018). (Semi-) enclosed systems provide opportunities to develop so called decoupled integrated systems. This term was introduced for aquaponics (Goddek et al. 2016) and refers to systems consisting of different compartments (i.e. fed species and extractive species) which are integrated as separate functional units. For these systems, spatial connection between the fish and extractive cultivation unit is not required, which provides the opportunity to collect organic wastes at the sea farm and cultivate extractive species on land or in separate sea tanks. To keep the diet for

the extractive species of sufficient quality in decoupled systems, preserving the organic wastes is recommended, since fish waste degrades quickly (Beristain 2005). Drying and acidification are relatively fast and easy to apply, which is important from a commercial perspective. Both methods are known to inactivate microbial activity (Luckstadt 2008, Betoret et al. 2016) and may therefore interfere with feeding activity of the polychaetes. Acidification is also known to change the content of the diet (Hardy et al. 1984, Özyurt et al. 2016), which may affect feeding activity, but also the body content of the polychaetes. For example, FA profiles of *Capitella* sp. eggs differed depending on the food source of the adults (Marsh et al. 1990). During digestion, FAs are generally released from lipid molecules, but they are not always completely broken down like other nutrients (e.g. proteins). Therefore, FAs can be incorporated and stored in the body tissue in their basic form. This can subsequently result in bioaccumulation of specific FAs and can reflect an animal's diet (Iverson 2009).

Compared to FA profiles, amino acid profiles of an organism are fairly constant. Amino acids are the building blocks of proteins, and their sequence is determined in the genome. Deficiencies of essential amino acids in an animal's diet reduce the rate of protein synthesis but do not lower the level of the amino acids in proteins, resulting in amino acid profiles that are relatively constant (Mente et al. 2002, Sissener 2018). Polychaete amino acid profiles are therefore assumed to remain similar irrespective of diet, but differences between species are to be expected (Limin et al. 2006).

In the current study, we evaluated the production rate and body composition of *Capitella* sp. and *O. craigsmithi* fed with fresh and preserved salmon waste in order to evaluate their application in coupled and decoupled IMTA systems. Growth and survival were determined to evaluate performance. Body composition, with the focus on FA profiles, was measured to evaluate the feasibility of including polychaetes in fish feed. Amino acid profiles of the 2 polychaete species were also measured and compared. Given the impact of drying and acidification on microbial activity and diet content, we hypothesized that feeding fresh salmon waste to *Capitella* sp. and *O. craigsmithi* would result in higher production and higher FA content compared to feeding preserved salmon waste. Due to the lack of data on both polychaete species with respect to their diet preference, possible differences in the response of both species to the dietary treatments were also tested.

2 Materials & Methods

2.1 Study site and species

Capitella sp. and *Ophryotrocha craigsmithi* were collected underneath a soft-bottom (125 m depth) and a hard-bottom (140 m depth) commercial Atlantic salmon (*Salmo salar*) farm, respectively, both located in the coastal area of western Norway. *Capitella* sp. was collected using a van Veen grab. To collect *O. craigsmithi*, 3 iron trays (1.2 × 1.2 × 0.1 m with a perforated base to allow water to pass through) covered with different plastic substrate were deployed underneath the salmon farm. Depth of tray deployment varied between 50 and

150 m. After being submerged for 3 wk, these trays where brought to the surface and all polychaetes present in the substrates were collected. Van Veen and benthic tray samples were gently rinsed and polychaetes were collected. Immediately after collection, polychaetes were placed in an aerated tank containing water from 200 m depth, ensuring similar salinity and water quality parameters as their natural habitat. The polychaetes were then transported to the experimental facilities at Austevoll Research Station (Institute of Marine Research, Norway). Two different *Ophryotrocha* species were collected, but in this experiment only the most abundant, *O. craigsmithi,* was included (species determination was confirmed using cytochrome c oxidase subunit I barcode data). The genus *Capitella* is represented by a group of morphologically similar (cryptic), but so far undescribed, species in Norwegian waters. The name '*Capitella capitata'* that is often used for capitellids under fish farms around the world (e.g. Hanson & Tenore 1981, Heip 1995) refers to a species described from Greenland (Blake 2009), and since genetic barcode data from the type locality of that species are not available, it is currently not known to what extent this species occurs in Norway. A taxonomic revision of *Capitella* species and other annelids in nutrient-rich habitats such as underneath fish farms, funded by the Norwegian species initiative, is ongoing (T. G. Dahlgren unpubl. data). We have therefore used the name '*Capitella* sp.' for the species investigated in this study.

Polychaetes were acclimatized for 2 d in flow-through, circular tanks receiving filtered (1 µm) seawater (34.8 ± 0.1 ‰, 8.7 ± 0.2°C) pumped from 200 m depth. During this acclimation period, they were fed with salmon faeces which were stored in a freezer (-20°C) and thawed before being fed.

At the end of the experiment, not enough polychaete material could be sampled to perform both FA and amino acid analyses. As amino acid profiles are not expected to be highly affected by the diets given, a new batch of individual *O. craigsmithi* and *Capitella* sp. were collected from the fish farming sites. These animals were not exposed to the experimental treatments and were solely analysed for amino acid profiles.

2.2 Treatments

A 3 × 2 factorial design was used to evaluate the effect of diet (i.e. fresh, acid-preserved and oven-dried salmon faeces) and polychaete species (i.e. *Capitella* sp. and *O. craigsmithi*) on growth, survival and final polychaete body composition (C, N and FAs).

For diet preparation, faeces were collected twice a week from salmon (individual weight ca. 2−4 kg) kept at the sea cage facility of Austevoll Research Station. Faeces were collected by stripping the fish. Using this method ensured that only faeces were collected and not uneaten feed or other particulate matter. After collection, faeces were directly centrifuged (6300 × *g* for 3 min; Eppendorf 5810R), and liquid was carefully removed. This process was repeated twice, after which the solid fraction was homogenized and used to formulate the experimental diets. The fresh centrifuged faeces diet was directly fed to the polychaetes. The acid diet was preserved by adding formic acid (80%) to the centrifuged faeces to create a pH

<4 (pH 3.4 ± 0.1). The acidified faeces were left for 24 h at room temperature, after which excessive liquid was carefully removed. Before feeding, the faeces were washed twice with seawater to reduce acidity. Washing was done by adding sea-water to the acidified faeces, centrifuging for 3 min (6300 × *g*) and removing leftover liquid. The dried diet was preserved by oven-drying the centrifuged faeces for 48 h at 100°C. Due to the difference in time span needed for the preservation of the diets (direct use, 24 h and 48 h), treatments started on 3 successive days, i.e. Day 1: fresh treatment, Day 2: acid treatment and Day 3: dried treatment.

2.3 Experimental design

At the start of the experiment, for each species, individuals of comparable lengths were selected (visually) from the acclimation tanks and divided over the experimental chambers. Simultaneously, polychaetes were collected as start sample to determine initial body weight and body composition (n = 3 × 30 individuals of each species). Polychaetes collected for the start samples were then placed in a tank with clean seawater for 2 d in order to clear their guts. Thereafter, samples were rinsed with deionized water and stored in the freezer (-20°C). Initial body composition and average individual weight were determined for the start samples and used in growth calculations (see *Section 2.4*).

Capitella sp. (initial weight 1.7 ± 0.1 mg ash-free dry weight [AFDW], mean ± SD) were kept in 1000 ml, cylindrical chambers (n = 66 ind. chamber^{-1}, n = 4 chambers treatment^{-1}). Inflow was at the top of the chamber, and outflow was positioned opposite to the inflow, approximately 1 cm above the bottom of the chamber. Glass marbles (5 mm, VWR) were added to each chamber (approximately 1 cm of the bottom was covered), to mimic natural substrates while providing the opportunity to observe the polychaetes during daily check-ups. Similar experimental chambers were used for *O. craigsmithi* (initial weight 1.9 ± 0.1 mg AFDW), but because of different behaviours (i.e. mucus production, community formation, active swimming in the water column) compared to *Capitella* sp., the design was adapted. Water inflow was positioned at the bottom of the chamber, and the outflow consisted of 2 larger openings (3 cm diameter) placed opposite each other at the top of the chamber and covered with mesh (250 µm) to prevent polychaetes escaping. To mimic natural substrate conditions, a pre-combusted stone was added to the chamber. To standardize between species, total biomass was kept similar using 50 individuals of *O. craigsmithi* per chamber (n = 4 chambers treatment^{-1}). Flow rate was set to 28 ± 2 ml min^{-1}, and the experimental chambers were kept in the dark during the experiment.

Polychaetes were fed 1 of the 3 experimental diets for a period of 4 wk. This time span was assumed to be long enough to distinguish potentially significant changes in FA profiles (Kirsch et al. 1998, Turner & Rooker 2005). Treatments were randomly assigned to the chambers using the random numbers function ('RAND()') in Microsoft Excel (2016). Animals were fed twice a week. Feed was provided in excess (~1.5 g chamber^{-1} feeding^{-1}), for which the amount was determined based on a pilot study. During the experiment, leftover feed was always observed,

confirming that feed was provided in excess. During each feeding, leftover feed was first removed and the chambers were cleaned, after which new feed was added. Flow was terminated for a minimum of 30 min to allow the feed to settle. During each feeding, diet samples were collected from each diet treatment (i.e. fresh, acid or dried faeces) (n = 6 samples $diet^{-1}$ $feeding^{-1}$) and stored in the freezer (-20°C up to 4 wk, following protocols described by Budge et al. 2006) before further analyses.
At the end of the experiment, polychaetes were counted to determine survival. Chambers were cleaned and polychaetes were left for 2 d in clean seawater, enabling them to empty their guts, before the final sample was collected. Sampled polychaetes were rinsed with deionized water and stored in the freezer (similar protocol as above) before further analyses.

2.4 Analytical analyses and calculations

Diet and polychaete samples were freeze-dried and then ground using a bullet mill. For each diet, samples collected over the time span of the experiment were pooled in such a way that each sample contained a replicate from each feeding, resulting in a total of 6 samples per diet for analyses.

2.4.1 Subsampling

Diets and polychaetes were analysed for dry matter (freeze-dried), ash (550°C for 6 h), gross energy (adiabatic bomb calorimetry; IKA-calorimeter C7000, ISO 9831), C and N content and FA profiles. Gross energy was expressed in kJ g^{-1} AFDW, and C, N and total FAs were expressed in g kg^{-1} AFDW. C and N samples were combusted with an element analyser (Flash 2000, Thermo Fisher) at 1020°C, in the presence of oxygen, to convert C and N to CO_2 and NOx, respectively. Thereafter, NOx was reduced to N_2 in a reduction column.
FA profiles were analysed according to the method described by Meier et al. (2006). Samples were methylated, and the respective FA methyl esters (FAMEs) were analysed on an HP-7890A gas chromatograph (Agilent) with a flame ionization detector (GC-FID). The FA 19:0 was added as an internal standard. Dry HCl in methanol (2.5 M) was used as a methylation reagent. FAMEs were extracted using 2 × 2 ml of hexane. The extracted hexane was diluted or concentrated to obtain a suitable chromatographic response. One µl was injected splitless (i.e. the split was open after 2 min), and the injection temperature was set to 280°C. The column was a 25 m × 0.25 mm fused silica capillary, coated with polyethylene-glycol of 0.25 µm film thickness, CP-Wax 52 CB (Varian-Chrompack). Helium (99.9999%) was used as mobile phase at 1 ml min^{-1} for 45 min and then increased to 3 ml min^{-1} for 30 min. The temperature of the FID was set at 300°C. The oven temperature was programmed to hold at 90°C for 2 min, increase from 90 to 165°C at 30°C min^{-1} and then to 240°C at 2.5°C min^{-1}, and was then held there for 35 min. Total analysis time was 75 min. Fifty-eight well-defined peaks in the chromatogram were selected and identified by comparing retention times with a FAME standard (GLC-463 from Nu-Chek Prep.) and retention index maps and mass spectral libraries (GC-MS) (www.chrombox.org/index.html) performed under the same chromatographic conditions as the GC-FID (Wasta & Mjøs 2013).

Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-463 mixture. The chromatograms were integrated using the EZChrom Elite software (Agilent Technologies). FAs are given as % of total FAs. Complete FA compositions (g kg^{-1} AFDW) of the 2 polychaete species fed the 3 different diets are provided in Appendix Table S5.1.
Additionally collected polychaete samples (i.e. not exposed to the experimental treatments, n = 4 × 30 individuals for *Capitella* sp. and n = 3 × 75 individuals for *O. craigsmithi*) were freeze-dried, and amino acid composition was determined after hydrolysis, using an ultra-performance liquid chromatochraphy system as described by Espe et al. (2014).

2.4.2 Calculations
Based on the number of individuals at the start and end of the experiment, survival (%) per chamber was calculated. AFDW of the polychaete samples was used to calculate total biomass gain per chamber. Average individual body weight (mg AFDW) at the start and end of the experiment was determined by dividing the total AFDW of a polychaete sample (either start or end) by the number of individuals in that sample. Specific growth rate (SGR, % d^{-1}) was calculated using the following formula:

$$SGR = ((\ln(W_f) - \ln(W_i)) / T) \times 100 \quad (1)$$

where W_f is the average individual final weight (mg AFDW), W_i is the average individual initial weight (mg AFDW), and T is the number of experimental days.

2.5 Statistical analyses
Statistical analyses were performed in R studio 3.4.0 and PRIMER v6. Prior to statistical analysis, residuals of the data were checked for homogeneity of variance and normality using Q−Q plots and Shapiro-Wilk and Levene tests. One-way ANOVA was used to test differences in composition between diets (C, N, energy, total FAs and FA profiles) and to test differences in growth (biomass gain and SGR) and survival within polychaete species when fed the 3 diets. Two-way ANOVAs were used to test the effect of diet, species and their interaction on polychaete final body composition (N, C, energy, total FAs and FA profiles). Due to the low sample size (n = 2), *O. craigsmithi* fed the dried diet was excluded from the 2-way ANOVA. Additionally, FA classes were compared, using all data, between the 2 polychaete species using a non-parametric Mann-Whitney *U*-test.
When assumptions of homogeneity of variance and normality where violated, data were transformed. If after transformation assumptions of homogeneity of variance and normality were still violated, we used a non-parametric Kruskal-Wallis test. If only the assumption of homogeneity of variance was violated, Welch's ANOVA was used. When the ANOVA tests were significant ($p < 0.05$), treatments were compared using Tukey's HSD post hoc multiple comparison tests. When results of Welch's ANOVA were significant, treatments were compared by the Games-Howell post hoc test. Significant results found with the non-parametric test were followed by Mann-Whitney *U*-test with Bonferroni correction.

To compare FA profiles of diets and polychaetes fed with these diets, a principal component analyses (PCA) was run on all data using PRIMER v6. Data were not transformed.

3 Results

3.1 Diets

Preservation affected diet composition (Table 5.1). Compared to the other 2 diets, preserving salmon faeces by acidification resulted in a significantly lower AFDW (Mann-Whitney *U*; $p < 0.01$), N (Tukey HSD; $p < 0.001$) and energy (Tukey HSD; $p < 0.001$) content, while the FA content was significantly higher (Tukey HSD; $p < 0.001$). Drying the salmon faeces at 100°C resulted in a significantly higher energy content (Tukey HSD; $p < 0.001$), but a lower FA content (Tukey HSD; $p < 0.001$), compared to the fresh and acid diets. C content did not differ among the 3 diets (Kruskal-Wallis; $p = 0.097$).

3.2 Growth and survival

The number of individuals in all experimental chambers with *Ophryotrocha craigsmithi* reduced over the experimental period. This was partly the result of mortality, but we cannot rule out that some animals might have escaped. Survival of *O. craigsmithi* was (mean ± SD) 80 ± 6, 75 ± 13 and 48 ± 39% for the fresh, acid-preserved and oven-dried diet, respectively, with no statistical differences between the dietary treatments (Welch's ANOVA; $p = 0.36$). Despite the reduced number of individuals at the end of the experimental period, total biomass of *O. craigsmithi* fed the fresh diet increased during the experimental period, resulting in a biomass gain of 133 ± 8 mg AFDW (Fig. 5.1).

Table 5.1
Composition of the experimental diets, i.e. fresh salmon faeces and salmon faeces preserved by acidification (formic acid, pH < 4) or by oven-drying at 100°C.

	Fresh	Acid	Dried	Significance
AFDW	777 ± 5^{a}	675 ± 47^{b}	774 ± 4^{a}	**
C	425 ± 12	424 ± 15	441 ± 15	ns
N	24 ± 1^{a}	15 ± 2^{b}	24 ± 1^{a}	***
Energy	18.0 ± 0.3^{b}	16.9 ± 0.4^{c}	18.5 ± 0.2^{a}	***
Total FA	62 ± 0.4^{b}	72 ± 0.6^{a}	59 ± 0.5^{c}	***

Value are given as mean ± SD (n = 6 samples treatment^{-1})
AFDW, Ash Free Dry Weight; C, Carbon; N, Nitrogen; FA, Fatty Acid
AFDW in g kg dry matter^{-1}, C, N and total FA in g kg AFDW^{-1}, energy in kJ g AFDW^{-1}
*** = P-value < 0.001, ** = P-value < 0.01, * = P-value < 0.05, ns = not significant
Means within a row lacking a common subscript letter (a, b, ..) differ significantly ($P < 0.05$)

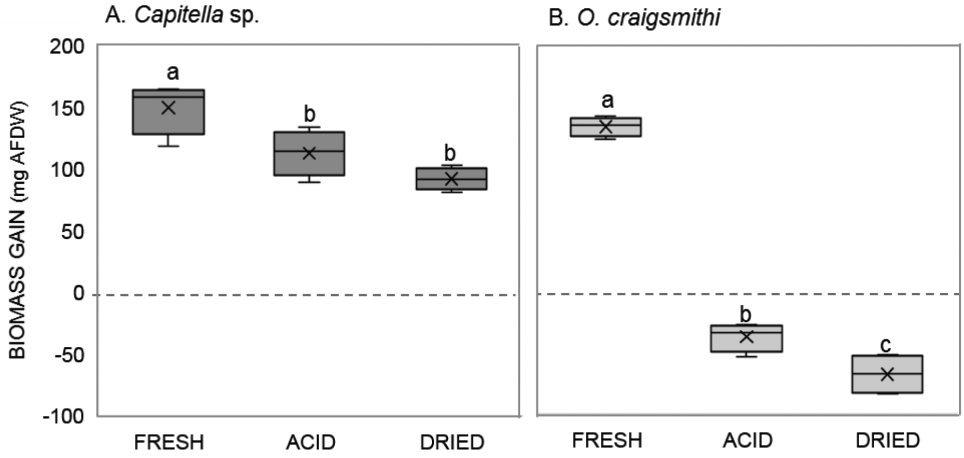

Figure 5.1
Biomass gain of (A) *Capitella* sp. and (B) *Ophryotrocha craigsmithi* fed 3 different diets (fresh, acid-preserved and oven-dried). Biomass gain was calculated based on total biomass at the start and end in each experimental chamber. Boxes represent the interval between the lower and the upper quartiles of the distributions. Crosses are the mean values (n = 4 tanks treatment^{-1}). Vertical bars show minimal and maximal values. Boxes lacking a common subscript letter differ significantly ($p < 0.05$)

Feeding *O. craigsmithi* the preserved diets resulted in a negative biomass gain (mg AFDW), which were both significantly lower compared to *O. craigsmithi* fed the fresh diet (Fig. 5.1; Tukey HSD; $p < 0.001$). The same pattern was observed for the SGR; *O. craigsmithi* fed the fresh salmon faeces had a significantly (Games-Howell; $p < 0.001$) higher SGR (mean ± SD; 3.56 ± 0.30% d^{-1}) than *O. craigsmithi* fed the preserved diets (mean ± SD; -0.70 ± 0.32 and -1.02 ± 1.06% d^{-1} for the acid and dried treatments, respectively). The greatest reduction in biomass was observed for *O. craigsmithi* fed the dried diet, which had a significantly lower biomass gain than both other diets (Fig. 5.1; Tukey HSD; $p = 0.001$).
In most experimental chambers with *Capitella* sp. (except for 2 chambers), the number of individuals increased, probably due to reproduction, resulting in >100% of individuals present at the end of the experiment compared to the start (respectively 104 ± 6%,121 ± 8% and 111 ± 9% for the fresh, acid and dried diets). At the end of the experiment, significantly more individuals were present in the chambers fed the acid diet compared to chambers assigned to the fresh treatment (Tukey HSD; $p < 0.05$). In all *Capitella* sp. treatments, biomass (mg AFDW) increased during the experimental period (Fig. 5.1). Total biomass gain (mg AFDW) was significantly higher in the fresh treatment compared to both other treatments (Fig. 5.1; Tukey HSD; $p < 0.001$). This was also reflected in the significantly higher SGR (Tukey HSD; $p < 0.01$) for *Capitella* sp. fed the fresh diet (2.56 ± 0.34% d^{-1}), compared to *Capitella* sp. fed the preserved diets (1.59 ± 0.36 and 1.58 ± 0.38% d^{-1} for the acid and dried treatments, respectively). Yet,

in contrast to *O. craigsmithi*, there was a net gain of *Capitella* sp. biomass with all diets tested.

3.3 Body composition

The initial and final body composition of *O. craigsmithi* and *Capitella* sp. are shown in Table 5.2. Except for the energy and total FA content, a species effect was observed for all other parameters (2-way ANOVA; $p < 0.001$). At the end of the experiment, *Capitella* sp. had a significantly higher AFDW, C and C:N ratio compared to *O. craigsmithi*, while the latter had a significantly higher N content (2-way ANOVA; $p < 0.001$).

Diets affected AFDW and FA content of the polychaetes (2-way ANOVA; $p < 0.05$). A significantly lower AFDW content was found when polychaetes were fed the acid diet, compared to polychaetes fed the dried diet (Tukey HSD; $p < 0.05$). Total FA content of polychaetes fed the dried diet (80 g kg^{-1} AFDW) was significantly lower compared to polychaetes fed the other 2 diets (Tukey HSD; $p < 0.05$). An interaction effect between diet and species was found for N and total FA body content (2-way ANOVA; $p < 0.05$). At the end of the experiment, *O. craigsmithi* fed the fresh or acid diet had a significantly higher N content compared to *Capitella* sp. fed the fresh, acid or dried diet (Tukey HSD; $p < 0.05$). Total FA content was highest in *Capitella* sp. fed the acid diet (125 g kg^{-1} AFDW), which was significantly higher compared to the other treatments (Tukey HSD; $p < 0.05$). Interestingly, *O. craigsmithi* fed the acid diet had one of the lowest FA contents (86 g kg^{-1} AFDW), which was significantly lower than the FA content of *Capitella* sp. fed the acid diet (Tukey HSD; $p < 0.001$). The lowest FA content was observed for *O. craigsmithi* fed the dried diet (73 g kg^{-1} AFDW).

Amino acid analyses showed that both species contained all essential amino acids (Fig. 5.2). Higher levels of each essential amino acid were observed for *O. craigsmithi* compared to *Capitella* sp. In particular, lysine and isoleucine were high in *O. craigsmithi* (respectively 32.7 ± 0.6 and 23.6 ± 1.2 mg g^{-1} dry matter) (Fig. 5.2).

3.4 FAs

FA composition of the diets was dominated by saturated FAs (SFAs, > 65%), followed by monounsaturated FAs (MUFAs, > 25%) (Table 5.3). PUFAs only had a minor contribution to the total FA composition of the diets (<10%), resulting in a low (< 0.15) PUFA:SFA ratio for the diets (Table 5.3). The main SFAs in the diets were 18:0, 16:0 and 22:0, while MUFAs were dominated by 18:1 (n-9) (Table 5.3).

Preservation of the salmon faeces affected the FA profile (Table 5.3). Fresh salmon faeces had a significantly lower SFA content compared to the preserved salmon faeces (Table 5.3; Mann-Whitney *U*; $p < 0.01$). MUFA and PUFA content, on the other hand, were significantly higher in the fresh faeces compared to the preserved faeces (Table 5.3; respectively Mann-Whitney *U* and Games-Howell; $p < 0.01$). The lowest PUFA content was measured in the faeces preserved by acidification, of which both total PUFAs and the n-6 PUFAs were significantly lower than in the fresh and dried faeces (Table 5.3; Games-Howell; $p < 0.001$).

Table 5.2
Analysed body composition of the two polychaete species (*Ophryotrocha craigsmithi* and *Capitella* sp.) fed three different diets (Fresh, Acid and Dried).

	O. craigsmithi				*Capitella* sp.				Significance		
		Final				Final					
	Initial	Fresh	Acid	Dried	Initial	Fresh	Acid	Dried	D	S	D*S
AFDW	508 ± 84	730 ± 41	705 ± 49	794 ± 69	922 ± 19	902 ± 17	841 ± 26	860 ± 24	*	***	ns
Carbon	396 ± 18	441 ± 14	428 ± 8	ND	469 ± 18	485 ± 9	488 ± 4	484 ± 4	ns	***	ns
Nitrogen	95 ± 5	112 ± 1^{a}	109 ± 3^{a}	ND	96 ± 3	99 ± 1^{b}	101 ± 3^{b}	99 ± 0.3^{b}	ns	***	*
C/N	4.2 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	ND	4.9 ± 0.1	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.0	ns	***	ns
Energy	ND	23 ± 2	21 ± 2	ND	15 ± 5	22 ± 1	22 ± 0.5	22 ± 0.2	ns	ns	ns
Total FA	ND	104 ± 11^{b}	86 ± 8^{b,c}	73	ND	97 ± 11^{b,c}	125 ± 4^{a}	80 ± 4^{c}	***	ns	***

Value are given as mean ± SD (For initial body composition n = 3 tanks treatment^{-1}. For final body composition n = 4 tanks treatment^{-1}, except for carbon and nitrogen content of *O. craigsmithi* acid, where n = 3 tanks treatment^{-1} and total FA of *O. craigsmithi* dried, where n = 2 tanks treatment^{-1}).
AFDW, Ash Free Dry Weight; FA, Fatty Acids; ND, Not Determined; D, Diet; S, Species
AFDW in g kg dry matter^{-1}, Energy in kJ gram AFDW^{-1}, Carbon, Nitrogen and Total FA in g kg AFDW^{-1}
*** = P-value < 0.001, ** = P-value < 0.01, * = P-value < 0.05, ns = Not Significant
Means within a row lacking a common subscript letter (a, b, ..) differ significantly (P < 0.05)

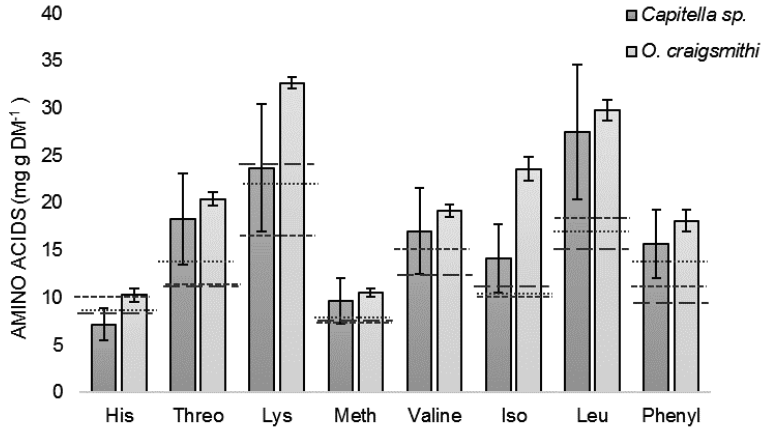

Figure 5.2
Essential amino acid body composition of *Capitella* sp. and *Ophryotrocha craigsmithi* (mg gram Dry Matter^{-1}) measured in this study (bars) and essential amino acid requirements (mg gram DM^{-1}) for Atlantic salmon (dashed line), tilapia (square dotted line) and Tiger shrimp (round dotted line) as reported in NRC (2013).
Bars are mean values, n = 4 samples for *Capitella* sp. and n = 3 samples for *O. craigsmithi*. Error bars represent standard deviations. His, histidine; Threo, threonine; Lys, lysine; Meth, methionine; Iso, isoleucine; Leu, leucine; Phenyl, phenylalanine.

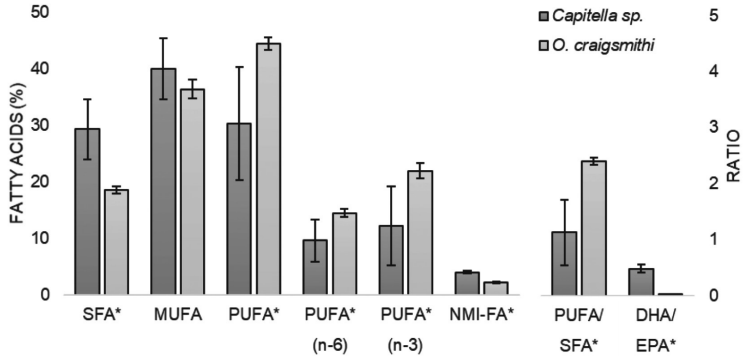

Figure 5.3
Fatty acid classes (% of total FA) and ratios (PUFA/SFA and DHA/EPA) per polychaete species (*Capitella* sp. and *Ophryotrocha craigsmithi*).
Bars are mean values, *Capitella* sp. n =12 samples, and *O. craigsmithi* n = 10 samples. Error bars represent standard deviations. Fatty acid classes or ratios with an asterisks (*) are significantly different between the two polychaete species ($p < 0.05$). All FAs (>0.1 % of total FA) were used to calculate the sum of SFA, MUFA, PUFA, PUFA (n-6), PUFA (n-3) or NMI. SFA, Saturated Fatty Acid; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; NMI-FA, Non-Methylene Interrupted Fatty Acid; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid.

Table 5.3
Fatty acid composition (% of total FA) of the diets.

FA (% of total FA)	Fresh	Acid	Dried	Significance
SFA	67 ± 2[a]	71 ± 1[b]	71 ± 1[b]	**
14:0	2.4 ± 0.2	2.6 ± 0.0	2.5 ± 0.2	ns
16:0	18.6 ± 0.6[b]	20.8 ± 0.2[a]	19.2 ± 0.9[b]	***
18:0	20.9 ± 0.9	21.8 ± 0.3	22.5 ± 0.9	ns
20:0	4.9 ± 0.2	5.2 ± 0.1	5.3 ± 0.2	ns
22:0	18 ± 1	18.4 ± 0.3	20 ± 1	ns
24:0	0.95 ± 0.03[b]	1.04 ± 0.01[a]	1.04 ± 0.02[a]	**
MUFA	33 ± 2[a]	29 ± 1[b]	29 ± 1[b]	**
18:1 (n-7)	1.33 ± 0.07	1.28 ± 0.03	1.20 ± 0.07	ns
18:1 (n-9)	14 ± 1	13 ± 1	12 ± 0.4	ns
20:1 (n-9)	2.1 ±0.09[a,b]	2.2 ± 0.04[a]	1.97 ± 0.06[b]	**
22:1 (n-11)	2.89 ± 0.06[a]	2.91 ±0.05[a]	2.73 ±0.03[b]	***
24:1 (n-9)	1.17 ± 0.01[c]	1.29 ±0.02[a]	1.20 ±0.02[b]	***
PUFA	9 ± 0.4[a]	5 ± 0.2[c]	6 ± 0.2[b]	***
PUFA (n-6)	4.7 ± 0.3[a]	2.3 ± 0.1[c]	3.6± 0.1[b]	***
18:2 (n-6)[1]	4.3 ±0.2[a]	2.0 ±0.2[c]	3.3 ±0.1[b]	***
20:4 (n-6)[2]	0.07 ± 0.004[a]	0.03 ± 0.005[c]	0.04 ± 0.003[b]	***
PUFA (n-3)	3.6 ± 0.1[a]	2.3 ±0.1[b]	2.4 ± 0.1[b]	***
18:3 (n-3)[3]	0.8 ± 0.08[a]	0.3 ± 0.02[c]	0.4 ± 0.02[b]	***
20:5 (n-3)[4]	0.6 ± 0.03[a]	0.4 ± 0.01[b]	0.4 ± 0.02[b]	***
22:6 (n-3)[5]	0.8 ± 0.01[a]	0.4 ± 0.02[b]	0.4 ± 0.02[b]	***
NMI	0.4 ± 0.02[a]	0.4 ± 0.01[a]	0.4 ± 0.03[b]	**
PUFA/SFA	0.13 ± 0.009[a]	0.07 ± 0.004[c]	0.09 ±0.004[b]	***
DHA/EPA	1.32 ± 0.09[a]	1.19 ± 0.02[b]	1.18 ± 0.04[b]	*

Only FA contributing more than 1 % of the total FA are considered here, and [1]LA, [2]AA, [3]ALA, [4]EPA, [5]DHA. All FAs (>0.1 % of total FA) were used to calculate the sum of SFA, MUFA, PUFA, PUFA (n-6), PUFA (n-3) or NMI.
Values are given as mean ± SD (n = 6 samples diet^{-1}, except for the acid diet where n = 4 samples diet^{-1}).
FA, Fatty Acid; SFA, Saturated Fatty Acid; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; NMI, Non-Methylene Interrupted; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic Acid; AA, Arachidonic Acid; ALA, α-Linolenic Acid.
*** = P-value < 0.001, ** = P-value < 0.01, * = P-value < 0.05, ns = Not Significant
Means within a row lacking a common subscript letter (a, b, ..) differ significantly (P < 0.05).

All FA classes except MUFAs were significantly different between the 2 polychaete species (Fig. 5.3; Mann-Whitney U; $p < 0.05$). FA composition of *Capitella* sp. was dominated by MUFAs (40 ± 5% of total FAs), followed by PUFAs (30 ± 10% of total FAs) and SFAs (29 ± 5% of total FAs; Fig. 5.3). *O. craigsmithi* was rich in PUFAs (44 ± 1% of total FAs) and MUFAs (36 ± 2% of total FAs; Fig.5.3). Both n-3 and n-6 PUFAs were significantly higher in *O. craigsmithi* than in *Capitella* sp. (Mann-Whitney U; $p < 0.01$). The difference in FA classes between the polychaete species is also reflected in their PUFA: SFA ratio, which was significantly higher for *O. craigsmithi* (Mann-Whitney U; $p < 0.001$).
An interaction effect between species and diet was found for all FA classes (Table 5.4; 2-way ANOVA; $p < 0.05$). This interaction effect revealed that except for non-methylene interrupted (NMI) FAs, *Capitella* sp. fed the fresh or dried diet significantly differed in FA classes from the other treatments (Table 5.4; Tukey

Table 5.4
Fatty acid composition (% of total FA) of the two polychaete species (*Capitella* sp. and *Ophryotrocha craigsmithi*) fed with the three different diets (Fresh, Acid and Dried).

		Capitella sp.			*O. craigsmithi*			Significance		
		Fresh	Acid	Dried	Fresh	Acid	Dried	D	S	D*S
SFA		30±3^{b}	23±1^{c}	35±2^{a}	18±0.4^{d}	19±0.5^{d}	19	***	***	***
	14:0	6.6±0.5^{a}	4.8±0.4^{b}	7.3±0.8^{a}	1.2±0.1^{c}	1.0±0.1^{c}	0.96	***	***	**
	16:0	13±1^{a,b}	10±1^{c}	14±1^{a}	12.6±0.4^{b}	12.9±0.3^{a,b}	12.9	***	ns	***
	18:0	5.0±0.6^{b}	4.0±0.3^{c}	6.3±0.3^{a}	3.2±0.1^{c}	3.5±0.2^{c}	4.0	***	***	**
	20:0	0.9±0.1^{b}	0.7±0.1^{c}	1.1±0.1^{a}	0.1±0.01^{d}	0.1±0.02^{d}	0.2	***	***	*
	22:0	1.2±0.6	1.3±0.2	2.4±0.5	0.08±0.03	0.2±0.1	0.4	**	***	ns
MUFA		45±1^{a}	33±1^{c}	42±2^{a}	38±2^{b}	36±1^{b,c}	35	***	***	***
	16:1 (n-7)	3.2±0.1	2.3±0.2	2.9±0.1	4.1±0.6	4.0±0.7	4.1	*	***	ns
	16:1 (n-9)	0.8±0.04^{c}	0.6±0.04^{c}	0.7±0.07^{c}	3.1±0.2^{a}	3.2±0.1^{a}	2.8	ns	***	*
	18:1 (n-7)	4.2±0.1^{b}	3.2±0.1^{b}	4.0±0.2^{b}	9.7±0.6^{a}	9.8±0.7^{a}	10.6	ns	***	*
	18:1 (n-9)	20±1^{a}	14±0.4^{c}	17±1^{b}	11±1^{d}	9±1^{d}	8	***	***	**
	20:1 (n-7)	0.9±0.04^{b}	0.6±0.02^{c}	0.8±0.02^{b}	0.9±0.03^{a,b}	1.0±0.08^{a}	1.3	***	***	***
	20:1 (n-9)	3.7±0.2^{a}	2.6±0.1^{c}	3.2±0.1^{b}	1.0±0.2^{d}	0.6±0.1^{d}	0.4	***	***	***
	20:1 (n-11)	4.9±0.5^{a}	3.7±0.1^{b}	5.4±0.3^{a}	4.0±0.1^{b}	4.1±0.2^{b}	4.6	***	***	***
	22:1 (n-9)	0.8±0.02^{b}	0.5±0.04^{c}	0.8±0.05^{b}	1.4±0.1^{a}	1.6±0.2^{a}	1.8	ns	***	***
	22:1 (n-11)	2.5±0.1^{a}	1.7±0.1^{c}	2.2±0.1^{b}	0.7±0.2^{d}	0.4±0.04^{e}	0.3	***	***	***
PUFA		25±2^{b}	43±2^{a}	23± 4^{b}	44±2^{a}	45±1^{a}	45	***	***	***
	16:4 (n-1)	3.5±0.4^{b,c}	3.1±0.1^{c}	4.3±0.4^{a}	3.5±0.4^{b,c}	4.1±0.3^{a,b}	4.2	***	ns	*
	18:5 (n-1)	0.7±0.1^{c}	0.7±0.01^{c}	0.8±0.1^{c}	1.9±0.2^{b}	2.2±0.2^{a}	2.5	*	***	*
PUFA (n-6)		8±1^{b}	14±0.4^{a}	6±2^{b}	15±0.5^{a}	14±0.2^{a}	16	***	***	***
	18:2 (n-6)[1]	4.6±0.8^{b}	7.8±0.2^{a}	3.4±1.0^{b,c}	3.2±0.4^{c}	2.3±0.2^{c}	2.6	***	***	***
	20:2 (n-6)	2.4±0.2^{b}	3.8±0.1^{a}	2.0±0.5^{b,c}	2.1±0.4^{b}	1.4±0.1^{c}	1.2	***	***	***
	20:4 (n-6)[2]	0.5±0.1	1.4±0.1	0.4±0.2	7.7±0.4	8.5±0.2	10.0	***	***	ns
PUFA (n-3)		8±1^{b}	21±1^{a}	7±2^{b}	22±1^{a}	23±0.3^{a}	20	***	***	***
	18:3 (n-3)[3]	1.1±0.2^{b}	2.6±0.2^{a}	1.0±0.4^{b}	1.0±0.2^{b}	0.6±0.1^{b}	0.4	***	***	***
	20:5 (n-3)[4]	3.1±0.2^{c}	9.4±0.6^{b}	2.9±1.1^{c}	18±2^{a}	20±0.5^{a}	18	***	***	***
	21:5 (n-3)	0.5±0.04^{b}	1.1±0.1^{a}	0.5±0.2^{b}	0.3±0.02^{c}	0.3±0.02^{c}	0.3	***	***	***
	22:5 (n-3)	0.6±0.1^{c}	1.6±0.1^{a}	0.5±0.2^{c}	0.9±0.1^{b}	0.9±0.02^{b}	0.7	***	ns	***
	22:6 (n-3)[5]	1.6±0.3^{b}	5.1±0.4^{a}	1.2±0.4^{b,c}	0.6±0.1^{c}	0.5±0.1^{c}	0.2	***	***	***
NMI		4.1±0.3^{a,b}	3.8±0.1^{b}	4.2±0.1^{a}	2.2±0.1^{c}	2.22±0.02^{c}	2.6	*	***	*
	22:2 NMI	1.8±0.2^{a}	1.6±0.1^{b}	1.9±0.1^{a}	1.0±0.1^{c}	1.0±0.03^{c}	1.2	**	***	**
PUFA/SFA		0.8±0.1^{c}	1.9±0.2^{a}	0.7±0.2^{c}	2.4±0.1^{b}	2.4±0.1^{b}	2.3	***	***	***
DHA/EPA		0.51±0.06	0.54±0.04	0.39±0.01	0.03±0.007	0.02±0.005	0.01	***	***	ns

Only FA contributing more than 1 % of the total FA are considered here, and [1]LA, [2]AA, [3]ALA, [4]EPA, [5]DHA. All FAs (>0.1 % of total FA) were used to calculate the sum of SFA, MUFA, PUFA, PUFA (n-6), PUFA (n-3) or NMI.
Values are given as mean ± SD (n = 4 tanks treatment^{-1}, except for *O. craigsmithi* dried where n = 2 tanks treatment^{-1}).
FA, Fatty Acid; SFA, Saturated Fatty Acid; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; NMI, Non-Methylene Interrupted; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic Acid; AA, Arachidonic Acid; ALA, α-Linolenic Acid.
*** = P-value < 0.001, ** = P-value < 0.01, * = P-value < 0.05, ns = Not Significant
D, Diet; S, Species. Means within a row lacking a common subscript letter (a, b, ..) differ significantly (P < 0.05).

HSD; $p < 0.05$); MUFA content was significantly higher (Tukey HSD; $p < 0.001$), while PUFA content was significantly lower (Tukey HSD; $p < 0.001$). This was also reflected in the n-3 and n-6 PUFAs, which were both lowest for *Capitella* sp. fed the fresh and dried diets (Tukey HSD; $p < 0.001$). An interaction effect between diet and species was also observed for the PUFA:SFA ratio (2-way ANOVA; $p < 0.05$). Feeding *Capitella* sp. with the acid diet significantly increased their PUFA:SFA ratio compared to *Capitella* sp. fed with the fresh or dried diets (Tukey HSD; $p < 0.001$). The lowest docosahexaenoic (DHA):eicosapentaenoic acid (EPA) ratio was observed in *O. craigsmithi* fed the dried diet (Table 5.4).

The PCA run on all samples (Fig. 5.4) separated the polychaete samples from the diet samples. In addition, samples from the 2 polychaete species were also separated from each other (Fig. 5.4). The first principal component (PC1) explained 81.2% of the sample distribution and was mainly driven by, in order of relative importance, 22:0, 18:0, 20:5 (n-3), 16:0 and 18:1 (n-7). PC2 explained 15.7% of the sample distribution and was driven by 20:5 (n-3), 18:1 (n-9), 20:4 (n-6), 14:0, 22:0, 18:0 and 18:1 (n-7). Furthermore, the PCA showed that FA profiles of *O. craigsmithi* were independent of the diets given, which was not the case for *Capitella* sp. *Capitella* sp. fed the acid diet differed in FA profile from *Capitella* sp. fed the fresh or dried diets.

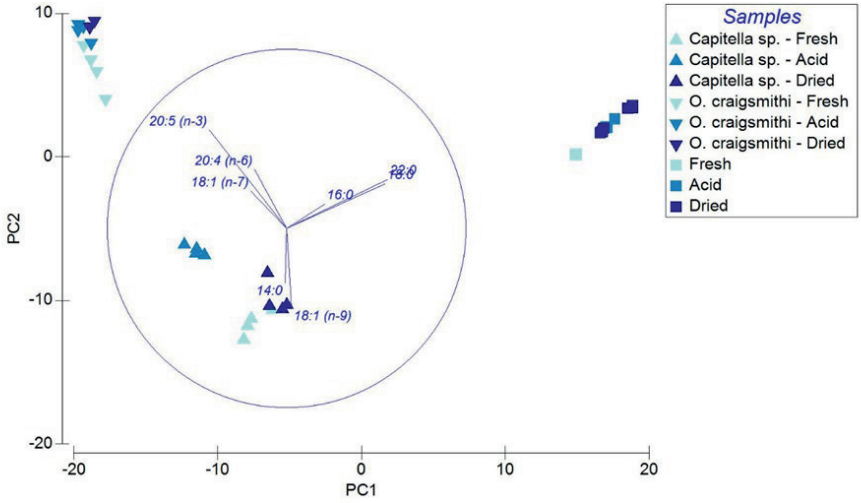

Figure 5.4

Principle component analyses (PCA) run with FA profiles of all samples, including the polychaetes (*Capitella* sp. and *Ophryotrocha craigsmithi*) and the diets (Fresh, Acid and Dried). PCA was run on untransformed data. Vectors show correlations of >0.2.

For 8 of the 9 FAs mainly driving the differences between the polychaete species in the PCA, a significant species effect was found (Table 5.4; 2-way ANOVA; $p < 0.05$); *Capitella* sp. had a significantly higher content of 14:0, 18:1 (n-9), 18:2 (n-6), 20:2 (n-6) and 22:6 (n-3), while *O. craigsmithi* was richer in 18:1 (n-7), 20:4 (n-6) and 20:5 (n-3). The 2 species did not differ in 16:0 (Table 5.4; 2-way ANOVA; $p = 0.067$). Interestingly, for 7 of the 9 FAs mainly driving the difference between species in the PCA, *Capitella* sp. fed the acid diet was significantly different from the other treatments (Table 5.4, Tukey HSD; $p < 0.05$).

4 Discussion

This study shows that, although diets fed to the polychaetes contained relatively low levels of PUFAs (5–9%), high levels were observed in the polychaetes (> 30%), including the FAs essential for fish. These results indicate the potential for producing high-quality marine resources using *Capitella* sp. and *Ophryotrocha craigsmithi* as extractive species in integrated aquaculture systems. Especially for *O. craigsmithi*, we observed a stable FA profile with high levels of EPA and arachidonic acid (AA). Feeding *Capitella* sp. with acid-preserved salmon feces enhanced their FA profile in terms of essential FAs. Both species showed highest growth when fed fresh salmon feces. These differences in growth and composition between diets and species have implications for application in coupled and decoupled IMTA systems.

4.1 Polychaetes as high-quality marine resources?

O. craigsmithi and *Capitella* sp. used in the current study were rich in PUFAs (> 30% of total FAs) and contained the essential FAs for fish, i.e. α-linolenic acid (ALA, 18:3 n-3), linoleic acid (LA, 18:2 n-6), AA (20:4 n-6), EPA (20:5 n-3) and DHA (22:6 n-3), making these polychaetes interesting candidates as alternative ingredients for fish diets. Freshwater fish and salmonids can produce AA, EPA and DHA when the precursors LA and ALA are provided via the diets. For freshwater fish and salmonids, part of their FA requirement can therefore be fulfilled when C_{18} PUFAs are present in the diet, but the C_{20} and C_{22} PUFAs are often nutritionally more effective. Atlantic salmon and other marine fish have a restricted activity of Δ5 and Δ6 desaturase and elongase and therefore AA, EPA and DHA are required in their diets (Ruyter et al. 2000, Izquierdo 2005, Tocher 2010, Tocher 2015). Various alternatives for fish-oil have been explored (reviewed by Gatlin et al. 2007). Most alternatives proposed from terrestrial origin do contain ALA and LA, but lack AA, EPA and DHA (Table 5.5), making them less suitable for diets of marine finfish, except for recent advances in the genetically modified crop *Camelina sativa*, which now has the potential to include EPA and DHA (Betancor et al. 2017) (Table 5.5). Alternatives proposed from marine origin, including marine polychaetes, often contain EPA and DHA (Table 5.5), and are therefore interesting for marine finfish diet formulation.

Table 5.5
Fatty acid composition in % of total FA (g kg DM^{-1} in parentheses) of fish-oil and potential alternatives for fish-oil reported in literature.

	SFA	MUFA	PUFA (n-6)	PUFA (n-3)	LA	ALA	AA	EPA	DHA	Ref.
Fish oil*	19-31 (170-277)	25-62 (223-554)	1.3-3	12-31 (107-277)	1.1-1.7 (10-15)	0.3-0.8 (3-7)	0.1-1.6 (0.9-14)	5-17	3-13	1
Vegetable oils*										
Crude palm oil	49 (463)	37 (350)	9 (85)	0.2 (1.9)	9 (85)	0.2(1.9)	ND	-	-	2
Soybean oil	14 (132)	23 (217)	51 (482)	7 (66)	51(482)	7 (66)	-	-	-	2
Rapeseed oil	5 (48)	63 (602)	20 (191)	12 (115)	20(191)	12(115)	-	-	-	2
Sunflower oil	10 (95)	20 (189)	66 (624)	0	66(624)	-	-	-	-	2
GM Camelina seed oil	16	20	31	33	18-21	13-15	2	6-11	5-8	3,4
Rendered animal products*										
Poultry	29 (260)	43 (385)	20 (179)	1 (9)	20(179)	1 (9)	-	-	-	2
Pork lard	39 (349)	44 (393)	10 (89)	1 (9)	10 (89)	1 (9)	-	-	-	2
Beef tallow	48 (429)	41 (366)	3 (27)	0.6 (5.4)	3 (27)	0.6(5.4)	-	-	-	2
Marine micro algae										
Schizochytrium sp.	9	2	18	46	ND	ND	1	0.8	43	5
Marine macro algae										
Ascophyllum nodosum	NI	NI	22 (10)	8 (4)	11 (5)	2 (0.7)	10 (5)	4 (2)	ND	6
Undaria pinnatifida	NI	NI	21 (4)	35 (6)	4 (0.8)	7 (1)	16 (3)	16 (3)	ND	6
Ulva lactuca	NI	NI	32 (7)	32 (7)	25 (6)	20 (4)	2 (0.4)	1 (0.2)	ND	6
Polychaetes										
Hediste diversicolour	NI	NI	NI	NI	3 (0.8)	2 (0.5)	5 (1)	39 (11)	4	7
Capitella sp.	23-35	33-45 (29-40)	6-14	7-21	3-8	1-3 (0.7-3)	0.5-1 (0.3-1.5)	3-9	1-5	8
Ophryotrocha craigsmithi	18-19 (10-14)	35-38 (19-29)	14-16 (8-11)	20-23 (11-17)	2-3 (1-2)	0.4-1 (0.2-1)	8-10 (5-6)	18-20 (10-14)	0.2-0.5 (0.1-0.5)	8

1. NRC (2013); 2. Turchini et al. (2009); 3. Ruiz-Lopez et al. (2014); 4. Betancor et al. (2017); 5. Sarker et al. (2016); 6. Van Ginneken et al.(2011); 7. Bischoff et al. (2009); 8. This study

* For conversion from % of total FA to g kg DM^{-1} FA concentrations stated in CVB (2018) were used.GM, Genetically Modified; NI, No Information; ND, Not Detectable; SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids; PUFA, Polyunsaturated Fatty Acids; LA, Linoleic Acid; ALA, α-Linolenic Acid; AA, arachidonic acid; EPA, eicosapentaenoic; DHA, docosahexaenoic acids

Although both polychaete species contained the FAs essential for fish, a distinctly different FA signature was observed based on the PCA and statistical comparison between the 2 species. It should be noted that statistical comparisons of 2 species are not always desirable, in particular when studying adaptation (Garland Jr & Adolph 1994). In the current study, species were statistically compared with the aim of evaluating their potential as ingredients for aquatic diets. *O. craigsmithi* was richer in both n-3 and n-6 PUFAs. In particular, high levels of EPA (19%) and AA (8.5%) were observed for *O. craigsmithi*. Salvo et al. (2015a) also reported high levels of EPA and AA in *O. cyclops* collected underneath aquaculture farms in Newfoundland (Canada), but reported lower values (EPA:<15% and AA: 4%) than observed in the current study. Similar to Salvo et al. (2015a), we also found relatively low levels of DHA (0.2−0.6%) for *O. craigsmithi*, resulting in a low DHA:EPA ratio (0.02). The optimal DHA:EPA ratio reported for several marine finfish (e.g. sea bream, sea bass, halibut and turbot) ranges between 1.3 and 2.08 (Rodŕiguez et al. 1998, Sargent et al. 1999, Xu et al. 2016), but for Atlantic salmon it has been shown that when diets lack DHA, this can be converted from EPA (Bou et al. 2017). In the current study, *Capitella* sp. was 6 times richer in DHA compared to *O. craigsmithi*. The DHA content of *Capitella* sp. was higher in our study compared to that of Marsh et al. (1990), who reported a DHA content of <1%. Nevertheless, they reported EPA contents of 22%, which is more than 4 times higher than the EPA content of *Capitella* sp. measured in this study (5%). One possible explanation for these differences is that the *Capitella* sp. used by Marsh et al. (1990) and the *Capitella* sp. in our study might be different species.

Diets fed to the polychaetes contained low levels (<1%) of EPA, DHA and AA, while high levels were observed in the polychaetes themselves. Salvo et al. (2015a) also observed high proportions of AA, EPA and 20:2 n-6 in the body of *O. cyclops*, while low levels were found in its food sources. To explain this result, Salvo et al. (2015a) proposed 3 hypotheses: (1) even though low levels of certain FAs are present in food sources, accumulation in body tissue may result in increased levels measured in the polychaetes; (2) polychaetes contain enzymes which enable *de novo* synthesis of particular FAs; and (3) these FAs could have been transferred from bacterial biomass. The diets used in the current study did contain EPA, DHA and AA, albeit at low levels, and therefore all 3 hypotheses suggested by Salvo et al. (2015a) could have played a role. Nevertheless, the high levels of AA and EPA in *O. craigsmithi* compared with *Capitella* sp. suggests an active enzyme system in *O. craigsmithi* that is able to strongly modify FAs from its diet through elongation and desaturation (Monroig et al. 2013), resulting in an enrichment of the salmon waste by *O. craigsmithi* into essential FAs. *De novo* synthesis of particular long-chain FAs, like EPA, have been demonstrated for some marine polychaetes, including *Arenicola marina* and *Nereis virens* (García-Alonso et al. 2008, Olive et al. 2009, Pairohakul 2013). In our study, FA profiles of polychaetes at the start of the experiment were lacking, and it is therefore unknown if the polychaetes collected underneath salmon farms may already have had enhanced levels of certain FAs, if *de novo* synthesis did take place and/or if FAs were taken up via bacterial biomass.

FA profiles of *O. craigsmithi* were less influenced by the diets compared to *Capitella* sp. The multivariate analyses showed that the FA profile of *Capitella* sp. changed when fed with the acid diet; in particular, the amount of long-chain PUFAs increased. From an aquaculture point of view, feeding *Capitella* sp. with acidified salmon faeces enhanced its FA profile, since a significant increase in ALA, LA, AA, EPA and DHA was observed. The influence of diet on *Capitella* sp. FA profiles was also shown by Marsh et al. (1990). They reported that FA profiles of *Capitella* sp. eggs could be distinguished based on the FA profile of diets fed to the individuals producing the eggs. Although in the current study a significantly higher total FA content was observed for the acid diet compared to the fresh and dried diets, the essential FAs were similar to the dried diet and even lower than the fresh diet (in terms of % of total FAs). Using formic acid to produce fish silage has also been shown to result in a decrease in PUFAs, including DHA, compared to fresh fish (Özyurt et al. 2016). It remains unclear why essential FAs (in terms of % of total FAs) increased in the *Capitella* sp. fed with the acid diet. Gene expression of the desaturation and elongation genes may give more insights, but was beyond the scope of this study.

4.2 Growth performance of polychaetes

Based on the FA profiles, it can be concluded that both *O. craigsmithi* and *Capitella* sp. are high-quality marine resources. Besides a suitable FA profile, growth performance on fish waste also determines the potential use of these polychaete species in integrated systems. This study shows that both polychaete species can grow on salmon waste. Other polychaete species studied in integrated aquaculture settings that can grow on fish feces are *Nereis virens* (halibut feces) (Brown et al. 2011), *Hediste diversicolor* (eel sludge) (García-Alonso et al. 2008) and *Perinereis nuntia vallata* (flounder feces) (Honda & Kikuchi 2002). Growth rates (SGRs) reported in those studies ranged between 0.45 and 2.09% d^{-1}, which were lower than the highest SGR of *O. craigsmithi* fed fresh salmon feces in our study (3.6% d^{-1}). Given the negative growth rates for *O. craigsmithi* fed the preserved diets, it is questionable if *O. craigsmithi* can be cultivated on salmon faeces preserved by drying or by acidification. This has implications for the potential use of *O. craigsmithi* in decoupled integrated aquaculture systems, since preservation of fish waste in these systems is recommended. In contrast, *Capitella* sp. seems to thrive on both preserved and fresh fish waste, as biomass increased in all treatments, although the highest growth rate for this species was also achieved with fresh salmon feces. Successful biomass production of *Capitella* sp. on fish wastes was also shown in a study by Tsutsumi et al. (2005), where *Capitella* sp. was cultivated underneath a fish farm in Kuusura Bay, Japan. The highest biomass production was observed in December and January, during which biomass gain was approximately 40−50 g wet weight m^{-2} mo^{-1}. Converting results of the current study gives a biomass gain of 58 g wet weight m^{-2} mo^{-1} for *Capitella* sp. fed with the fresh diet, indicating that the results of the current study are comparable to biomass production obtained in the field. Interestingly, in our study, reproduction of *Capitella* sp. was observed in all treatments. Members of the genus *Capitella*

are opportunistic, and depending on sediment quality they can start reproduction at the age of 4 wk, after which they are known for continuous reproduction (Grassle & Grassle 1976, Levin et al. 1996, Linton & Taghon 2000). They are described as r-strategists, being able to reproduce fast under unstable conditions (Tsutsumi et al. 2005). In our study, highest reproduction was observed in the acid treatment. The acid diet had the lowest quality in terms of N and energy, and this contradicts with studies reporting fast reproduction for *Capitella* sp. in organically enriched sediments (Bridges et al. 1994, Levin et al. 1996, Linton & Taghon 2000). It remains unclear why we observed the highest reproduction of this species in the acid diet.

We had hypothesized that preserving salmon faeces would inactivate microbial activity. Although their exact role is not fully understood, there are indications that microbes play a role in the diets of the 2 polychaete species used in the current study (Findlay & Tenore 1982, Wiklund et al. 2009a), and this might be an explanation for the lower performance on preserved waste. The difference in performance between *O. craigsmithi* and *Capitella* sp. may indicate that the role of microbes in the diet of *O. craigsmithi* is more profound. This is supported by Wiklund et al. (2009a) who suggested, based on the P-type jaw structure of *O. craigsmithi*, that microbes, in particular mat-forming large filamentous bacteria, make up an important part of the diet of *O. craigsmithi*. Also, it was shown that underneath fish farms in Canada, part of the diet of *O. cyclops* consists of bacterial mats, but the interaction between polychaete communities, chemoautotrophic bacteria and organic waste underneath fish farms is not fully understood (Salvo et al. 2015a). Furthermore, acidification of waste changed the quality of the diet. Although deterioration can be avoided, acidification is also known to break down proteins in short-chain peptides, and free amino acids (Hardy et al. 1984), which could have disappeared from the preserved waste, decreasing the N and energy content of the acid diet. The growth of *Capitella* sp. is related to both organic N and caloric content of diets (Tenore 1983), which may explain the low growth performance of the polychaetes. On the other hand, the dried diet had an even higher energy content than the fresh salmon faeces, which seems to contradict the lower growth performance. However, the hard, condensed structure of this diet may have made it more difficult to consume by the polychaetes (visual observations).

4.3 Implications for integrated aquaculture

Results of this study demonstrate that *Capitella* sp. and *O. craigsmithi* are promising species for converting fish waste into a valuable product with potential for aquatic diets. To assess the full potential of *Capitella* sp. and *O. craigsmithi* as extractive species in integrated aquaculture, additional aspects should be considered. The current study focused on FA profiles of the polychaete species, and contributes to the exploration for alternatives for fish-oil in aquatic diets. Not only fish-oil alternatives, but also fish meal replacers are needed (Tacon & Metian 2015), and it would be interesting to explore if, based on protein levels and amino acid profiles, *Capitella* sp. and *O. craigsmithi* cultivated in integrated systems could

be regarded as fishmeal alternatives. Amino acid profiles of *Capitella* sp. and *O. craigsmithi* collected under the fish farms indicated that based on the essential amino acid levels, both polychaete species would fulfil the amino acid requirement reported by the National Research Council (NRC 2013) for Atlantic salmon, tilapia *Oreochromis* spp. and tiger shrimp *Penaeus monodon* (Fig. 5.2). Especially the methionine and lysine content of the polychaete species is of great interest, since these are often the first limiting amino acids reported for plant-based alternatives to replace fish meal in aquatic diets (Van Der Meer & Verdegem 1996, Furuya et al. 2004). The species-to-species ban of the EU (European Commission 2001) prohibits the re-use of polychaetes cultivated on salmon waste for salmon diets. Nevertheless, based on their amino acid profiles and FA profiles, *Capitella* sp. and *O. craigsmithi* show potential as a high-quality marine resource for other products as well. Next steps in the realization of a polychaete-based aquatic diet should focus on digestibility, attractability and palatability of *Capitella* sp. and *O. craigsmithi* for fish and shrimp.

Technical issues for integrated cultures should also be considered (Fang et al. 2017). To our knowledge, cultivation and harvesting techniques for marine cultivation of polychaetes have not been developed, although some steps have been taken. Jansen et al. (2019) described a pilot study in which benthic cultivation trays were used to enhance and collect *Ophryotrocha* spp. underneath salmon farms.

Besides enhancing the productivity of an aquaculture production system, the other major goal of the IMTA approach is to mitigate wastes produced by the fed cultures via extractive species (Chopin 2013a, Hughes & Black 2016). The biomitigation potential of both *Capitella* sp. and *O. craigsmithi* should thus be studied to evaluate the overall efficiency and success as extractive species for IMTA systems.

In conclusion, the presence of essential FAs makes both *Capitella* sp. and *O. craigsmithi* highly valuable as alternative resources for fish feed formulation. In particular, *O. craigsmithi* is an excellent candidate due to its stable FA profile and high levels of EPA and AA. Feeding *Capitella* sp. with acid-preserved fish faeces enhanced its FA profile. Potential for incorporation in integrated aquaculture systems is not only based on FA content but also the production (growth) potential of polychaetes fed with fish wastes. Fresh faeces is the best diet for both species, suggesting that microbes play an important role in the diet of polychaetes. Interestingly, *O. craigsmithi* showed poor growth performance on preserved fish waste, while *Capitella* sp. showed some growth, albeit less compared to the fresh diet. Despite the increased FA profiles for *Capitella* sp. fed on acid diets, growth, and thus production potential, was lower. Overall, *O. craigsmithi* seems suitable for use in integrated open-water systems, where fresh fish faeces are continuously supplied to the benthic system, but less for decoupled systems. *Capitella* sp. could be an interesting species for both open-water and decoupled integrated systems, given that growth was observed on all diets.

Acknowledgements

We thank Lerøy Vest AS and Marine Harvest AS for access to the farms and help with collection of the polychaetes. We thank the technical staff of Austevoll Research Station for experimental set-up and laboratory assistance. We also thank the editor and 3 anonymous reviewers for their comments and suggested changes to earlier versions of this paper. This research was supported by Ocean Forest AS (Research and Development license FD0036-1608763/Institute of Marine Research), the Norwegian species initiative and carried out as part of the PhD study of MAJ Nederlof (IPOP project TripleP@Sea KB-24-001-019 at Wageningen University).

Supplementary material

Table S5.1

Total fatty acid profile (g kg AFDW^{-1}) of the two polychaete species (*Capitella* sp. and *Ophryotrocha craigsmithi*) fed with the three different diets (Fresh, Acid and Dried).

		Capitella sp.			*O. craigsmithi*		
		Fresh	Acid	Dried	Fresh	Acid	Dried
SFA		29±2	29±1	28±2	19±2	16±1	14
	14:0	6.4±0.5	6.0±0.6	5.9±0.5	1.3±0.3	0.9±0.2	0.7
	Iso 15:0	0.09±0.04	0.08±0.03	0.09±0.03	0.02±0.01	0.02±0.01	0.01
	Antiso 15:0	0.35±0.01	0.27±0.004	0.28±0.01	0.07±0.01	0.06±0.002	0.05
	15:0	0.64±0.04	0.59±0.04	0.57±0.04	0.13±0.02	0.11±0.01	0.10
	Iso 16:0	0.30±0.02	0.30±0.02	0.29±0.03	0.02±0.004	0.02±0.004	0.01
	16:0	12±1	12.2±0.7	11.3±0.6	13±1.0	11±1.0	9
	Iso 17:0	0.34±0.03	0.35±0.03	0.31±0.02	0.13±0.01	0.12±0.01	0.10
	Antiso 17:0	0.54±0.01	0.56±0.02	0.55±0.04	0.05±0.003	0.04±0.002	0.04
	17:0	0.55±0.03	0.49±0.02	0.47±0.03	0.13±0.004	0.12±0.01	0.11
	Iso 18:0	0.12±0.01	0.08±0.01	0.12±0.02	0.01±0.001	0.01±0.001	0.01
	18:0	4.8±0.7	4.9±0.2	5.0±0.4	3.3±0.3	3.0±0.2	2.9
	Antiso 19:0	0.16±0.01	0.19±0.02	0.15±0.02	0.19±0.02	0.21±0.02	0.16
	Antiso 20:0	0.14±0.08	0.16±0.07	0.09±0.02	0.08±0.01	0.07±0.01	0.06
	20:0	0.82±0.12	0.84±0.07	0.89±0.09	0.10±0.02	0.11±0.01	0.13
	22:0	1.2±0.6	1.7±0.3	1.9±0.4	0.09±0.04	0.18±0.09	0.27
	24:0	0.14±0.02	0.16±0.02	0.15±0.03	0.02±0.01	0.02±0.004	0.02
MUFA		44±6	42±1	33±2	39±6	31±4	26
	14:1 (n-7)	0.21±0.03	0.21±0.01	0.12±0.01	0.10±0.02	0.10±0.02	0.07
	15:1 (n-x)	0.25±0.04	0.12±0.03	0.24±0.05	0.02±0.001	0.02±0.001	0.04
	15:1 (n-6)	0.29±0.02	0.28±0.02	0.21±0.02	0.03±0.01	0.02±0.01	0.01
	16:1 (n-11)	0.23±0.01	0.23±0.02	0.20±0.02	0.06±0.02	0.05±0.01	0.05
	16:1 (n-9)	0.76±0.06	0.80±0.06	0.58±0.06	3.2±0.2	2.7±0.2	2.0
	16:1 (n-7)	3.1±0.5	2.8±0.2	2.3±0.1	4.3±1.0	3.4±0.9	3.0
	16:1 (n-5)	0.22±0.02	0.25±0.02	0.18±0.03	0.12±0.02	0.13±0.03	0.07
	17:1 (n-10)	0.10±0.01	0.11±0.01	0.09±0.01	0.05±0.01	0.04±0.01	0.04
	17:1 (n-6)	0.11±0.01	0.11±0.01	0.09±0.01	0.02±0.004	0.02±0.004	0.02
	17:1 (n-8)	0.30±0.03	0.21±0.02	0.24±0.03	0.07±0.01	0.06±0.01	0.06
	17:1 (n-7)	0.41±0.02	0.46±0.04	0.39±0.01	0.26±0.03	0.23±0.02	0.18
	18:1 (n-11)	0.65±0.12	0.67±0.08	0.54±0.02	0.43±0.12	0.28±0.06	0.25
	18:1 (n-9)	19±4	18.2±0.3	14±1	12±2	8±1	6
	18:1 (n-7)	4.1±0.5	4.1±0.2	3.2±0.2	10±2	8±1	8
	18:1 (n-5)	0.29±0.03	0.35±0.02	0.27±0.02	0.19±0.05	0.17±0.04	0.16
	20:1 (n-11)	4.7±0.1	4.7±0.2	4.4±0.1	4.2±0.5	3.5±0.3	3.3
	20:1 (n-9)	3.6±0.6	3.2±0.1	2.6±0.2	1.0±0.3	0.5±0.1	0.3
	20:1 (n-7)	0.85±0.06	0.79±0.01	0.67±0.03	0.94±0.08	0.84±0.04	0.98
	20:1 (n-5)	0.27±0.01	0.31±0.02	0.24±0.03	0.10±0.01	0.08±0.01	0.08
	22:1 (n-11)	2.5±0.4	2.1±0.1	1.7±0.1	0.70±0.26	0.34±0.03	0.21
	22:1 (n-9)	0.80±0.11	0.67±0.04	0.63±0.05	1.41±0.03	1.34±0.04	1.29
	22:1 (n-7)	0.34±0.02	0.46±0.07	0.30±0.01	0.23±0.01	0.21±0.01	0.23
	24:1 (n-9)	0.69±0.05	0.57±0.03	0.57±0.07	0.05±0.01	0.04±0.005	0.03
PUFA		24±4	55±3	19±4	46±4	39±3	33
	16:2 (n-4)	0.10±0.01	0.17±0.02	0.09±0.02	0.05±0.02	0.03±0.01	0.02
	18:2 (n-4)	0.16±0.07	0.18±0.02	0.11±0.01	0.20±0.05	0.14±0.02	0.11
	18:2 (n-7)	0.08±0.03	0.07±0.01	0.03±0.003	0.16±0.03	0.13±0.03	0.15
	16:4 (n-1)	3.4±0.1	3.9±0.2	3.5±0.3	3.60±0.09	3.45±0.08	3.05
	18:5 (n-1)	0.65±0.03	0.83±0.03	0.67±0.05	1.94±0.09	1.86±0.08	1.81

Continued

Table S5.1 - *Continued*

	Capitella sp.			*O. craigsmithi*		
	Fresh	Acid	Dried	Fresh	Acid	Dried
PUFA (n-6)	8±2	18±1	5±2	15±2	12±1	11
18:2 (n-6)[1]	4.5±1.3	9.8±0.6	2.8±0.9	3.3±0.7	2.0±0.3	1.9
20:2 (n-6)	2.4±0.4	4.8±0.1	1.7±0.4	2.2±0.7	1.2±0.2	0.9
20:3 (n-6)	0.26±0.04	0.68±0.09	0.14±0.04	0.50±0.06	0.40±0.04	0.40
20:4 (n-6)[2]	0.51±0.09	1.70±0.16	0.31±0.14	7.9±0.8	7.3±0.7	7.3
22:2 (n-6)	0.24±0.04	0.44±0.03	0.19±0.05	0.20±0.01	0.18±0.01	0.17
22:4 (n-6)	0.09±0.01	0.37±0.04	0.06±0.03	0.86±0.04	0.76±0.06	0.70
22:5 (n-6)	0.04±0.01	0.21±0.01	0.02±0.02	0.06±0.02	0.03±0.004	0.02
PUFA (n-3)	8±2	27±2	6±2	22±1	20±2	15
16:4 (n-3)	0.16±0.02	0.13±0.002	0.11±0.02	0.02±0.01	0.02±0.01	0.01
18:3 (n-3)[3]	1.1±0.3	3.3±0.4	0.8±0.3	1.1±0.3	0.5±0.1	0.3
18:4 (n-3)	0.60±0.08	0.53±0.03	0.47±0.04	0.32±0.05	0.21±0.08	0.06
20:3 (n-3)	0.38±0.08	1.16±0.09	0.29±0.13	0.27±0.07	0.15±0.02	0.10
20:4 (n-3)	0.10±0.03	0.34±0.04	0.06±0.02	0.21±0.03	0.14±0.02	0.10
20:5 (n-3)[4]	3.0±0.5	12.0±1.1	2.4±0.9	18.7±0.8	17.2±1.5	13.2
21:5 (n-3)	0.51±0.07	1.36±0.08	0.41±0.14	0.30±0.02	0.25±0.02	0.20
22:5 (n-3)	0.58±0.09	1.99±0.19	0.41±0.14	0.96±0.10	0.75±0.08	0.53
22:6 (n-3)[5]	1.55±0.42	6.39±0.61	0.94±0.39	0.60±0.14	0.40±0.08	0.15
24:5 (n-3)	0.03±0.01	0.08±0.01	0.03±0.02	0.02±0.001	0.02±0.002	0.02
24:6 (n-3)	0.06±0.03	0.12±0.02	0.10±0.02	0.02±0.01	0.02±0.003	0.02
NMI	4.0±0.2	4.8±0.1	3.4±0.2	2.3±0.3	1.9±0.2	1.9
20:2 Δ5,11 NMI	0.87±0.08	0.86±0.06	0.70±0.05	0.34±0.08	0.24±0.05	0.21
20:2 Δ5,13 NMI	0.27±0.01	0.32±0.02	0.24±0.03	0.10±0.01	0.08±0.01	0.08
20:3 Δ5,11,14 NMI	0.34±0.04	0.80±0.03	0.26±0.08	0.22±0.05	0.18±0.02	0.16
20:4 Δ5,8,11,14 NMI	0.34±0.06	0.34±0.04	0.25±0.04	0.03±0.002	0.02±0.002	0.02
22:2 Δ7,13 NMI	1.75±0.07	1.98±0.04	1.55±0.07	0.99±0.10	0.87±0.07	0.84
22:2 Δ7,15 NMI	0.41±0.01	0.47±0.02	0.38±0.02	0.61±0.06	0.50±0.04	0.56

FA contributing less than 0.1% are not considered. All FAs (>0.1 % of total FA) were used to calculate the sum of SFA, MUFA, PUFA, PUFA (n-6), PUFA (n-3) or NMI.

Values are given as mean ± SD (n = 4 tanks treatment^{-1}, except for *O. craigsmithi* dried where n = 2 tanks treatment^{-1}).

[1]LA, [2]AA, [3]ALA, [4]EPA, [5]DHA.

FA, Fatty Acid; SFA, Saturated Fatty Acid; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; NMI, Non-Methylene Interrupted; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; LA, Linoleic Acid; AA, Arachidonic Acid; ALA, α-Linolenic Acid.



Chapter 6

General discussion

1 Introduction

This thesis explores nutrient fluxes in marine IMTA systems that differ in their degree of openness, with the aim to gain more insights in the (re-)cycling of fed nutrients through different IMTA systems. Focus is on quantifying N, P and C retention of fed nutrients by extractive species and waste valorisation. The thesis starts with a literature review (*Chapter 2*) aiming to establish a generic framework that provides quantitative insights in nutrient retention efficiencies in a range of IMTA systems. This review highlights the variability in nutrient dynamics through IMTA under different conditions of system openness, as different factors influence nutrient retention in each system. *Chapter 2* also identifies knowledge gaps of which some are addressed in *chapters 3, 4* and *5*.

In a world where resources become scarce and pressure on the environment is growing, nutrient retention and efficient use is a general policy goal. IMTA aims to extract excess nutrients by harvesting commercially valuable extractive species, thus reducing nutrient accumulation and keeping the production environment healthy and prosperous. In other words, in IMTA a linear farming system is expanded towards a more circular farming system, producing additional valuable crops that benefit from nutrients not retained by fed species. It can also be considered to what extent ecosystem services may profit from the nutrient flows generated by the IMTA approach, especially in open-water farming systems. The value of IMTA is therefore wider than mitigating waste nutrients or producing valuable extractive species, but rather is the integrated value of economic, ecological and social benefits.

In this chapter the research outcomes are discussed in the framework of wider marine IMTA applications, including ecological and economic merits, ecosystem services and perspectives for circularity.

2 Ecological effects of IMTA

IMTA systems can be designed in a way that maximum retention efficiencies will be realized, given the different degrees of system openness. This thesis investigates nutrient retention efficiencies in marine IMTA systems, to get a better understanding of realistic retention efficiencies among different IMTA systems. Based on the framework presented in *Chapter 2* we demonstrate that nutrient retention efficiencies of 40-75% seem realistic in salmon-kelp-mussel-polychaete IMTAs. It should be acknowledged that retention of nutrients by the farmed fish is included in this conceptual model. Compared to fish monocultures (~43% N, 24% P and 38% C retention), in IMTA systems an additional 7-32% N, 16-41% P and 2-37% C of the fed nutrients can be retained via the extractive components (*Chapter 2*). In the further discussion focus is on the waste retention by extractive species. As shown, waste retention shows a large variation, depending on the openness of the system. Obviously, the highest nutrient retention can be achieved in closed systems. The challenge lays therefore, in the first place, in understanding the factors that determine retention efficiencies in open-water systems.

2.1 Open-water IMTA

In open-water IMTA systems, connectivity between waste nutrients and extractive species depends on environmental factors, like water residence time, ambient nutrient levels, seasonal variation and spatial scale. These factors are responsible for the difference in retention efficiency between open-water and closed IMTA systems; we calculate that in closed IMTA systems 22-32% N, 41% P and 7-37% C of fed nutrients can be recycled by extractive species, while in open-water systems this is 7% for N, 16% for P and 2-12% for C (*Chapter 2*).

The waste retention efficiency of IMTA can be defined as the fraction of waste from the fish farm that is retained by extractive species (e.g. Cranford et al. 2013). However, in open-water systems, a balance approach seems more realistic. In this approach the net effect of nutrient extraction is the basis for calculating the retention efficiency, no matter whether inorganic and (particulate) organic material comes from the fish farm or from the environment. IMTA performance is in this approach calculated on the basis of the farm input data (feed), fed species nutrient retention data and the extraction data of harvested extractive species. Using this approach, the ambient nutrient level is no longer used to quantify retention efficiencies, as the origin of the nutrients retained by the extractive species does not matter. The main discussion points regarding the balance approach are (1) spatial scale; how to define the boundaries of the ecosystem where the IMTA's are located, and (2) impact; how to avoid adverse impacts on the local ecosystem.

The first discussion point relates to the actual boundaries of open-water IMTA systems. Extractive species need to be located in the area where most of the waste nutrients are dispersed, and this differs per waste type (Sanz-Lazaro & Sanchez-Jerez 2020). While solid wastes sink and accumulate underneath fish cages (Holmer et al. 2007, Bannister et al. 2016), hydrodynamics result in dilution and dispersal of dissolved and suspended particulate waste in the water column (Sanderson et al. 2008, Brager et al. 2015, Jansen et al. 2018). Therefore, species that extract dissolved or suspended particulate material from the water column, e.g. seaweeds and bivalves, do not necessarily have to be cultivated in close vicinity of the fish farm, and instead the bioremediation potential of seaweeds and bivalves could be evaluated at a more regional scale (Sanz-Lazaro & Sanchez-Jerez 2020).

Still the question remains where to cultivate the seaweeds and bivalves, and establish the boundaries to evaluate IMTA performance. Sanz-Lazaro and Sanchez-Jerez (2020) suggested that in relatively enclosed water bodies (e.g. lakes, fjords, lochs) water-column IMTA could be practiced at small water body scale, e.g. 1-10 km, while in off-coast and offshore water-column IMTA could be practiced at regional water body scale, e.g. 10-100 km (Fig. 6.1). Placing seaweed and bivalve cultivation not directly besides the fish farm, reduces the concerns related to logistic issues, like the space for boats and infrastructure required for optimal operation of the fish farm (Hughes & Black 2016).

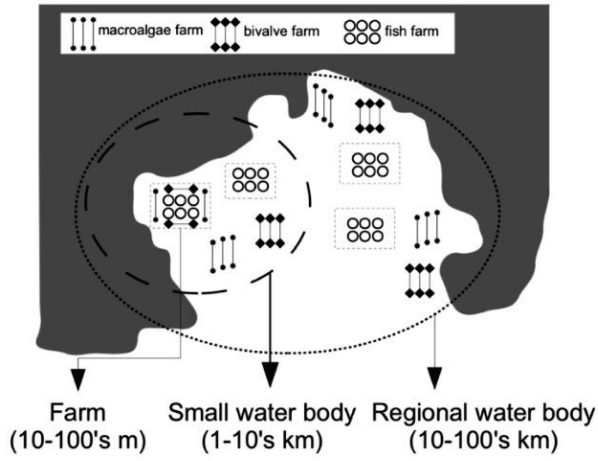

Figure 6.1
Spatial scale of open-water IMTA systems. Derived from Sanz-Lazaro and Sanchez-Jerez (2020).

In addition, as mentioned in *Chapter 2*, and highlighted in literature (Broch et al. 2013, Reid et al. 2013, Holdt & Edwards 2014), large areas of seaweed and bivalve cultivation will be required in open-water IMTA. Using the balance approach helps to locate these large cultivation areas. Enhanced growth stimulated by the waste nutrients resulting from fish cages, is only reported for seaweeds and bivalves cultivated within tens to hundreds of meters from the fish cages (Kerrigan & Suckling 2016, Fossberg et al. 2018). When IMTA is practiced at small (1-10 km) or regional (10-100 km) water body scale, this enhanced growth of seaweeds and bivalves is less likely, and similar growth as in monocultures of seaweeds and bivalves are expected.

The second discussion point using the balance approach relates to avoidance of adverse impacts on the local ecosystem. Positive bioremediation effects occur when the amount of nutrients removed by extractive species equals the waste released by the fish farm. However, if the amount of nutrients removed by the extractive species exceeds the waste released by the fish farm, there is a risk of stripping ambient nutrients. Nutrient competition between large-scale seaweed farms and natural phytoplankton may result in nutrient shortage for phytoplankton, which directly affects filter feeders and other herbivorous species food availability and eventually the entire food web (Campbell et al. 2019, van der Meer 2020). Temporal issues (*Chapter 2*) are important to include in the balance approach; a 'seasonal mismatch' between waste release and nutrient uptake may strip ambient nutrients during particular seasons, while during other seasons waste release results in nutrient enrichment (Broch et al. 2013).
Insights in potential impacts on the local ecosystem due to extractive species waste production, is also required. Bivalves create a vertical biodeposition flux,

additional to the flux directly from the fish farm, thereby reinforcing the risk of local benthic anoxia (Cranford et al. 2013). This could be mitigated by additional deposit feeders underneath the bivalve cultivation. Successful integration of sea cucumbers with bivalve cultures have been reported (reviewed in Zamora et al. 2018), and the same approach could be applied to bivalve-polychaete integration. Seaweed cultivation result in DOM release, and in *Chapter 2* we estimate that 38-131 kg C ton^{-1} feed (7-25% of total C input) is released as seaweed-derived DOM in the conceptual IMTA. In Sanggou Bay (China), characterized by IMTA practices and large-scale seaweed cultivation, higher concentrations of DOM have indeed been reported near the seaweed cultivation areas (Mahmood et al. 2017). To mitigate potential impacts, sponges can be included in IMTA systems to extract excess DOM (Fu et al. 2007, Osinga et al. 2010, Gökalp et al. 2020). Yet, sponges produce waste in the form of POM. Gökalp et al. (2020) suggests that the POM produced by sponges can be converted to sea cucumber biomass. It was estimated that in total 49% of DOM released by seaweeds is retained in a sponge–sea cucumber combination, increasing the retention efficiency of IMTA systems.

Main impacts of cage farms are observed on the benthic ecosystem in comparison to water column impacts, as organic solid wastes sink and accumulate underneath fish cages. Sediments underneath fish monocultures have shown to be 4 to 27 times more enriched than unaffected sites (Hall et al. 1990, Holmer 2010). The organic waste footprint depends upon the fish farm location, and ranges from up to 30m from the farm in shallow areas (Findlay et al. 1995, Kempf et al. 2002), to 500m from the farm in deeper areas, like fjords (Bannister et al. 2016). These footprints result in local impacts, highlighting the importance of developing benthic IMTA on farm scale.

In this thesis we demonstrate the bioremediation potential of two polychaete species, *Capitella* sp. and *Ophryotrocha craigsmithi*, fed salmon faeces (*Chapter 4*). These species were selected, as they are naturally present underneath salmon cages, among others in Norwegian fjords (Kutti et al. 2007b, Bannister et al. 2014) and along the Canadian coast (Salvo et al. 2015a). Polychaetes of the genus *Capitella* are found in many parts of the world and are well known bio-indicators for organic polluted sediments, as they are highly associated with organically enriched benthic ecosystems (Pearson 1978). *Ophryotrocha* species are also associated with organically enriched sediments (Dahlgren et al. 2001). They are, however, mainly found in the deep sea on whale-falls (Dahlgren et al. 2006, Wiklund et al. 2009a) and on hard substrates underneath fish farms in Norway and Canada (Murray et al. 2012, Salvo et al. 2014). In this thesis we show that both polychaete species feed and grow on salmon faeces, and we highlight their potential as extractive species to be included in IMTA (*Chapter 4 & 5*). Since they are highly efficient fish waste convertors, in recent years different polychaete species gained attention as potential extractive component for IMTA systems (Honda & Kikuchi 2002, Bischoff 2007, Fang et al. 2017, Jerónimo et al. 2020). These studies, and in addition the results reported in this thesis highlight that polychaete-driven benthic IMTA seem promising to mitigate benthic impacts resulting from fed aquaculture.

The development of the deposit feeder component of open-water IMTA is still in its infancy, and practical large-scale cultivation and harvesting techniques are to the best of our knowledge not yet developed. In *Chapter 4* we calculate that densities required to convert the daily organic waste flux deposited below an average salmon farm, fall within ranges for natural populations of *Capitella* sp. and *O. craigsmithi*. Nevertheless, these are large densities to cultivate (65 000−95 000 ind. m^{-2} of *Capitella* sp. or 36 000−194 000 ind. m^{-2} of *O. craigsmithi*) and it raises practical questions, like where to place these benthic cultivation units and how to harvest them efficiently? In particular in deep Norwegian fjords, this will be challenging. Pilot studies have been done, including the introduction of *Capitella* spp. juveniles underneath cages (i.e. sea ranching) (Kinoshita et al. 2008), or the enhancement of indigenous polychaete species, among others *Ophryotrocha* spp., by providing additional surface via benthic trays (Jansen et al. 2019). This latter method has the advantage that production of juveniles is not required.
The development of decoupled integrated systems, as defined in *Chapter 4 & 5*, may also provide opportunities for deposit feeder cultivation. Technological advances resulting in enclosed or semi-enclosed sea cages for fed species, create possibilities to collect and upgrade solid waste before being fed to deposit feeders. This alternative IMTA concept was initially introduced for aquaponics to optimize species-specific conditions for the separate components of aquaponics, i.e. fish and plants (Goddek et al. 2016). In decoupled IMTA systems spatial connection between fed and extractive cultivation units is not required, avoiding impracticalities in the combined cultivation at the same location (Zamora et al. 2018). As spatial connection is not required, this could mean that collected solid waste is not immediately fed to the deposit feeders, but is first stored. To maintain the nutritional value during storage, in decoupled IMTA systems proper preservation of the organic waste collected is recommended. In this thesis it was chosen to use the common preservation techniques drying and acidification, as these methods are relatively fast and easy to apply. Both methods show a reduced growth and bioremediation potential for both *Capitella* sp. and *O. craigsmithi* (*Chapter 4 & 5*). For the development of decoupled fish-polychaete IMTA systems it is recommended to seek for different preservation methods, which are commercially applicable, but improve bioremediation potential.

In general, further application of the balance approach in open-water IMTA can learn from nutrient fluxes in closed and semi-open IMTA systems. Processes and pathways involved in waste retention by extractive species in these latter systems are more easy to quantify. Insights gained can be used in models for open-water IMTA, and provide a better understanding of factors that define the environmental benefits of open-water IMTA.

2.2 Closed system IMTA

Compared to open and semi-open IMTA systems, in closed IMTA systems it is important to define all waste fluxes separately, as waste is the sole food source for the extractive species. A good understanding of on the one hand waste fluxes (quantity and quality) and on the other hand nutrient requirements of the

extractive species, is therefore needed. For mussels there is the indication that fish faeces alone is insufficient (Both et al. 2011). In this thesis we demonstrate that both *Capitella* sp. and *O. craigsmithi* show high growth rates when fed fresh salmon faeces (*Chapter 5*), indicating that fresh salmon faeces can serve as a sole food source for these polychaete species. For other polychaete species it is also shown that they can grow on organic fish waste (Bischoff 2007), but growth can be lower compared to commercial polychaete diets, as was shown by Brown et al. (2011).

A point of attention in closed systems are high nutrient concentrations, as a result of nutrient accumulation due to minimum water exchange. In this thesis we demonstrate that orthophosphate concentrations of 0.9 mM are toxic for the seaweed *Ulva* spp. (*Chapter 3*). In closed systems these high nutrient concentrations may not only result from fed species cultivation, but waste production by the extractive species also accumulate in the system, if not treated. In *Chapter 2* we calculate that of the total input to the conceptual IMTA (i.e. feed), 2-5% N, 5-8% P and 8-13% C was non-retained, and excreted or respired as inorganic nutrients by the bivalves and deposit feeders. If included in the farm design, these non-retained inorganic nutrients could serve as an additional food source for autotrophic extractive species. If not included in the farm design, these nutrients may accumulate and could reach toxic concentrations or escape as greenhouse gases.

3 Economic effects of IMTA

IMTA systems are man-made. Farmers will most likely co-culture different species in an IMTA setting with the aim to extract a higher fraction of fed nutrients than realized in monoculture, if this is economically feasible. This requires that the value generated by extractive species in the IMTA does not compromise or should even improve the value of the production of fed species compared to in a monoculture setting (Hughes & Black 2016). Possible benefits include increased farm profits, risk reduction due to product diversification or a healthier production environment and better market acceptance of IMTA products (Ridler et al. 2007, Barrington et al. 2010). The probability to reach economic sustainability will be enhanced if ecological and social externalities are internalized in a way that rewards farming in an IMTA setting (Knowler et al. 2020). In this section, potential production benefits are explored, focusing on how IMTA systems upcycle the non-retained low-quality nutrients from the fish farm into high-quality and high-value extractive species. A full picture of the economic feasibility of IMTA would also require analysing the potential financial benefits, but this is not within the scope of this thesis.

3.1 Increased production

In an IMTA system, non-retained fed nutrients are partially recycled by extractive species, which raises total production and improves feed efficiency of the whole system. In Europe, however, this does not necessarily lead to financial benefits for fish farmers, in part explaining a limited IMTA adoption rate by the industry, in spite of a high positive interest among researchers and public institutions (Hughes

& Black 2016). In China, large-scale implementation of IMTA focusses mainly on shellfish and seaweed, with a relatively small contribution from fed species (Sun et al. 2020). This is very different from IMTA development in Europe, which mainly involves small-scale pilot trials centered around fed marine finfish as the principle culture species group. In Western countries, where extractive species have a relatively low value, until today, proof of financial profitability of IMTA is inconclusive, while in China income from shellfish and seaweed generates sufficient profit to support large-scale implementation of IMTA systems (Hughes & Black 2016). There might, however, be other scenarios that could play a role in the development of IMTA in areas where proof of direct financial profits is not yet clear, such as in Europe.

High expectation exist for the expansion of marine aquaculture, in face of the growing demand for human food (Duarte et al. 2009, Gentry et al. 2017). Currently, only 1-2% of our food is produced in the seas and oceans (Duarte et al. 2009), even though they make up 70% of the earth's surface. Based on available area, expansion of marine aquaculture seems therefore promising (Gentry et al. 2017), provided all necessary resources to support aquaculture at sea are available. The latter cannot be taken for granted. The majority of finfish and shrimp aquaculture (70%) consist of fed aquaculture (Tacon & Metian 2015), relying on external feed input, and expansion of marine finfish production will put pressure on (scarce) resources. Besides, feed represents the major production cost in monoculture of fish and shrimp (Rana et al. 2009). It would thus be beneficial to achieve high nutrient use efficiencies and recycle as much as possible of the non-retained nutrients by the fed species via the harvest of extractive species, creating additional resources (Knowler et al. 2020).
There are also high expectations for the expansion of low trophic aquaculture, not relying on external feed inputs, like seaweeds and bivalves (SAPEA 2017). Nevertheless, as seas and oceans are relatively nutrient poor, nutrient limitation is a major concern for offshore low trophic aquaculture (van der Meer 2020). In IMTA systems, excess nutrients from fish farms may act as fertilizers for extractive species, enhancing local productivity.

Chapter 2 presents an estimate of the biomass of extractive species that can be produced in the conceptual four-species marine IMTA system. On a wet weight basis, one metric ton of salmon feed (98% dry matter content), yields 0.89 ton salmon (FCR of 1.1), 3 to 23 ton seaweed, 0.08 to 0.6 ton mussel and 0.6 to 3.0 ton polychaete biomass (Table 2.3, Table 6.1). In 2019, globally 8.0 million ton (Mt) of marine finfish was produced (FAO/FishStatJ 2021). Assuming an FCR of 1.1, then approximately 9.0 Mt of feed was fed. If all marine finfish would be integrated into a four-species IMTA setting, and additional 27 to 207 Mt seaweed, 1 to 5 Mt mussels and 5 to 27 Mt of polychaetes could have been produced in 2019 (Table 6.1).
In 2019, 18 Mt of molluscs and 35 Mt of seaweeds were produced through aquaculture, practically all in the marine environment (FAO/FishStatJ 2021). For seaweeds, the global output of dissolved inorganic waste from marine finfish

farming alone is sufficient to support a 0.8 to 6 times higher production than today's production, if all marine finfish is produced in the conceptual four-species IMTA described in *Chapter 2*. For molluscs, the global output of particulate suspended waste from marine finfish farming would only contribute to a 0.04 to 0.3 time higher production. It should be noted that these are maximum productions achievable in IMTA systems, as maximum retention efficiencies reported in literature were used in *Chapter 2*. Based on the global marine finfish production, a minimum of 5 Mt of polychaetes can be produced under fish cages, which is much higher than today's production of deposit feeders through aquaculture. This highlights a non-fulfilled niche, which could contribute to an enhanced production in the marine environment.

Table 6.1
Biomass input and output of a conceptual four-species marine IMTA (Fish-Seaweed-Mussel-Polychaete) per ton of feed and extrapolated to global aquaculture marine finfish production in 2019 (FAO/FishStatJ 2021). Biomass estimates per ton salmon feed are based on highest (second column from left) and lowest (third column from left) tissue contents reported in literature. For calculations, see *Chapter 2* (Mt: million metric ton). The values from column 2 and 3 are extrapolated to the amount of feed fed globally to finfish at sea estimated for 2019, and shown in column 4 and 5, respectively.

	based on 1 ton salmon feed (ton)		***based on global marine finfish production (Mt)***	
	Input (ww)			
Feed	1		9.0	
	Output (ww)			
Fed species	0.9		8.0	
Seaweed	3.0	23.0	27	207
Mussel	0.08	0.6	1	5
Polychaete	0.6	3.0	5	27
Total biomass	4.6	27.5	41	247
Biomass extractive species	3.7	26.6	33	239

3.2 Waste valorisation

Besides the production of additional biomass, extractive species can upcycle low-quality resources (i.e. fed aquaculture waste products) into high quality products (i.e. extractive species biomass), whereby scarce and valuable nutrients can be regained. The results of this thesis highlight two strategies to regain valuable and scarce nutrients namely, essential fatty acids via polychaetes (*Chapter 5*) and phosphorus via seaweeds (*Chapter 3*).

Omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), in particular eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acid, are essential components of the human diets (Simopoulos 2008, Tocher 2015). Deficiencies have been observed in large parts of the world, which are expected to increase with the increasing human population (Hamilton et al. 2020). Fish and seafood are the primary sources for EPA and DHA in human diets, not because fish themselves are efficient producers of n-3 fatty acids, but they play an important role in bioaccumulation of n-3 LC-PUFA through the food web, making them available to humans (Hamilton et al. 2020). As capture fisheries are stagnating, the aquaculture industry needs to increase to fulfill the supply. Nevertheless, many farmed fish species also require essential fatty acids via their diets, to satisfy their own needs and to result in an optimal profile for human diets (Tocher 2015). Fish-oil has traditionally been used in formulated fish diets due to its richness in the for fish essential n-3 LC-PUFAs, but production has stagnated. In addition, the use of fish-oil in aquaculture is unsustainable due to feed-food competition, as most of the fishmeal and fish-oil produced is based on human edible products (Cashion et al. 2017).
IMTA could play a role in increasing the n-3 LC-PUFA use-efficiency of aquaculture systems, by using polychaetes as extractive component. Results of this thesis indicate that although salmon faeces fed to *Capitella* sp. and *Ophryotrocha craigsmithi* contained relatively low levels of PUFAs, high levels of essential fatty acids were measured in the polychaetes themselves (*Chapter 5*). These results highlight that polychaetes do not only have the ability to recuperate otherwise wasted nutrients, but also upcycle low-quality resources into a high quality product. The potential of polychaetes to convert fish culture by-products (i.e. spilled feed and faeces) into valuable biomass in terms of fatty acids, has also been demonstrated by other studies (García-Alonso et al. 2008, Bischoff et al. 2009, Brown et al. 2011, Marques et al. 2018, Jerónimo et al. 2020). Studies have shown that polychaetes species (i.e. *Arenicola marina, Nereis virens*) actively biosynthesise essential long chain polyunsaturated fatty acids (Olive et al. 2009, Pairohakul 2013). Combining their high bioremediation potential (e.g. *Chapter 2 & 4*), with their potential to valorize waste (e.g. *Chapter 5*), polychaetes are interesting candidates to be included in IMTA systems, if practicalities related to culture and harvest are resolved (*Section 6.2.1*).

Phosphorus is also an important element, essential for all life on earth, and phosphorus products are used in a wide range of industries (Desmidt et al. 2015). Agriculture is the highest consumer of phosphorus, mainly due to fertilizer use and

the inclusion of phosphorus in animal feed. In aquafeeds, in particular in plant-based diets phosphorus is added to avoid dietary phosphorous deficiency. Due to the high demand of phosphorus-based products, natural reserves decline, and become scarce (Desmidt et al. 2015). In aquaculture research, focus is therefore on formulating feed with a high P retention use efficiency (Maas 2021). Nevertheless, even under maximum P retention, some P losses are inevitable, and there is a need to develop better strategies to improve the phosphorus use efficiency. In *Chapter 3* of this thesis we show that *Ulva* spp. converts inorganic P into a harvestable product with a P content ranging from 0.30–0.35% P in dry matter. Seaweeds could thus convert difficult to harvest inorganic P into easy to harvest seaweed, and thus play a role in minimizing the loss of phosphorus, which is a limited resource.

4 Ecosystem services

IMTA is driven by the aim to mitigate excess nutrients released by fed aquaculture, in order to keep the environment healthy and prosperous. Proper application of the IMTA concept may promote several additional ecosystem services, besides the bioremediation of waste nutrients. Ecosystem services are benefits people obtain from the ecosystem. Using The Economics of Ecosystems and Biodiversity (TEEB) framework (TEEB 2010), ecosystem services can be divided in 4 categories; (*i*) provisioning services, meaning the production of goods, (*ii*) regulating services, which relates to benefits that are received from regulating ecosystem processes, (*iii*) habitat and supporting services, which encompasses habitat provisioning and maintaining diversity, and (*iv*) cultural services, which are non-material benefits obtained by humans in their relationship with the ecosystem (Gentry et al. 2020). The concept of ecosystem services has been adopted as a policy tool for the Convention on Biological Diversity, at the Earth Summit in Rio in 1992 and was applied to natural environments, to support nature conservation. Recently, the idea that human modified systems, like aquaculture practices, do not only consume, but also provide ecosystem services, gains attention (Alleway et al. 2019, Gentry et al. 2020). This section discusses potential ecosystem services provided by IMTA systems. Of course, the ecosystem services of IMTA are the sum of the services provided by the different species at different trophic levels. This discussion focuses on synergies realized across trophic levels in IMTA. For the ecosystem services of the constituents of IMTA see for example: Kim et al. (2017), Hasselström et al. (2018), Bruschetti (2019) and Smaal et al. (2019).

4.1 Provisioning services

All aquaculture practices provide provisioning services via the biomass generated, as this is their principle commercial objective. Compared to monocultures of fed species, in IMTA systems the production per unit input is increased, due to the additional production of extractive species (Hughes & Black 2016). Extractive species can be more productive in IMTA systems as a result of the fertilizing role of fed species. IMTA-produced seaweeds in land-based systems generally have higher and less variable productivity and protein content compared to seaweeds harvest from the wild (Schuenhoff et al. 2003, Abreu et al. 2011). For bivalves in

open-water IMTA systems, enhanced productivity compared to monocultures of bivalves, is predominantly the case in areas or during seasons with low ambient food quality or quantity (*Chapter 2*, Peharda et al. 2007, Handå et al. 2012a). It remains an important feature, as nutrient limitation is a major constraint for upscaling (offshore) aquaculture of extractive species as required for the future sustainable provisioning of human nutrition (SAPEA 2017, van der Meer 2020).
Biomass produced in IMTA systems (i.e. fed and extractive species) can be used for different applications, like food, feed, raw materials and medicinal resources. Several extractive species show potential as high-quality feed ingredients, for example as fishmeal or fish-oil alternatives in fish feed. Polychaetes, which are a natural food source for several fish species (Darnaude 2005, Ende et al. 2018), have been proposed as high-quality feed ingredient, among others due to their FA profiles (*Chapter 5*; Bischoff et al. 2009, Brown et al. 2011). Seaweeds are studied as potential protein source for fish diets, and inclusion levels up to 10% seem feasible, depending on the seaweed species (Valente et al. 2006). The recycling of fed species waste nutrients back into feed ingredients, increases the feed efficiency of the system, but legal constraints should also be considered (Hughes & Black 2016).

4.2 Habitat and supporting services

Open-water IMTA systems can facilitate a wide variety of wild species, as they provide habitat and shelter, which could enrich biodiversity in and around the IMTA systems. Although fouling of nets is undesirable, artificial structures like racks, cages and ropes that are used to cultivate fed and extractive species, provide substrates which can be colonized by organisms like bacteria, algae, ascidians, molluscs and cnidarians (Costa-Pierce & Bridger 2002, Callier et al. 2018, van der Schatte Olivier et al. 2018). Bivalve shells provide hard substrates for organisms to attach to, and shelter in between the shells (van der Schatte Olivier et al. 2018, Smaal et al. 2019). Seaweed cultivation offers refuges areas, and the holdfast, the stipe and the lamina of kelp provide different habitat (Hasselström et al. 2018, Langton et al. 2019).
In general, artificial structures in the water column tend to aggregate fish, and several fisheries rely on the deployment of floating objects, which are referred to as Fish Aggregating Devices (FADs) (Castro et al. 2002). IMTA systems may act as FADs, as fish cages, bivalve and seaweed culture structures facilitate aggregations of wild fish (Callier et al. 2018). There is a variety of mechanisms that play a role in the attraction of wild fish, as they can provide shelter, shade, substrate for egg deposition or food sources such as fouling communities (Beveridge 1984, Callier et al. 2018). In addition, fish farms may attract wild species due to feed spillages, while bivalve, seaweed and, likely also deposit feeders may attract other species as a food source (Callier et al. 2018, Theuerkauf et al. 2021). It is not always clear if fish aggregations near aquaculture practices are the result of additional production or simply an increased concentration due to attraction (Theuerkauf et al. 2021).

4.3 Regulating services

Mitigation of eutrophication and organic enrichment, the main driver behind the use of IMTA, is a regulating service. Addition of extractive species to fed cultures results in enhanced nutrient cycling, as nutrients are either converted into harvestable biomass or are converted to other nutrient forms. Consequently, not only water quality and water transparency are improved, also, nutrient regeneration may stimulate primary production (e.g. mineralization by deposit feeders). This is particularly relevant under nutrient limited conditions commonly found offshore, which limits the scope for large-scale development of low-trophic aquaculture at sea (van der Meer 2020). Controlled uptake of fed nutrients in an IMTA setting in oligotrophic areas can therefore reduce nutrient limitations.

Uptake and assimilation of carbon by seaweed and bivalves contributes to carbon sequestration (Krause-Jensen & Duarte 2016, Jansen & van den Bogaart 2020). Carbon dioxide uptake by seaweeds may also have a positive effect on ocean acidification (Gentry et al. 2020). The exact potential is not fully understood, but Mongin et al. (2016) reported that on a local scale seaweed cultivation could increase the aragonite saturation state, indicating the potential of seaweeds to mitigate the effects of increased acidification on a local level.

Above mentioned regulating services are all connected to nutrient cycling. There are, however, more regulating services that can be provided by IMTA systems. Seaweeds are net producers of oxygen, providing oxygen-rich habitats, which could potentially benefit the immediate environment around seaweed farms and may play a role in addressing de-oxygenation as a result of global warming (Duarte et al. 2017). Both seaweed and bivalve cultivation can play a role in coastal protection, as large seaweed and bivalve cultivation structures dampen wave energy, which in turn controls erosion and stabilize sediments (Alleway et al. 2019). Due to their filter capacity, bivalves may play a role in reduction of pathogens, like sea lice, as studies in the laboratory have shown that bivalves take up larvae from salmon lice (Webb et al. 2013, Montory et al. 2020).

4.4 Cultural services

Seafood, either captured or cultivated, plays in important role in several cultures, religions and communities, which is often based on cultural heritage. Aquaculture, including the related industries such as feed producers, fish processors and sellers, contribute to many -mostly rural- livelihoods providing income, employment and food (Alleway et al. 2019). The development of IMTA systems could provide additional jobs, as the addition of extractive species to fed species increases complexity, which require different designs, management and regulations compared to monocultures (Hughes & Black 2016). IMTA may also play a role in improving the general perspective on aquaculture, as the rapid increase of the aquaculture sector drives environmental, welfare and health concerns (Froehlich et al. 2017). Barrington et al. (2010) showed that participants of their study had a positive perspective towards IMTA, considering it a more sustainable practice than fish monoculture. Incorporating IMTA systems in tourism, for example via

ecotourism, or education, may help to further enhance the positive perception towards aquaculture.

Overall, above mentioned services, which are the result of coupling extractive species to fed aquaculture, indicate that the value of IMTA is more than only the food trading values of fed species + extractive species.

5 Perspectives on circularity

Our current food system is under pressure, as the way we produce food nowadays contributes to approximately a quarter of the human's induced greenhouse gas emissions (Vermeulen et al. 2012), and plays a considerable role in land degradation, water pollution, climate change and biodiversity loss (Foley et al. 2011, Springmann et al. 2018). In a world with an expanding human population, where resources become scarce and the pressure on the environment is increasing, we need to rethink our food production system and consumption pattern. Circular food systems are seen as a promising way to feed the growing world population within the planetary boundaries, and circular strategies gain momentum (Jurgilevich et al. 2016, Van Zanten et al. 2019, Muscat et al. 2021).

For the transition towards a circular bioeconomy, Muscat et al. (2021) presents five ecological principles for circular biomass use. The first principle states to *safeguard* and regenerate the health of ecosystems, and therefore promote production practices that contribute to conservation and regeneration of ecosystems and the resources they provide. The second principle is to *avoid* the production of non-essential products and the loss and waste of essential ones, thereby reducing overexploitation of natural resources and focus on renewable resources. The third principle addresses to *prioritize* biomass for basic human needs, in the way that feed-food competition is avoided. As farming, including aquaculture, results in inevitable nutrient losses and by-products, the fourth principle advocates the *recycling* of these nutrients and by-products back in the food system. The fifth, and last principle relates to *entropy* and states to minimize energy uses.

Fed species aquaculture currently operates mostly linearly. IMTA could play a role in the transition of aquaculture from linear to more circular systems, as the philosophy of the IMTA concept aligns with some of the ecological principles described by Muscat et al. (2021). Fed species aquaculture is associated with the release of nutrients, not retained for growth, which can have detrimental effects on the marine ecosystem (Holmer 2010). IMTA contributes to a reduction of these excess nutrients to the environment (*principle 1; safeguard*), by recycling fed species waste nutrients into extractive species biomass (*principle 4; recycling*). Bioremediation potential of IMTA is still mainly conceptually described, but in this thesis we made a first step to quantify nutrient retention efficiencies in different IMTA systems. Although we show that a substantial fraction of nutrients released by the fed species is retained by extractive species (*Chapter 2*), 100% recycling of these nutrient losses and by-products is an utopia, among others due to

inevitable waste production by the extractive species themselves. To safeguard marine ecosystems, it is therefore important to pay attention to the regenerative capacity of the ecosystem itself in the design of IMTA systems. Via the recycling of nutrients in extractive species biomass, IMTA systems can also contribute to the recuperation of essential nutrients otherwise lost from fed species aquaculture (*principle 2; avoid*), like phosphorus and essential fatty acids. Seaweeds may for example, play a role in recuperation of scarce phosphorus (*Chapter 3*), while polychaetes may play a role in the recuperation of essential fatty acids (*Chapter 5*).

The ecological principles described by Muscat et al. (2021) also highlight improvements for IMTA systems in the transition towards circular food systems. A wide variety of species have already been studied for their potential inclusion in IMTA systems (*Chapter 2*) and research is ongoing. Based on the third principle (*prioritize*), cultivation of extractive species that directly contribute to basic human needs should be prioritized, like bivalves and sea cucumbers. Seaweeds also play a role in human diets, but care have to be taken on excessive intake of salt, iodine and heavy metals (Cherry et al. 2019). Another point of attention is the input required for the fed compartment of IMTA systems. In circular food systems the role of animals is to upcycle low-quality by-products unsuitable for human consumption into high-quality food sources (van Hal 2020). According to the *prioritize* principle, this would mean that the input of IMTA only consist of human in-edible biomass (Muscat et al. 2021). Currently, most of the fishmeal and fish-oil is produced from food-grade fish, resulting in feed-food competition (Cashion et al. 2017). Alternative source, like polychaetes, may avoid this feed-food competition (*principle 3; prioritize*). As it is questionable if polychaetes will become part of human diets, they may play a role in the reduction of fishmeal and fish-oil in aquatic diets, as both their FA and AA profile seem promising (*Chapter 5*). This development requires further research on harvesting and cultivation techniques, but also on the potential to use polychaetes in fish diets. It should also be noted that currently based on EU regulations it is forbidden to re-use extractive species in diets for the same species as the fed species in the IMTA system.

6 Concluding remarks

This thesis provides insights in the (re-)cycling of fed nutrients through different IMTA systems and their waste valorisation potential. In addition, wider marine IMTA applications are discussed, including potential ecosystem services provided by IMTA systems and the potential of IMTA to contribute towards more circular food systems. The main conclusions of this thesis are:

- Based on current literature, it is feasible to recycle 22-32% N, 41% P and 7-37% C from fed nutrients via extractive species in a four-species marine land-based closed IMTA systems, while in an open-water IMTA system this is 7% for N, 16% for P and 2-12% for C.
- Under moderate to high nitrogen (0.5-5 mM) and phosphorus (0.01-0.1 mM) concentrations, *Ulva* spp. performance is not influenced by

(unfavourable) stoichiometry and high nitrate concentrations do not limit phosphorus uptake. This is promising for closed IMTA systems with *Ulva* spp. as extractive species. Orthophosphate concentrations of 0.9 mM are toxic for *Ulva* spp., and these conditions should be avoided in the design and management of closed IMTA systems.

- *Capitella* sp. could be of interest for decoupled IMTA systems, as feeding acid-preserved faeces result in an enriched fatty acid profile of the polychaete.
- *Capitella* sp. and *Ophryotrocha craigsmithi* are interesting species to include in coupled IMTA systems, as they show good bioremediation and growth potential when fed fresh salmon faeces and they are able to convert low-quality fish faeces into a high-quality resource, containing for fish essential fatty acids and amino acids.

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Summary (English)

Samenvatting (Nederlands)

Summary

With the increasing demand for fish and seafood, further growth of the aquaculture sector is foreseen. This growth associates with ecological concerns, including pressure on natural resources, a growing demand for high-quality aquafeeds and an increased waste production (i.e. uneaten feed, metabolic waste and faeces) with potential detrimental effects if discharged into the environment. Therefore, sustainable aquaculture approaches are needed, to keep up with the increasing demand for food and resources while minimizing adverse impacts on the environment. Integrated Multi-Trophic Aquaculture (IMTA) is an approach with the ambition to fulfil this need. In IMTA systems, *fed species* aquaculture (i.e. dependent on external feed supply) is combined with *extractive species* aquaculture (i.e. extract nutrients from their environment), so that waste resulting from fed species is recycled in extractive species biomass. This approach strives to reduce aquaculture waste losses by improving resource use efficiencies and transforming linear monocultures into circular systems.

The general concepts and principles of IMTA have been extensively addressed in the literature. Still, the environmental benefits of IMTA are mainly conceptually described and insufficiently quantified. The aim of this thesis is to investigate nutrient retention efficiencies in different marine IMTA systems (i.e. open-water sea cages, land-based flow-through and recirculating aquaculture systems [RAS]) by quantifying the fluxes involved in nitrogen (N), phosphorus (P) and carbon (C) retention and exploring the impact of biological and environmental factors on retention efficiency and waste valorisation.

The thesis starts with a literature review of nutrient retention efficiencies in IMTA systems (*Chapter 2*). The observed variability in nutrient dynamics within different IMTA systems was compiled into a generic framework showing nutrient retention efficiencies. First a conceptual four-species marine IMTA system was defined composed of the following functional groups: fish, seaweed, bivalve filter feeders and benthic deposit feeders. Considering the nutrient requirements and reported use efficiencies of a salmon-kelp-mussel-polychaete IMTA it was calculated that 79-94% of N, P and C supplied with fish feed can theoretically be retained. In practice various biological and environmental factors influence the bioremediation of IMTA systems, and therefore biological (extractive species waste production, stoichiometry in nutrient requirements) and environmental factors (temporal and spatial connectivity) were assessed against the theoretical reference frame, indicating that nutrient retention efficiencies of 45-75% for closed systems and 40-50% for open systems are more realistic. Compared to fish monocultures (43% N, 24% P and 38% C retention), an additional 22-32% N, 41% P and 7-37% C of fed nutrients can be recycled by extractive species in closed IMTA systems, while in open-water IMTA systems this is 7% for N, 16% for P and 2-12% for C.

The literature review of *Chapter 2* indicated that most studies using seaweed as extractive species in integrated aquaculture systems focus on bioremediation under low to moderate nutrient conditions. As a result of nutrient accumulation,

these conditions are not always representative for the high nutrient concentrations in RAS. In *Chapter 3* the hypothesis was tested that in RAS systems unfavourable stoichiometry and/or toxic conditions reduce seaweed performance. Therefore, growth, tissue content and nutrient removal rates of *Ulva* spp. exposed to moderate to high nitrogen (0.5-5 mM) and phosphorus (0.01-0.9 mM) concentrations were examined. It was shown that under these nutrient concentrations; (I) tissue content of the seaweeds were above the critical threshold for maximum growth, while unfavourable stoichiometry (N:P ratio < Atkinson atomic ratio of 30:1) did not limit seaweed growth; (II) an orthophosphate concentration of 0.9 mM was toxic for *Ulva* spp.; (III) high nitrate concentrations did not inhibit phosphorus uptake; (IV) growth performance of *Ulva* spp. did not change when nitrate was exchanged for a similarly high ammonia concentration although the tissue nitrogen content was higher when exposed to ammonia. The latter suggests that ammonia was stored faster in the tissue and that growth was limited by other factors. Overall, the results of this chapter contribute to a better understanding of the application of *Ulva* spp. as extractive species for closed IMTA systems.

The literature review of *Chapter 2* also highlighted that compared to seaweeds and bivalves, less research is done on benthic species in IMTA. The next two chapters (*Chapter 4 & 5*) therefore focussed on the benthic part of IMTA. We selected two polychaete species, *Capitella* sp. and *Ophryotrocha craigsmithi*, that naturally occur in high densities under open-water fish cages and studied their bioremediation potential (*Chapter 4*) and their ability to upgrade fish waste into a high-quality marine resource (*Chapter 5*). We investigated their potential role in coupled and decoupled IMTA systems. While coupled IMTA refers to the more conventional IMTA approach whereby extractive species are integrated with fed species within the same (eco)system, in decoupled IMTA the fed and extractive species become separate functional units. For decoupled IMTA, preservation of fish faeces is recommended, and the hypotheses that preservation of fish faeces affects the bioremediation potential (*Chapter 4*), production potential and body composition of the polychaete species (*Chapter 5*) were tested. In *Chapter 4*, respiration and ammonia excretion rates were measured for polychaetes fed fresh, oven-dried or acidified salmon faeces and combined with nutrients incorporated into tissue growth, to estimate nutrient requirements. Nutrient requirements were then used to evaluate bioremediation potential. Nutrient requirements ranged from 5 to 26 mg C and from 2 to 6 mg N g^{-1} AFDW d^{-1}, with highest values observed for *O. craigsmithi* when fed fresh faeces. Preservation of the faeces reduced the bioremediation potential of both polychaete species. Overall, the nutrient requirements measured in this study were comparable with or higher than other polychaete species, highlighting the potential for organic fish waste bioremediation by *Capitella* sp. and *O. craigsmithi*.

In *Chapter 5,* the production rate and body composition (focus on fatty acid [FA] profiles) were determined for polychaetes fed fresh, oven-dried or acidified salmon faeces. Albeit diets contained relatively low polyunsaturated FA (PUFA) levels (5-

9% of total FA), both polychaete species were rich in PUFAs (>30% of total FA), including the for fish essential FAs. *Capitella* sp. fed the acidified diet enriched its FA profile. Overall, these results show the potential of *Capitella* sp. and *O. craigsmithi* to convert fish waste into a valuable product. For both polychaete species, the highest growth was observed when feeding fresh faeces. Oven-dried and acidified faeces resulted in negative growth for *O. craigsmithi*.

Combined *Chapter 4 & 5* highlight that both *Capitella* sp. and *O. craigsmithi* are interesting species to include in IMTA systems, whereby *O. craigsmithi* seems more suitable for integration in coupled systems, while *Capitella* sp. is interesting for both coupled and decoupled integrated systems.

In the general discussion (*Chapter 6*) the wider potential ecosystem services of IMTA and circularity aspects, are discussed. This shows that the value of IMTA is much more than mitigation of excess nutrients or producing valuable extractive species, but rather is the integrated value of economic, ecological and social benefits. The main conclusions of this thesis are:

- Based on current literature, it is feasible to recycle 22-32% N, 41% P and 7-37% C from fed nutrients via extractive species in a four-species marine land-based closed IMTA systems, while in an open-water IMTA system this is 7% for N, 16% for P and 2-12% for C.
- Under moderate to high nitrogen (0.5-5 mM) and phosphorus (0.01-0.1 mM) concentrations, *Ulva* spp. performance is not influenced by (unfavourable) stoichiometry and high nitrate concentrations do not limit phosphorus uptake. This is promising for closed IMTA systems with *Ulva* spp. as extractive species. Orthophosphate concentrations of 0.9 mM are toxic for *Ulva* spp., and these conditions should be avoided in the design and management of closed IMTA systems.
- *Capitella* sp. could be of interest for decoupled IMTA systems, as feeding acid-preserved faeces result in an enriched fatty acid profile of the polychaete.
- *Capitella* sp. and *Ophryotrocha craigsmithi* are interesting species to include in coupled IMTA systems, as they show good bioremediation and growth potential when fed fresh salmon faeces and they are able to convert low-quality fish faeces into a high-quality resource, containing for fish essential fatty acids and amino acids.

Samenvatting

Door de toenemende vraag naar voedsel uit zee, is de verwachting dat de aquacultuursector blijft groeien. Deze groei wordt echter geassocieerd met potententiele negatieve ecologische impact, onder andere doordat de druk op natuurlijke grondstoffen toeneemt, er meer vraag komt naar hoogwaardig visvoer en de groei resulteert in een toename van afval, zoals voedselresten, excretie en feces, welke kunnen resulteren in schadelijke milieueffecten. Er is dus een noodzaak voor duurzame aquacultuur, waarmee voldaan kan worden aan de toenemende vraag naar voedsel en grondstoffen, maar met slechts een minimale impact op het milieu. Geïntegreerde Multi-Trofische Aquacultuur (IMTA, Integrated Multi-Trophic Aquaculture) heeft de ambitie om hieraan te voldoen. In IMTA-systemen wordt de kweek van soorten die gevoerd worden gecombineerd met de kweek van extractieve soorten, waarbij afval gerecycled wordt via geoogste extractieve soorten. Deze aanpak beoogt de milieu-impact van aquacultuur te verminderen door efficiënter gebruik te maken van de afvalnutriënten afkomstig van de kweek van aquatische soorten, door lineaire monoculturen te integreren in circulaire systemen.

De algemene principes van IMTA zijn veelal geaccepteerd, maar de milieuvoordelen van IMTA zijn slechts conceptueel beschreven en onvoldoende gekwantificeerd. In dit proefschrift wordt de nutriëntenretentie-efficiëntie in verschillende marine IMTA systemen (kooien in zee, doorstroom- en recirculatie-aquacultuur systemen [RAS] op land) geëvalueerd. Dit wordt gedaan door het kwantificeren van fluxen die een rol spelen bij stikstof- (N), fosfor- (P) en koolstofretentie (C) en het beter begrijpen van de invloed van verschillende biologische- en omgevingsfactoren op de retentie-efficiëntie en afvalvalorisatie in IMTA.

Dit proefschrift start met een literatuurstudie naar nutriëntenretentie-efficiëntie in IMTA-systemen (*Hoofdstuk 2*). De in de literatuur gevonden variabiliteit in nutriëntendynamiek binnen verschillende IMTA-systemen is samengebracht in een generiek kader voor nutriëntenretentie-efficiëntie. Daarvoor is allereerst een conceptueel marine IMTA gedefinieerd met vier-soorten; vis, zeewier, filterende schelpdieren en benthische deposit feeders. In *Hoofdstuk 2* is berekend dat in een zalm-suikerwier-mossel-worm IMTA theoretisch 79-94% van de N, P en C uit het visvoer vastgelegd kan worden in biomassa. In de praktijk zullen echter verschillende biologische- en omgevingsfactoren invloed hebben op de bioremediatie van IMTA-systemen en deze factoren zijn daarom getoetst aan het theoretisch kader, waaruit blijkt dat nutriëntenretentie-efficiëntie van 45-75% voor gesloten systemen op land en 40-50% voor open systemen in zee realistischer zijn. Vergeleken met pure viskweek (43% N, 24% P en 38% C retentie), kan een additionele 22-32% N, 41% P en 7-37% C uit het visvoer gerecycled worden via de extractieve soorten in een gesloten IMTA, terwijl in een open systeem slechts 7% N, 16% P en 2-12% C gerecycled kan worden.

De literatuurstudie in *Hoofdstuk 2* laat tevens zien dat de meeste studies naar zeewier in IMTA focussen op bioremediatie bij lage tot gematigde nutriëntencondities. Door nutriëntenophoping zijn deze condities niet altijd representatief voor de hoge nutriëntenconcentraties zoals deze voorkomen in gesloten systemen op land (RAS – Recirculating Aquaculture Systems). In *Hoofdstuk 3* is daarom de hypothese getest dat in RAS ongunstige stoichiometrie (verhouding tussen N en P) en/of toxische condities de groei en kwaliteit van het zeewier negatief kunnen beïnvloeden. Daarvoor zijn groei, weefselsamenstelling en nutriëntenopname van *Ulva* spp. onder gematigd tot hoge stikstof (0.05-5 mM) en fosfor (0.01-0.9 mM) concentraties onderzocht. Aangetoond is dat onder deze omstandigheden; (I) de weefselsamenstelling van het zeewier boven de kritische waarde voor maximale groei ligt, en dat ongunstige stoichiometrie (N:P ratio < Atkinson atomische ratio van 30:1) groei niet limiteert; (II) een fosfaat concentratie van 0.9 mM toxisch is voor *Ulva* spp.; (III) hoge nitraatconcentraties geen remmend effect hebben op fosforopname; en (IV) groei van *Ulva* spp. niet verandert wanneer nitraat uitgewisseld wordt voor ammonium, maar dat het stikstofgehalte in het zeewier wel hoger is in de ammonium behandelingen. Dit laatste suggereert dat ammonium sneller opgeslagen wordt in het weefsel en dat groei geremd wordt door andere factoren. In het algemeen dragen de resultaten van dit hoofdstuk bij aan een beter begrip over de toepassing van *Ulva* spp. als extractieve soort in gesloten IMTA systemen.

Uit de literatuurstudie in *Hoofdstuk 2* blijkt ook dat er relatief weinig onderzoek gedaan is naar benthische soorten die een rol kunnen spelen in IMTA. De volgende twee hoofdstukken (*Hoofdstuk 4 & 5*) focussen daarom op het benthische deel van IMTA. Twee wormsoorten zijn hiervoor geselecteerd, *Capitella* sp. en *Ophryotrocha craigsmithi*, welke beide in hoge dichtheden voorkomen onder viskooien. We onderzochten van deze twee soorten de bioremediatie-potentie (*Hoofdstuk 4*) en de potentie om visfeces om te zetten in een hoogwaardig product (*Hoofdstuk 5*). Ook is voor beide soorten bekeken hoe deze toegepast kunnen worden in zowel gekoppelde als ontkoppelde IMTA. Gekoppelde IMTA verwijst naar de traditionele IMTA waarbij gevoederde en extractieve soorten onderdeel zijn van hetzelfde (eco)systeem, terwijl in ontkoppelde IMTA de gevoederde en extractieve soorten in gescheiden systemen worden gekweekt. In ontkoppelde IMTA is conserveren van visfeces aangeraden, en daarom zijn de hypothesen getest dat conservatie van visfeces een effect heeft op de bioremedatie (*Hoofdstuk 4*), en op de productie en lichaamssamenstelling van de twee wormsoorten (*Hoofdstuk 5*). In *Hoofdstuk 4* zijn respiratie en ammonium-excretie gemeten van de wormen gevoerd met verse, gedroogde of aangezuurde zalmfeces. Daarnaast zijn de nutriënten vastlegging in weefselgroei gemeten om de nutriëntenbehoefte te berekenen. Deze nutriëntenbehoefte zijn vervolgens gebruikt om de bioremediatie-potentie te evalueren. De nutriëntenbehoefte variëren van 5 tot 26 mg C en 2 tot 6 mg N g^{-1} AFDW d^{-1}, met de hoogste waarde voor *O. craigsmithi* gevoerd met verse feces. Fecesconservatie vermindert de bioremediatie. Algemeen genomen zijn de nutriëntenbehoefte bepaald in *Hoofdstuk 4* vergelijkbaar met of zelfs hoger dan bij

andere wormsoorten, wat aangeeft dat de mogelijkheid van *Capitella* sp. en *O. craigsmithi* om visafval om te zetten goed is.

In *Hoofdstuk 5*, zijn de productie en lichaamssamenstelling (focus op vetzuur [FA] profielen) bepaald van de twee wormsoorten gevoerd met verse, gedroogde of aangezuurde zalmfeces. Ondanks dat de diëten relatief lage concentraties van meervoudig onverzadigde vetzuren (PUFA) hebben (5-9% van totaal FA), hebben de wormen hoge PUFA gehaltes (>30% of totaal FA), inclusief de voor vis essentiële FAs. Algemeen genomen geven deze resultaten weer dat *Capitella* sp. en *O. craigsmithi* visfeces om kunnen zetten in een hoogwaardig product. Beide wormen groeien het best wanneer ze gevoerd worden met verse visfeces. Gedroogde en aangezuurde zalmfeces veroorzaakt een negatieve groei bij *O. craigsmithi* wat aangeeft dat preserveren van visafval niet gunstig is voor deze soort.

Samen laten *Hoofdstuk 4 & 5* zien dat zowel *Capitella* sp. als *O. craigsmithi* interessante soorten zijn voor IMTA, waarbij *O. craigsmithi* meer geschikt lijkt voor gekoppelde systemen, terwijl *Capitella* sp. voor zowel gekoppeld als ontkoppelde IMTA geschikt is.

In de algemene discussie (*Hoofdstuk 6*) wordt een bredere kijk gegeven op mogelijke ecosysteemdiensten en circulariteitsaspecten van IMTA, wat laat zien dat de waarde van IMTA meer inhoudt dan alleen het recyclen van afvalnutriënten of de productie van waardevolle extractieve soorten. De belangrijkste conclusies uit dit proefschrift zijn:

- Gebaseerd op de huidige literatuur is het mogelijk om 22-32% N, 41% P en 7-37% C van de gevoerde nutriënten te recyclen via extractieve soorten in een gesloten IMTA systeem met vier soorten, terwijl dit 7% N, 16% P en 2-12% C is in een open-systeem.
- Onder gematigde tot hoge stikstof- (0.5-5 mM) en fosfor- (0.01-0.1 mM) concentraties, wordt *Ulva* spp. groei niet beïnvloed door (ongunstige) stoichiometrie en hoge nitraatconcentraties limiteren fosforopname niet. Dit is veelbelovend voor gesloten IMTA-systemen met *Ulva* spp. als extractieve soorten. Fosfaat concentraties van 0.9 mM zijn toxisch voor *Ulva* spp. en deze condities moeten voorkomen worden door middel van het ontwerp en management van gesloten IMTA-systemen.
- *Capitella* sp. kan interessant zijn voor ontkoppelde IMTA, doordat het eten van aangezuurde zalmfeces resulteert in een verrijkt vetzuurprofiel.
- *Capitella* sp. en *Ophryotrocha craigsmithi* zijn beide interessante voor gekoppelde IMTA, omdat ze goede bioremediatie- en groeipotentie laten zien wanneer ze gevoerd worden met verse zalmfeces. Daarbij wordt laagwaardige visfeces omgezet in een hoogwaardig product, welke de vetzuren en aminozuren bevatten die interessant zijn voor bijvoorbeeld visvoer.

About the author

Marit Aleida Jantien Nederlof was born on the 2nd of November 1986 in Utrecht, The Netherlands. She completed her VWO degree at Niftarlake College in Maarssen. Thereafter, she went to England to improve her English, and worked as an au pair. As she has always been interested in nature and animals, she started in 2006 with a Bachelor Biology, followed by a Master Biology both at the Wageningen University. During her bachelor she got fascinated with the aquatic world and in particular aquatic organisms. This resulted in a MSc major thesis on the effects of altering the group composition (homogenous versus heterogeneous) regarding sex class and size class on feed intake, growth and (non-)feeding behaviour of Dover sole (*Solea solea*) (at Wageningen University), followed by a MSc minor thesis on the effects of different aquaculture related stressors on growth, feed intake, oxygen consumption, stress- and feed behaviour of juvenile gilthead sea bream (*Sparus aurata*) (at Institute of Marine Research, Norway). After obtaining her MSc degree in 2013, Marit started in the same year with her PhD project at the Aquaculture and Fisheries Group of the Wageningen University, under the supervision of Aad Smaal, Marc Verdegem and Henrice Jansen. During her PhD, Marit went to the Institute of Marine Research in Norway for four months to perform several experiments. The PhD project resulted in this thesis. While finishing her PhD thesis, in 2017 Marit started to work as a research assistant at the Aquaculture and Fisheries Group. From January 2021 she continued working as an aquaculture lecturer and researcher at the Aquaculture and Fisheries Group, with a focus on (shell)fish adaptation physiology.

List of publications

Nederlof MAJ, Verdegem MCJ, Smaal AC, Jansen HM (2021) Nutrient retention efficiencies in integrated multi-trophic aquaculture. Reviews in Aquaculture 00: 1-19

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Completed training and supervision

The Basic Package	**3 ECTS**
WIAS Introduction Course	2013
Research Integrity and Ethics in Animal Sciences Course	2017
Scientific exposure	**9 ECTS**
International conferences	
Seagriculture, Den Helder/Texel, The Netherlands	2013
Aquaculture Europe, Donostia-San Sebastián, Spain	2014
Aquaculture Europe, Rotterdam, The Netherlands	2015
Seminars and workshops	
WGS PhD Workshop Carousel, Wageningen, The Netherlands	2014
Presentations	
Poster at Int. Conf. Aquaculture Europe	2014
Poster at WIAS Science day	2015
Oral at the workshop of the Nibi conference	2015
Oral at EnAlgae Int. seminar	2015
Oral at conference Visfederatie	2015
Oral at Int. Conf. Aquaculture Europe	2015
In-depth studies	**10 ECTS**
Stable isotopes: analysis and application in food web ecology, Doctoral Schools University Ghent, Belgium	2014
WIAS course Design of experiments	2013
MSc course Aquaculture Production Systems, Wageningen University	2013
Professional skills support course	**3 ECTS**
WGS course PhD competence assessment	2013
WGS course Techniques for writing and presenting a scientific paper	2016
WGS course Project and time management	2017
Didactic skills training	**9 ECTS**
Supervising practicals and excursions	
Life history of Aquatic Animals (AFI-31306)	2013

Sustainability in Fish and Seafood Production (AFI-33306)	2014
Sustainability in Fish and Seafood Production (AFI-33306)	2015
Nutrition, Welfare and Reproduction in Aquaculture (AFI-32306)	2014
Nutrition, Welfare and Reproduction in Aquaculture (AFI-32306)	2015
Supervising theses	
Bram Bloks, MSc major thesis	2014
Jori de Kok, MSc major thesis	2015
Boris van Troost, BSc thesis	2014
Maarten Rutting, BSc thesis	2014
Preparing course material	
Aquaculture Production Systems (AFI-31806)	2013
Management skills training	**3 ECTS**
Organisation PhD-trip	2016-2017
Selection committee for full professor Marine Animal Ecology, Wageningen University	2014-2015
Education and Training total:	**37 ECTS**

Completion of the training activities is in fulfilment of the requirements for the education certificate of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS). One ECTS equals a study load of 28 hours.

Acknowledgements

Writing this last chapter makes me realize that this PhD journey has come to its end. It has been a long journey, in which I learned a lot, both on a professional and personal level. This thesis would not have been accomplished without the help and support of others, and I would like to express my thanks to each of you, who contributed in one way or another to this PhD, from helping out with the research, to the mental support, encouragements, and distractions that are highly needed and appreciated. Some of you, I would like to thank in particular.

First of all my supervisors Aad, Marc and Henrice; thank you for starting and finishing this PhD journey with me. It was a long journey, but the three of you always gave me the encouragement that I was going to make it, and I highly appreciate that. I learned a lot from you and I am grateful for that. **Aad**, thank you for expressing your confidence in me. Your feedback, critical comments and discussions have improved the work, and in the last months of my PhD you really helped me to stay focussed. I highly appreciate that. As your last PhD, I hope you continue to enjoy your retirement, but I am sure that several of your activities are still bivalve related (I am sorry that bivalve were only a minor part in this PhD). **Marc**, as my daily supervisor in Wageningen I would like to thank you for all that I have learned from you, from nutrient balances, to production systems, to writing skills. Your kind and encouraging way of supervising, really helped me settle at AFI. You always made time for questions and discussions, even when you were busy. I highly appreciate that. You once called me erudite (although I immediately attenuated that by asking; 'what does that mean?'), but you have definitely expanded my vocabulary. As it won't be long until your retirement, I hope you find all the time to spend with your grandchildren. **Henrice**, simply saying thank you is not enough, as I do not think this thesis would have been finished without your help and support. Thank you for introducing me to IMTA, which started with participation in one of your field studies in Norway, to which I have great memories. Thank you for the opportunity to go to Norway for the polychaete work; I really enjoyed that. But above all, thank you for all your knowledge, structured way of working and thinking (I am still learning from this), your extra hours of work, friendship and support (including many pep talks). I am very happy that you are now also a part of the AFI family.

I would like to express my gratitude to all who have taught me about seaweeds and helped me with my seaweed experiments. **Willem Brandenburg**, thank you for introducing me to the seaweed world. Your enthusiasm about seaweeds is contagious. **Willem de Visser**, thank you for always providing me with new *Ulva*, and thinking along when it was (again) dying. **Amir Neori**, we only met for a couple of weeks in Wageningen, but the discussions and your ideas for the

seaweed manuscript are highly appreciated. I would also like to thank you for your regular check-ups on a personal level, including my well-being and my horse; he finally gained a spot in the acknowledgement as you would see towards the end of this chapter ;). **Job Schipper**, **Freek van den Heuvel** and **Alexander Ebbing**, thank you for the sampling possibilities and the help at the pilot farm of Hortimare.

I would like to thank **Menno** and all other biotechnicians at Carus; **Wian**, **Emily**, **Sander**, **Tom**, **Gerrit**, **Manoek** & **Truus**. As seaweed was not a common organism in Carus, I would like to thank you for all the help in designing the cultivation tanks, during the experiments and to maintain the seaweed stock. Special thanks to Emily, who was the first responsible to take care of the seaweeds. I would also like to thank **Ronald** and **Tino** for all their help in the lab. Special thanks to Tino, who finished my lab work while I was in Norway. **Saskia** and **Michel** from the ANU lab, thank you for all the IRMS measurements.

During my PhD I was lucky to go to Norway and perform several experiments with polychaetes. I would like to thank all who made this possible and helped me during my time at Austevoll Research Station (IMR); I really enjoyed my time there including the "Krabbefest" and "Julebord". Thanks to all the staff of Austevoll Research Station to make me feel welcome. **Oivind Strand** and **Harald Sveier**, thank you to provide me with this opportunity and I hope you keep being enthusiastic about polychaetes. **Fang**, thank you for all your help during the experiments, but also the dinners, fishing, hikes and Chinese tea. **Samuel**, thank you for your help with the respiration measurements. **Sonnich Meier**, thank you for the fatty acid analyses. **Thomas Dahlgren**, thank you for the species analyses, but also your enthusiasm about polychaetes. Thanks to the guys from the Austevoll sea cages for all their help with stripping fish (sorry for all the shit picking). **Svanhild**, **Sara** and **Jeanette**, thank you for your help with the lab work; without the three of you I could not have finished before going back to the Netherlands. **Sara** & **Martin**, thank you for the nice dinner. **Margareth**, thank you for always checking up on me, and your bright personality. **Cathinka**, a special thanks for you and your family; thank you for taking care of me, baking me a surprise birthday cake, and inviting me over for dinner and games; I highly appreciate that.

I would also like to thank my students for their hard work and input into this project; **Bram**, **Jori**, **Boris**, **Maarten** and **Timo**.

I would like to thank all my colleagues at AFI for the nice chats, coffee breaks, support and social part of work: **Ronald**, **Tino**, **Erik**, **Samara**, **Frank**, **Johan V.**, **Geert**, **Eugenia**, **Gera**, **Annet**, **Marjon**, **Miranda**, **Geertje**, **Fotini**, **Maria**, **Esther**, **Leo**, **Paul**, **Jan-Jaap**, **Ep**, **Johan S.**, **Jules**, **Roel**, **Killian**. **Johan Verreth**; thank you for making me feel welcome in your group and for the

possibility to organize the Japan trip. **Eugenia** and **Denise** (as adopted AFI colleague) also you contributed to a warm welcome at AFI; thanks for the coffee moments, but also the social activities outside working hours. **Annet** and **Marjon**, thank you for all the support over the last years, not only as the secretariat, but also for all the mental support. You two are special! **Miranda**, thank you for proofreading my work. **Geert** and **Johan Schrama**, I am very happy that you provided me the opportunity to stay at AFI and that you gave me the time that I needed to finish this PhD, which is highly appreciated. **Jules**, thanks for the lay-out of several figures in this thesis and of course for the nice talks that we had while sharing an office. **Erik**, thank you for introducing me to DNA isolation. Although it was too late to add to this thesis, it is highly appreciated. **Roel**, thanks for being my 'hulplijn' already for several years, all the nice chats, including the stories about your chickens, but above all, thank you for being my paranymph.

Thanks to the AFI-PhD's for the nice coffee breaks and regular chats. I especially want to thank the PhD's who I have met during my PhD time; **Joost**, **Kim**, **Thomas**, **Twan**, **Davood**, **Yale**, **Vivi**, **Vinna**, **Tu**, **Kabir**, **Tinh**, **Thuat**, **Gauthier**, **Christos**, **Mahmoud**, **Devi**, **Folasade**, **Shah**, **Desrina, Olivier**. **Joost**, we started around the same time, and it was nice to have someone around with whom I could share the start-up struggles of a PhD. Also thank you for helping me out with the design of my seaweed tanks. **Mahmoud**, we have shared an office for quite some time, and it was always nice to have you around for some mental support. And of course the nice lunches that you and your wife provided us! **Kim** and **Devi**, we shared a lot of moments together, including ups and downs, but you two were always there for me; thank you for that! **Thomas**, thank you for being such nice and fun company during conferences; I am sure that in the future we will keep seeing each other at conferences. **Sade**, to you I would like to say; I am proud of you! **Anne-Jo**, special thanks to you for the beautiful paintings for my cover; they really made it an exclusive design.

Graag wil ik ook nog het woord richten aan mijn familie en vrienden; jullie steun en support, *no matter what*, is zeer waardevol geweest voor mij de afgelopen jaren. Lieve kleproosjes: **Debbie**, **Peer** (Erika) en **Jullie-Anne**, dank jullie dat jullie er altijd voor me zijn. Ook al is iedereen zijn eigen weg bewandeld, als we samen zijn is het altijd gezellig en ik ben blij met zulke waardevolle vriendinnen! Lieve Pink Lotusjes: **Debbie**, **Ellen**, **Arnoud**, **Lizzy**, **Rob**, **Rianne**, **Aad**, betere huisgenoten had ik mij niet kunnen wensen en als we elkaar zien voelt het altijd als vanouds. El, dank voor alle gezellige lunchwandelingen en je betrokkenheid bij mijn PhD project (Yes, I did it). Lieve stalgenootjes: **Judith**, **Laura**, **Simone**, **Tess**, **Marieke**, **Herlinde**, **Sanne** (1), **Sanne** (2), **Danielle**, **Sinead**, **Renate**, **Annabel**, dank jullie wel dat jullie altijd voor me klaar hebben gestaan (en nog steeds) om voor Diablo te zorgen, dank jullie wel voor de gezelligheid en

buitenritten voor de ontspanning die ik nodig had (en heb)! **Jeroen**, bedankt voor het rijden van Renate en mij naar verschillende clinics of de hei. In het bijzonder wil ik **Jolanda van de Craats** bedanken; dank je wel dat jij altijd voor mij en Diablo hebt klaar gestaan (en staat), en voor de fantastische zorgen voor Diablo toen ik in Noorwegen zat. Dit betekend enorm veel voor mij. Ook wil ik in het bijzonder de familie Meurs bedanken, dank jullie wel dat ik altijd bij jullie terecht kan. **Jolan** bedankt voor de zorgen voor Diablo als het even te druk is, maar ook zeker de zorgen voor mij. **Henry**, dank je wel voor alle kopjes thee, en onze gesprekken over piranha's en (Hollandse) garnalen.

Lieve **Jan**, helaas kun je mijn verdediging niet meer maken, maar bedankt voor alle mooie, gekke en gezellige avonturen die we hebben mogen beleven op stal. De verstrooide professor is nu eindelijk klaar. Lieve **Maaike**, dank je wel dat we alles samen hebben kunnen regelen voor Jan, maar bovenal dat je zo'n fijne en waardevolle vriendin bent.

Mam, **Pap** en **Lilian**, dank jullie wel voor jullie onvoorwaardelijke steun, vertrouwen en support. Ik kan met alles bij jullie terecht, en ik weet dat jullie trots op me zijn. Ik ben dan ook heel trots dat jullie mij de basis hebben gegeven die ervoor heeft gezorgd dat ik hier nu sta. **Aileen**, **Esmee**, **Johnny**, **Sven**, **Joppe**, **Joyce**, **Lotte**, **Eelco**, **Fenne**, **Nick**, **Willem**, **Laura**, **Wim**, **Dini** en alle kids; dank jullie wel voor jullie interesse, steun en het begrip van de afgelopen jaren. Lieve **Aileen**, dank je wel dat jij als mijn oudste zus aan mijn zij wil staan als paranymph. Lieve **Essie**, dank je wel dat jij als mijn jongste zus mijn cover en uitnodiging hebt ontworpen. Heel bijzonder!

Mijn beestjes: **Johnny Walker**, **Fanny Blankers-Koen**, **Doos**, **Klara Bella Pluis**, **Pannekoek**, **Henk**, **Haas**, **Bernhard** en **Diablo (Aapie)** dank jullie wel voor alle ontspanning die jullie mij bezorgen. In het bijzonder wil ik Klara en Pannie bedanken; jullie zijn mijn meest geïnteresseerde publiek bij het oefenen van presentaties en hebben regelmatig geprobeerd mee te typen aan dit proefschrift en Aapie; dank je wel voor alle fijne ritjes, wandelingen en ontspannen momenten.

Lieve **Kas**, dank je wel dat je er altijd voor me bent, accepteert wie ik ben, dat je me de vrijheid geeft die ik nodig heb en dat je voor me zorgt. Deze PhD heeft ook impact gehad op jou leven en ik kan je niet genoeg bedanken hoe goed je hiermee om bent gegaan. Dit boekje is dan ook voor jou. Ik hou van je!

Marit

Colophon

The research described in this thesis was financially supported by The IPOP project TripleP@Sea KB-24-001-019 at Wageningen University, The Graduate School Wageningen Institute of Animal Sciences (WIAS) and Wageningen Marine Research (WMR). Ocean Forest AS (Research and Development licence FD0036-1608763/Institute of Marine Research) partly supported research described in this thesis.

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design
Anne-Jo van Riel and Esmee Nederlof

Printing
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