

**Microencapsulated diets to improve the productivity of
bivalve shellfish aquaculture for global food security**

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This thesis is submitted for the degree of Doctor of Philosophy

Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee

Abstract

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David Frederick Willer

Food is the single strongest lever to optimise human health and environmental sustainability on earth. However, food production and consumption patterns today threaten both people and planet. We face a double-burden of malnutrition and overconsumption, with two billion people micronutrient deficient and over two billion people overweight or obese, and the global food system is now the single largest greenhouse-gas-emitting sector. Bivalve shellfish – including mussels, clams, and oysters – could be an invaluable component of our global food solution. Bivalves are nutrient rich, production has a lower environmental footprint than that of all other animal foods, and developing just 1% of the coastline suitable for bivalves worldwide would provide over one billion people with all their protein needs. To realise global potential there is an urgent need for innovation to enable increased bivalve production and consumption. This thesis aimed to test whether new innovations in microencapsulated feeding technology could be used to overcome major bivalve industry bottlenecks and help drive a step change in our global food system.

The initial research focus was to assess the potential of microencapsulated feeds as a problem-solving tool in bivalve aquaculture and test viability via laboratory experiments. I performed a critical review to assess key industry challenges and identify where microencapsulation technology could most effectively be applied. Experimental tests then allowed me to demonstrate that microencapsulated feeds could be ingested by a commercially farmed bivalve, the blue mussel (*Mytilus edulis*), providing a foundation for research on specific industry challenges.

The second phase of the thesis assessed the effectiveness of microencapsulated feeds to tackle major bivalve hatchery bottlenecks in juvenile growth and broodstock conditioning. I undertook research both in the laboratory and at a commercial hatchery to reveal that microencapsulated feeds could increase the growth and survivorship of European oyster (*Ostrea edulis*) juveniles relative to conventional live algal diets. I then demonstrated

that the feeds could facilitate improved sexual development in *O. edulis* broodstock and enable this stage of bivalve production to become an order of magnitude more sustainable and economically efficient.

The final phase of the thesis aimed to use both experimental and literature analyses to explore how microencapsulated diets and bivalve aquaculture could help tackle broader nutritional problems and contribute towards food security goals. I identified an optimal dosing strategy to fortify Pacific oysters (*Crassostrea gigas*) with vitamin A and D, and outlined how tailoring the micronutrients encapsulated and bivalve species reared could provide a low cost mechanism to tackle regional nutritional deficiencies directly through the food supply. I then built on this global perspective and reviewed how expansion of bivalve aquaculture could help tackle food security challenges in the developing world. Key components of the value chain requiring further research, industry investment and policy changes were identified, alongside the importance of a multifaceted strategy to stimulate increased consumer demand.

To summarise, my work in this thesis has demonstrated how applying new innovations in microencapsulation technology to bivalve aquaculture can provide powerful solutions to a range of industry challenges. Scale up and further application of the research breakthroughs I have made can contribute towards a global revolution in food production with widespread benefit to human and planetary health.

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I would like to pay my special regards to Professor Howard Griffiths, who worked beyond all expectations to facilitate a range of vacation research opportunities that developed my scientific passion and skills, provided encouragement to pursue postgraduate research, and for his continued enabling support as I have developed a research focus on food security. Dr Andrew Tanentzap must be commended for facilitating an engaging rotation project and a wealth of analytical training. I also wish to show my gratitude to Professor Andrew Balmford and Professor Bill Sutherland who have provided expert and tailored guidance both during the PhD and in my planning of future academic research.

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Chapter 1: Introduction

1.1. The global food security goal

Food is the single strongest lever to optimise human health and environmental sustainability on earth. However, food is currently threatening both people and planet (Figure 1.1.)¹. We face a double-burden of malnutrition and overconsumption; worldwide 800 million people are malnourished and without enough food to meet their energy demands, two billion people receive adequate or excessive calories but are deficient in key nutrients, and at the same time over two billion are overweight or obese². In parallel, we have now exceeded the safe limit for four of the nine planetary boundaries that define the environmental limits within which humans could continue to develop and thrive for generations to come³. Agricultural activities emit 35% of greenhouse gases, occupy 40% of Earth's land, account for 70% of freshwater usage, and drive deforestation, habitat fragmentation, environmental eutrophication and biodiversity loss^{3,4}. Without concerted action the environmental impacts of the global food system will increase by 90% by 2050, and 50% of the global population will be overweight⁵. The USD \$127 trillion yr⁻¹ value of our ecosystems would halve, and the already high economic costs of obesity, 3% of global GDP, would skyrocket^{6,7}.

The global community now has the urgent goal to provide planetary health diets for an increased population of 10 billion people by 2050¹. Planetary health refers to 'the health of human civilization and the state of the natural systems on which it depends'⁸. Planetary health diets must meet the following requirements; they should contain healthy nutrition, tackle human health challenges, and be built upon sustainable food production. A strategy for action to provide these diets is needed^{1,3,6}.

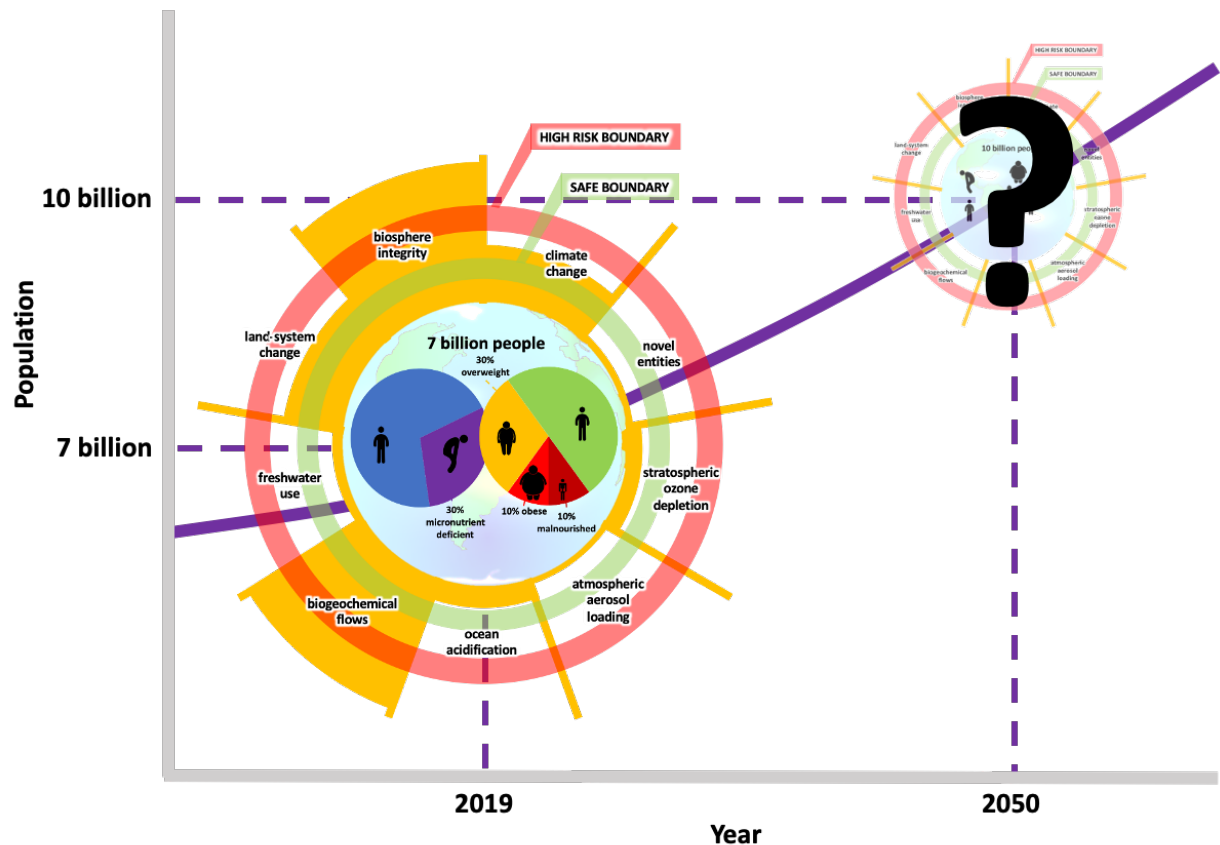


Figure 1.1. Pressures on planet Earth to tackle by 2050. For 2019, the proportion of the global human population who are overweight, obese, malnourished, and micronutrient deficient are shown in the planet centre. The extent to which we have exceeded the nine environmental planetary boundaries surround the planet. Figure developed from sources ^{2,3}.

1.2. Global strategies for action

The global strategy to provide planetary health diets has two key objectives. Firstly, a shift in agricultural priorities from producing large quantities of energy rich-food to producing healthy nutrient-rich food. Secondly, sustainable intensification of food production to increase high quality output ¹. Today, unsustainably high levels of red meat, sugar, and starch consumption are driving human health and environmental problems, and there is a need to cut consumption of these by at least 50%, while also increasing the production of vegetables, nuts, legumes and fish ¹. Sustainable intensification will require a minimum 75% reduction in yield gaps, improvements in fertiliser and water use efficiency, and crucially changes in the human food crops, feed crops, and animal foods we choose to produce ^{1,4,5}.

In order to meet the objectives and provide planetary health diets we must understand what the diet looks like at the consumer level. The planetary health diet is primarily plant-based, containing a diverse mix of vegetables alongside a smaller quantity of fruit, nuts, legumes, and complete grains (Figure 1.2.)¹. With meat production causing 60% of agricultural greenhouse gas emissions, this shift to primarily plant-based diets will reduce environmental impact⁹. However, consumption trends indicate people prefer meat, limiting potential impact for purely vegetarian solutions. In addition, nutrients key to human health are far less bioavailable in plants than meat, and rising atmospheric CO₂ is reducing the absolute content of these nutrients in plants^{10,11}. Therefore, a smaller but equally important proportion of the optimal diet will contain animal products to provide a rich and highly bioavailable source of key nutrients (Figure 1.2.)¹. As a result, there is now an important need to increase and intensify production of nutrient rich animal products that can be reared sustainably.

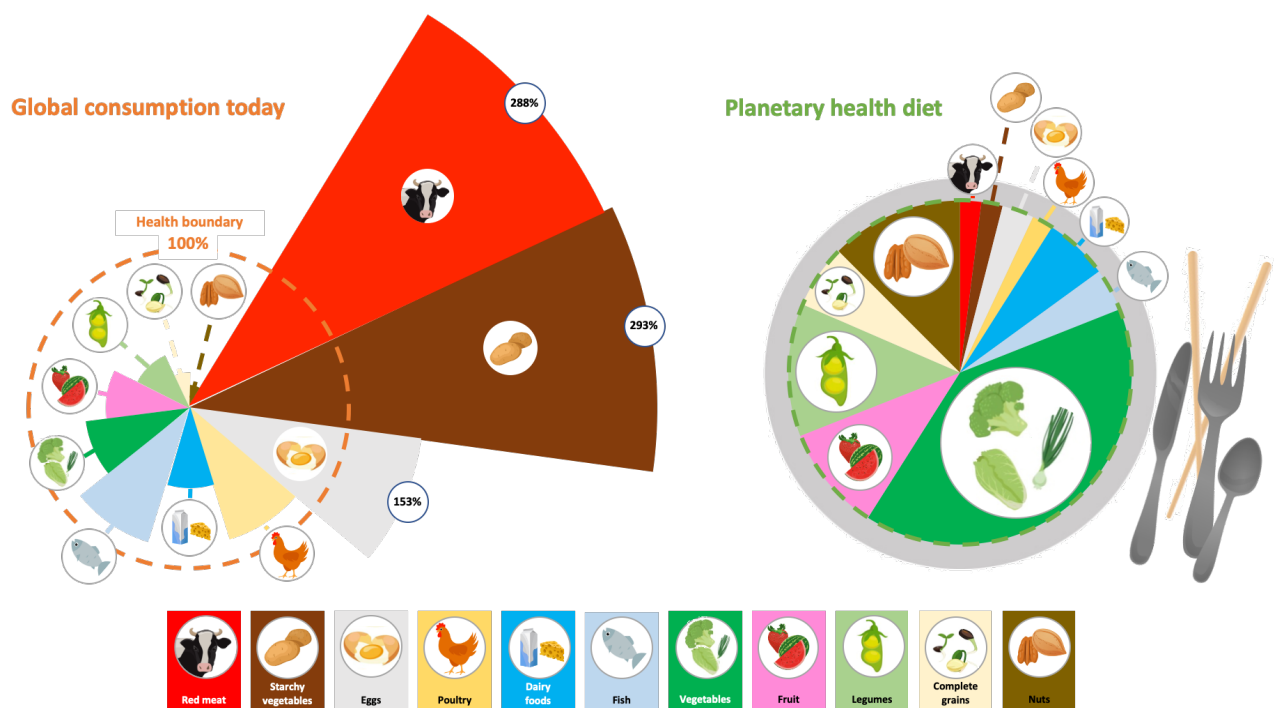


Figure 1.2. The planetary health diet. Comparing global food consumption today (2020) with optimal consumption for planetary health. The figure portrays the global overconsumption of red meat and starchy vegetables and a need to transform our dietary choices. The size of the wedges indicate consumption level relative to the 100% optimal health boundary. Figure developed from source¹.

1.3. Intensifying production of nutrient rich animal products

In order to sustainably intensify production of nutrient rich animal products, two important considerations need to be made. Firstly, we must carefully select the variety of animal products we improve the production of, based upon the health and environmental merits of each product. Secondly, we must optimise the mechanisms by which we sustainably improve the production of these products.

Terrestrial meat will remain a large component in the global food system, but changes are required to provide sustainability. Meats including beef, pork, and chicken are valuable nutrient sources, containing high levels of protein, zinc, iron, and vitamin A in particular (Figure 1.3.), with over 315 million tonnes currently produced worldwide ^{12,13}. However, their environmental footprint is also large, with beef having the highest greenhouse gas emissions, land use, freshwater use and eutrophication potential of all major food types (Figure 1.4.). Life Cycle Analysis (LCA) is used throughout this thesis in order to evaluate the environmental impacts of different food systems. An overview of the practical process of LCA, with specific regard to aquaculture, and the developments which have occurred since several chapters of this thesis were published, is outlined in Box 1.1.

There are several options to sustainably intensify terrestrial meat production. Efforts can be made to increase production of insect meat. Insects such as mealworms, crickets, and termites are rich in protein and vitamin B12 (Figure 1.3.), and have a lower environmental footprint than all other terrestrial meats (Figure 1.4.). A major challenge here will be in finding ways to shift consumer preferences towards these foods, with a relative minority of global cultures traditionally consuming insects ¹⁴. Changing the mechanism by which we produce beef, pork, or chicken is also an option. Laboratory grown meat has been shown to have a similar or improved nutritional profile to conventional meat. However, studies currently suggest that the environmental footprint of laboratory meat is indifferent to that of conventional meat. Significant improvements in production systems will be required to make sustainable laboratory meat a reality ^{14,15}.

Aquatic and marine animal meats will also continue to play a vital role in the global food system. Fish, crustaceans, and bivalves are among the most nutrient-rich food sources, containing exceptionally high levels of protein, omega-3 fatty acids and key micronutrients including selenium (Figure 1.3.). However, as with terrestrial meats, the environmental footprint of aquatic meats can be large. Today 90% of large predatory fish stocks, such as

sharks, tuna, marlin, and swordfish, have disappeared. Over 80% of the planet's remaining wild fish stocks are fully exploited, over-exploited, depleted, or in a state of collapse ¹⁶. Additionally, the farming of species such as shrimp and prawn also results a high level of carbon emissions and land use compared to other foods (Figure 1.4.).

Sustainable intensification of aquatic food production requires several key steps to be made. Firstly, there is a need to increase consumption of low trophic level wild fish species such as sprats, herring, and anchovies in replacement of higher trophic level species such as tuna and cod. Today just 3% of fish caught come from low trophic levels and there is significant room for improvement ¹⁷. Secondly, with annual catch of wild fish static over the past 30 years despite a doubling of fishing effort since 1950, there is a major need to change the mechanism by which we provide aquatic food products ¹⁶. Aquaculture is the alternative mechanism, and is already the world's fastest growing food sector, growing at 8% per annum over the past 30 years ¹⁶. There is great potential for further growth; worldwide there are 13 million km² of currently undeveloped coastline suitable for highly productive fish or bivalve aquaculture (Figure 1.5.) ¹⁸. Developing just 1% of this coastline could yield 340 Mt of fish and bivalve meat annually, enough to meet the annual protein needs of 2.8 billion people ¹⁸. There is need for transformation across the aquaculture sector to meet this potential ¹⁹.

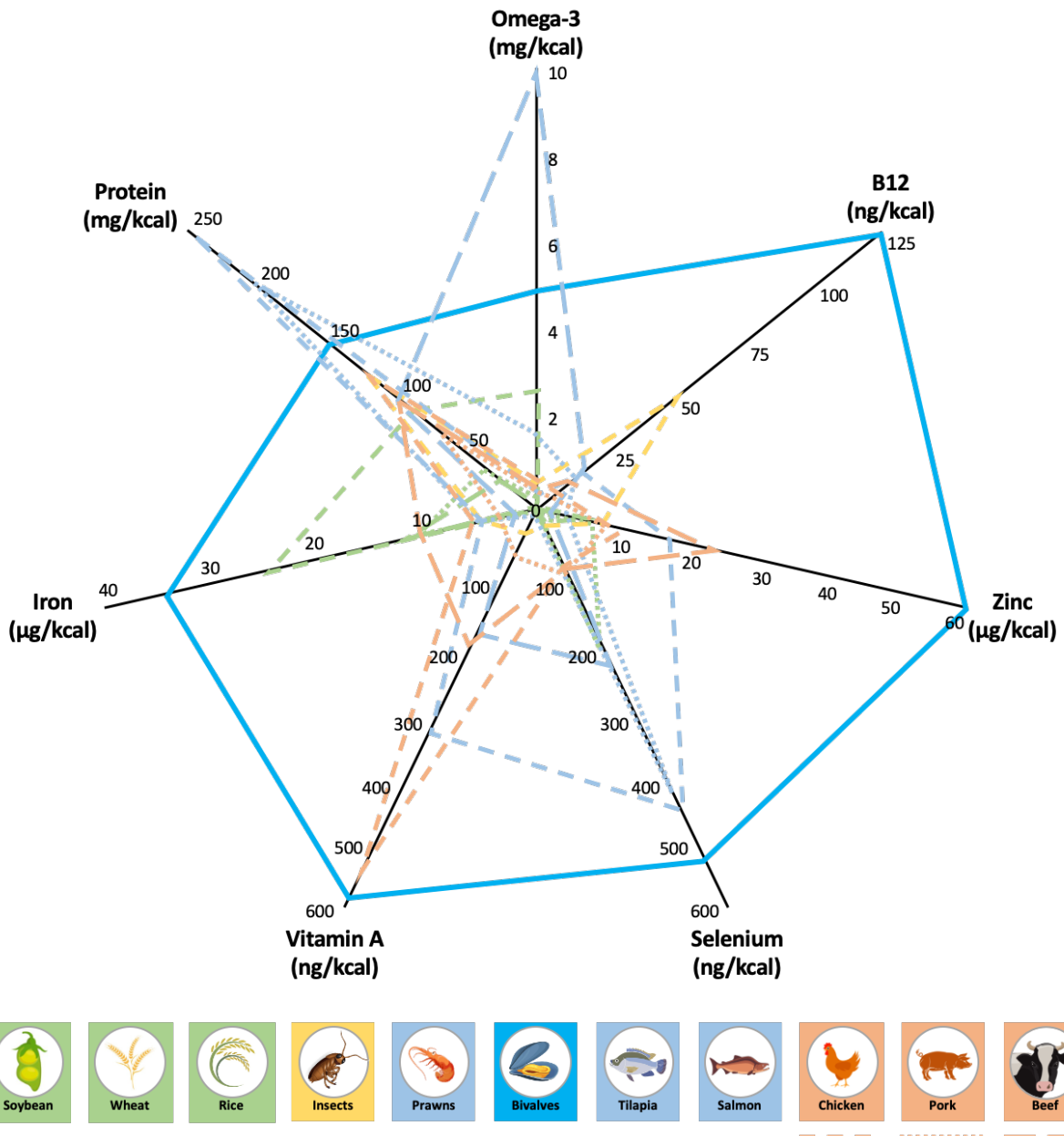


Figure 1.3. Nutritional value. Starplot comparing the content of key nutrients in major global food types. Aquatic and marine meats have exceptionally high levels of protein and omega-3 fatty acids and are the best source of many key micronutrients. Figure developed from source ¹³.

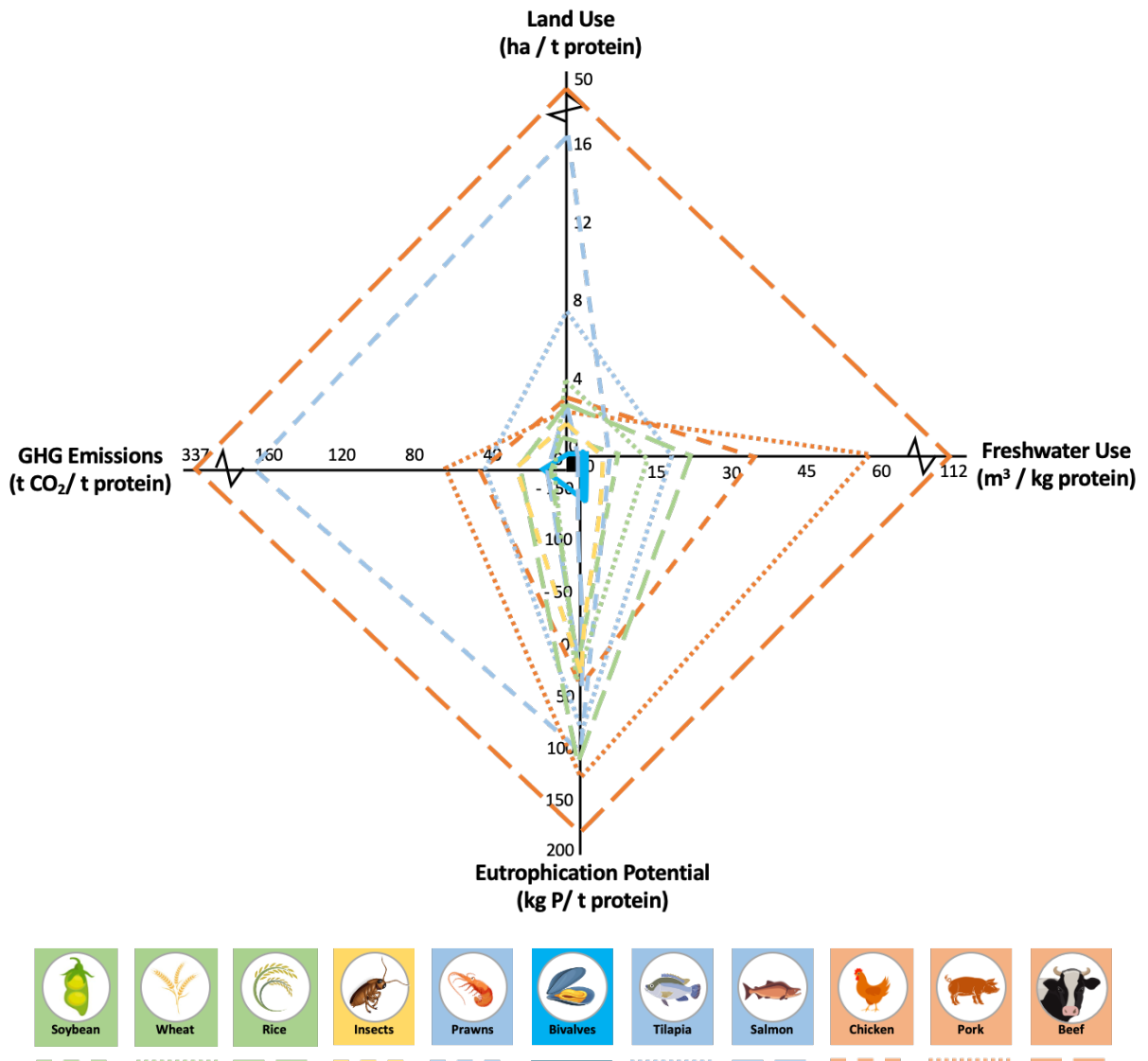


Figure 1.4. Environmental footprint. Starplot comparing the environmental footprint of major global food types. The environmental footprint of bivalves is lower than that of all other animal meats, and in many cases also lower than the plant crops. Note the broken axes for beef. Figure developed from sources ^{4,13,14,19,20}.

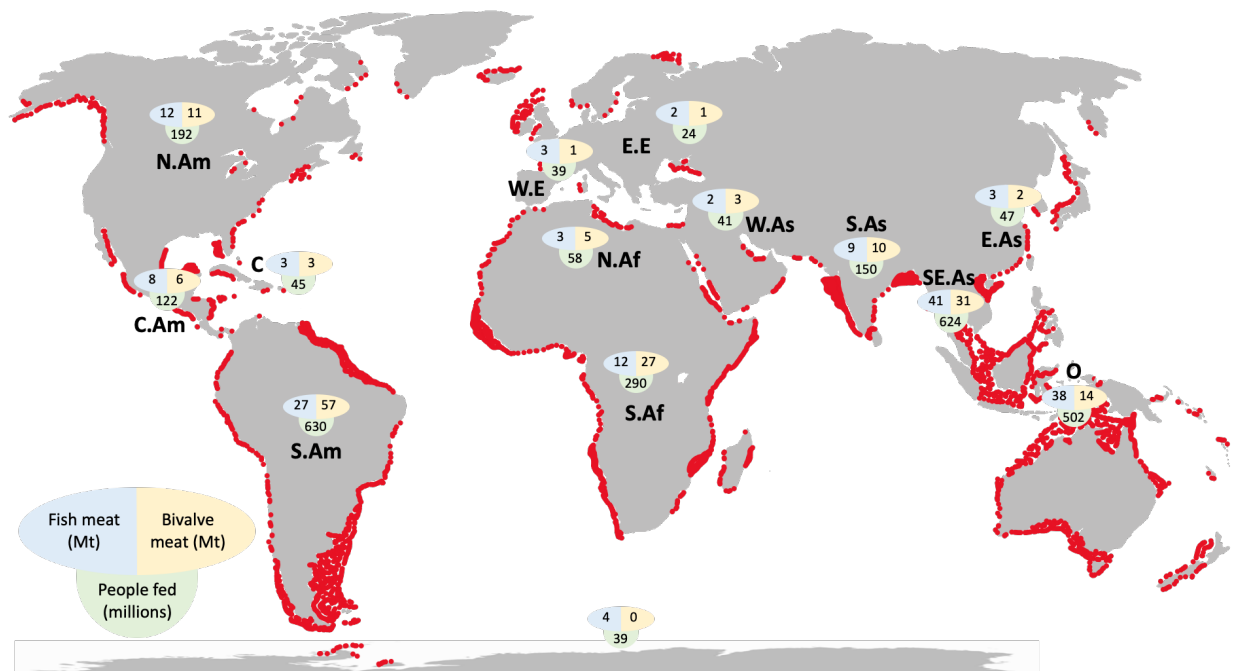


Figure 1.5. Global potential for aquaculture. Areas of coastline suitable for the development of productive fish or bivalve farming are shown in red. The quantity of fish meat and bivalve meat (Mt) that could be produced by developing just 1% of the suitable coastline is shown for each global region, alongside the number of people (millions) that could be fed with this meat as their only protein source. Regions are as follows: North America (N.Am), Central America (C.Am), Caribbean (C), South America (S.Am), Western Europe (W.E), Eastern Europe (E.E), Northern Africa (N.Af), Sub-Saharan Africa (S.Af), Western Asia (W.As), Southern Asia (S.As), Eastern Asia (E.As), South-Eastern Asia (SE.As), Oceania (O). Figure developed from source ¹⁸.

Box 1.1. Life Cycle Analysis and applications in aquaculture

Life Cycle Analysis (LCA) aims to identify and quantify the environmental impact of a process or product over the entire life cycle. In the context of food production systems, environmental impact categories within this analysis commonly include climate change, aquatic eutrophication, water use and land use ²¹. LCA is used throughout this thesis and in the production of display items such as Figure 1.4.

It is important to note that the output values of any LCA should be read with caution, as there are numerous factors that can influence the value displayed. Factors include the modelling approach and assumptions made in the analysis, the case-specificity of a given

analysis and its relevance for wider application, and importantly potential changes in a process over time that will affect its environmental impact ²¹.

To illustrate this point with specific regard to aquaculture one can consider Fish in : Fish out (FIFO) ratios. (FIFO) ratios are a principle metric used in life cycle analysis to assess the impact aquaculture may have on wild fish stocks. FIFO represents the amount of fish used to produce 1kg of farmed fish. Numerous calculation approaches have been used over the past three decades, and several developments have been made since many of the chapters of this thesis were published. One of the most recent approaches by Kok et al. 2020 is economic Fish in: Fish out (eFIFO) ratios, which include economic allocation to avoid double counting of fish resources ²². In eFIFO, the amount of fish required depends on the amount of feed needed to support 1kg of growth, the fraction of the feed that is fish meal and fish oil and the embodied fish per kg of fish meal and fish oil ²².

Consider salmon production, which is referred to in Chapter 2 of this thesis. Using Tacon & Metian's 2008 approach, which doesn't include economic allocation, the FIFO for salmon in 1995 is 7.8 ²³, yet when using Kok et al.'s 2020 approach the 1995 eFIFO value is 3.8 ²². These values differ greatly, and depending on which is reported very different outcomes could result regarding the selection of food production systems and policies we choose to make, emphasising the importance of considering the modelling approach. Chapter 2 of this thesis reports data using the Tacon & Metian approach, using a value of 4.9 for salmon ²⁴. FIFO values also change over time; the 2020 eFIFO value for salmon is just 1, due to advances in feeding practices. This emphasises the importance of using comparable approaches and time-relevant data when assessing global food solutions towards 2050.

1.4. The need to develop sustainable aquaculture

Aquaculture has the potential to play a pivotal role in providing nutrient rich food for planetary health diets, but must be done with minimal environmental costs ^{1,5,9,16,17,19}. To enable the industry to grow sustainably, improvements are needed across the entire value chain, from breeding to production to marketing ¹⁹. Improved selective breeding efforts are needed, to promote efficient resource use and lower production costs ¹⁹. New technologies and management practices are needed for sustainable disease control ^{19,25}. Feeding systems

need to be rethought; today over 3.6 Mt of fishmeal and fish oil, primarily from wild-caught fish, are used annually, and there is a need to shift to more sustainable sources such as algae^{19,25–27}. Better waste management and side stream recycling are needed to reduce resource usage and cut production costs¹⁹. Finally there is a need for careful selection of the aquaculture species we choose to farm, with a specific focus on low trophic level fish and bivalve shellfish, in order to optimise human health and environmental benefits (Figures 1.3., 1.4.). Effective marketing solutions will be needed to facilitate changes in consumer preferences towards these species^{1,19}.

This doctoral thesis focusses on developing bivalve shellfish aquaculture as a key component of sustainable aquaculture for global food security. Bivalve shellfish have a lower environmental footprint than all other meats and many plant crops, and are a rich source of key nutrients including protein, omega-3, selenium, zinc, iron, vitamin A and B12 (Figures 1.3., 1.4.). With over 1,500,000 km² of available coastline suitable for bivalve aquaculture worldwide there is outstanding potential for industry growth, and developing just 1% of this area would feed over 1 billion people with all their protein needs²⁸. In order for the industry to grow and meet its global potential there is a need to overcome problems and bottlenecks in bivalve production. New forms of microcapsules, recently developed as a mechanism to deliver toxins to biofouling shellfish²⁹, could have highly valuable application in aquaculture (Box 1.2.). This thesis explores the exciting opportunity to use these microcapsules to deliver nutritional products instead of toxins to bivalves, improve the quantity and quality of bivalves that can be produced, and help provide a solution to key bivalve industry problems.

Box 1.2. The development of microencapsulation technology

Microencapsulation technology enables compounds to be contained within tiny spheres known as microcapsules, with each having a diameter as small as 1 µm, to several hundred micrometres. A wide variety of active ingredients can be encapsulated including drugs, enzymes, vitamins, flavourings, and catalysts, with applications ranging from pharmaceuticals and food production to hazard control and defence³⁰. As discussed in Chapter 2 microcapsules offer a crucial advantage over other active nutrient delivery systems, allowing ingredients to be released at a precise and appropriate location and timepoint dependent upon the formulation^{25,30}. Capsules can take a range of forms, from a matrix formulation, to capsules with multiple cores, to monocore capsules with a single interior chamber and surrounding envelope³⁰.

The microcapsules used in this thesis are classed as monocoresh microcapsules, and were first developed under the name 'BioBullets' as a way of safely and effectively controlling invasive zebra mussels *Dreissena polymorpha*^{29,30}. The capsules contained the active ingredient KCl, and their specific size, buoyancy, and palatability allowed them to be preferentially filtered and ingested by the mussels. As a result, the mussels would accumulate a toxic dose of KCl over time, causing mortality, whilst the surrounding ecosystem would be unaffected due to the low concentration of the capsules in the water²⁹. This thesis aimed to leverage the microencapsulation technology to feed bivalves instead of eliminating them, allowing intensification of key stages within the bivalve production system³¹.

The thesis is presented as a set of published papers, outlined below. I took the lead on all pieces of work. My supervisor Dr David Aldridge contributed to study design, interpretation and reviewed each chapter before publication. Dr Samuel Furse performed the experimental component of the fatty acid and lipid analyses in Chapter 5.

Chapter 2: Microencapsulated diets to improve bivalve shellfish aquaculture for global food security³¹

There is a global need to sustainably increase aquaculture production to meet the needs of a growing population. Bivalve shellfish aquaculture is highly attractive from a human nutrition, economic, environmental and ecosystem standpoint. However, bivalve industry growth is severely constrained by problems throughout the value chain. New advances in microencapsulation technology have great potential to tackle these problems. Microencapsulated diets could efficiently deliver high-quality nutrients, disease control agents, and quality enhancers to bivalves. Microencapsulation has the potential to drive a step change in bivalve production, reduce production costs, enhance human nutrition and minimise impacts on the environment. If 25% of the protein we currently obtain from fish aquaculture was obtained from bivalves, we could spare an area of land larger than Wales, annual CO₂ emissions equal to half New Zealand's emissions, and annually 11.8 billion litres of freshwater.

Willer, D. F. & Aldridge, D. C. (2019). Microencapsulated diets to improve bivalve shellfish aquaculture for global food security. *Global Food Security* 23, 64–73. doi: 10.1016/j.gfs.2019.04.007

Chapter 3: Microencapsulated diets to improve bivalve shellfish aquaculture ³²

Bivalve production is heavily constrained by problems throughout the value chain, in particular poor feeding approaches, and new microencapsulation technology can provide a solution. This study demonstrated that a new formulation of microencapsulated diet we have developed known as BioBullets could be ingested by a commercially farmed bivalve; the blue mussel *Mytilus edulis*. Microparticles could be captured by mussels with similar efficiency to natural foods. Microparticles too large for ingestion were rejected in pseudofaeces. Microparticles were successfully ingested and broken down by the gut. Further work is required to assess the impact of microencapsulated diets on bivalve growth.

Willer, D. & Aldridge, D. C. (2017). Microencapsulated diets to improve bivalve shellfish aquaculture. *Royal Society Open Science* 4, 171142. doi:10.1098/rsos.171142

Chapter 4: Microencapsulated diets to improve growth and survivorship in juvenile European flat oysters (*Ostrea edulis*) ³³

Oysters represent 54% of the global bivalve market by value, with propagation of juveniles within hatcheries critical to allow the industry to grow. Growth and survival of juvenile oysters in hatchery systems is constrained by suboptimal feed. The live algal feed currently used is expensive, of variable quality, contamination prone, and the high level of skill and equipment required limits where hatcheries can be located. We demonstrate how a novel microencapsulated diet can increase the growth and survivorship of *Ostrea edulis* (European flat oyster) juveniles in both the laboratory and hatchery setting. *O. edulis* larvae fed a combined diet of microcapsules and algae for 8 days had a 46% greater increase in maximum size, 171% greater increase in minimum size, and 5% higher survival than larvae fed algae alone. *O. edulis* spat of 4 mm fed the combined diet for 7 weeks also had significantly greater survivorship (16% greater in hatchery, 58% greater in laboratory) and growth comparable (hatchery) or better (laboratory experiments) than algae alone.

Willer, D. & Aldridge, D. C. (2019). Microencapsulated diets to improve growth and survivorship in juvenile European flat oysters (*Ostrea edulis*). *Aquaculture* 505, 256–262. doi: 10.1016/j.aquaculture.2019.02.072

Chapter 5: Microencapsulated algal feeds as a sustainable replacement diet for broodstock in commercial bivalve aquaculture³⁴

The global bivalve shellfish industry relies upon a supply of juvenile bivalves produced by adult broodstock in hatcheries. Today large quantities of live algae are grown to feed broodstock at \$220 kg⁻¹, driving highly unsustainable energy and resource use. New advances in algal and microencapsulation technology provide solutions. We developed microencapsulated *Schizochytrium* algae diets, which can be produced sustainably at < \$2 kg⁻¹ from organic side-streams, and are shelf-stable to minimise waste. Physiological, histological, and cutting-edge metabolomic analyses demonstrate that in commercial settings sustainable microencapsulated diets facilitate improved sexual development and 12x greater omega-3 levels in oysters relative to conventional live algal diets. Further research into microencapsulated diets could support bivalve industry expansion, and contribute towards a step-change in sustainable global food production.

Willer, D. F., Furse, S. & Aldridge, D. C. (2020). Microencapsulated algal feeds as a sustainable replacement diet for broodstock in commercial bivalve aquaculture. *Scientific Reports*. doi: 10.1038/s41598-020-69645-0

Chapter 6: Vitamin bullets. Microencapsulated feeds to fortify shellfish and tackle human nutrient deficiencies²⁸

Over 2 billion people worldwide are micronutrient deficient, with regionally specific deficiencies, and a need for targeted micronutrient delivery to people through the food supply. Bivalve shellfish provide the most sustainable source of animal protein on the planet and are unique in that nutrients consumed at their end-life stage will be digested by humans, as humans consume the entire organism including the gut. We developed a novel microencapsulated vehicle for delivering micronutrients to bivalves to realise the potential of fortified bivalves to tackle human nutrient deficiencies. We demonstrated that oysters fed vitamin A and D microcapsules at a 3% initial dosage for just 8 hours had elevated tissue vitamin content. A serving of just two such bivalves provides enough vitamin A and D to meet human dietary RDAs. Scale-up of this technology and application to other bivalve species could provide a low-cost and highly sustainable mechanism to contribute towards tackling nutrient deficiencies globally.

Willer, D. F. & Aldridge, D. C. (2020). Vitamin bullets. Microencapsulated feeds to fortify shellfish and tackle human nutrient deficiencies. *Frontiers in Nutrition*, 7:102. doi: 10.3389/fnut.2020.00102

Chapter 7: Sustainable bivalve farming can deliver food security in the tropics³⁵

Suboptimal global food production is directly related to poor diets, nutrition-related disease and environmental pressure. The tropical regions bear the brunt of this crisis. Bivalve shellfish production is severely underdeveloped in the tropics, yet bivalves are one of the most sustainable and nutrient-rich food sources available, and there is outstanding potential to expand production. Efficient and effective development of bivalve aquaculture in the tropics will require several key components, with the implementation of new innovations and technologies across the value chain. New feeding and breeding approaches, grow-out and food safety investment, and novel approaches to seafood distribution and marketing will all play vital roles. Applying these approaches to develop just 1% of the potential production area in the tropics could feed nearly a billion people by 2050.

Willer, D. F. & Aldridge, D. C. (2020). Sustainable bivalve farming can deliver food security in the tropics. *Nature Food*, 1(7): 384-388. doi: 10.1038/s43016-020-0116-8

Chapter 8: Conclusion

The thesis aimed to apply breakthrough microencapsulated feed technology to research aiming to significantly improve the productivity of bivalve shellfish aquaculture, and thus help the bivalve industry to grow and play a leading role in meeting global food security goals. Novel microencapsulated feeds were developed, characterised, and proven digestible by commercially farmed bivalve species. Feeds enabled increased growth and improved survivorship in bivalve juveniles, enhanced the quality of broodstock and accelerated sexual development, and enabled fortification of adult bivalves with key nutrients for improved human health – results demonstrated in both laboratory experiments and industry trials. The thesis highlights the outstanding benefit of bivalves as a sustainable food source for a growing global population and the incredible open opportunity for industry expansion that could feed billions of people with nutrient rich food. Further research and stakeholder engagement to scale-up the use of microencapsulation technology, develop the bivalve value chain, and stimulate increased consumer demand can enable this opportunity to be realised.

Appendices

While not at the core of this Thesis, I include as appendices three additional papers that I authored during the course of this PhD. Appendix 1 explores wider opportunities for market expansion of bivalve aquaculture, Appendix 2 results from the obligatory BBSRC Professional Internship for PhD Students placement, and Appendix 3 results from the obligatory BBSRC Rotation Project.

Appendix 1: From pest to profit – The potential of shipworms for sustainable aquaculture

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We face a food crisis. Suboptimal diet is the biggest cause of death worldwide, food production the biggest greenhouse gas emitting sector, and by 2050 an extra 2.5 billion people need affordable nutrition. Current farming systems will fail to tackle this crisis, and there is an urgent need to diversify global food production and find effective solutions in currently underexploited food sectors. Shipworms, or shell-less *Teredo* clams, could prove a highly valuable component of such solutions. Historically viewed as a marine pest, they have unique physiological characteristics which make them an ideal food source, including exceptionally fast growth rates, the ability to feed on waste wood or sustainable microalgae, and a high protein and omega 3 content. Today only a select few traditional cultures in the Philippines consume shipworms, but there is considerable opportunity to develop mechanisms to farm shipworms and provide a sustainable, nutrient rich, affordable food source. This will require significant challenges to be overcome, ranging from fundamental research to industry development to food processing and marketing. Leveraging new innovations in breeding, aquaculture feeds, growth systems, food processing methodologies and consumer engagement can however offer powerful solutions, and could help turn what was once a maritime villain into a nutritional saviour.

Willer, D. F. & Aldridge, D. C. (2020). From pest to profit - The potential of shipworms for sustainable aquaculture. *Frontiers in Sustainable Food Systems*, 4:575416.
doi:10.3389/fsufs.2020.575416

Appendix 2: Matches and Mismatches Between Global Conservation Efforts and Global Conservation Priorities³⁷

Species extinctions are occurring 1000 times faster than background rates and conservation efforts must focus limited resources on prioritised threats and habitats. To protect biodiversity conservation organisations mobilise funds; effort realised in staff time, research and public engagement. There is need to understand whether global conservation effort is distributed appropriately across Threats and Habitats to protect the greatest number of high extinction risk species. In this study three major measures of global conservation effort across Red List Threats and Habitats were assessed; staff time spent by the largest cluster of conservation organisations in the world - Cambridge Conservation Initiative, efforts by international NGOs through social media, and global conservation research publications since the year 2000. We find global conservation effort is generally aligned with global conservation priorities, but there are important outliers. Shrublands and rocky areas receive disproportionately little investment across all effort measures relative to the number of high extinction risk species, threats from residential and commercial development receive relatively low research and time investment despite social media attention, while marine areas and climate change receive more attention than expected. Governments and society must make critical conservation decisions in the context of rapid global change, and there is potential for key Threats or Habitats to receive less attention than required. The global conservation community would be wise to carefully consider and improve its understanding of effort-priority mismatches if the greatest number of high extinction risk species are to be protected.

Willer, D. F., Smith, K. & Aldridge, D. C. (2019). Matches and Mismatches Between Global Conservation Efforts and Global Conservation Priorities. *Frontiers in Ecology and Evolution* vol. 7 297. doi: 10.3389/fevo.2019.00297

Appendix 3: Feasting on terrestrial organic matter: Dining in a dark lake changes microbial decomposition³⁸

Boreal lakes are major components of the global carbon cycle, particularly because of sediment-bound heterotrophic microorganisms that decompose terrestrially derived organic matter (t-OM). The ability for sediment bacteria to break down and alter t-OM may depend on environmental characteristics and community composition. However, the connection between these two potential drivers of decomposition is poorly understood. We tested how

bacterial activity changed along experimental gradients in the quality and quantity of t-OM inputs into littoral sediments of two small boreal lakes, a dark and a clear lake, and measured the abundance of operational taxonomic units and functional genes to identify mechanisms underlying bacterial responses. We found that bacterial production (BP) decreased across lakes with aromatic dissolved organic matter (DOM), but the process underlying this pattern differed between lakes. Bacteria in the dark lake invested in the energetically costly production of extracellular enzymes as aromatic DOM increased in availability in the sediments. By contrast, bacteria in the clear lake may have lacked the nutrients and/or genetic potential to degrade aromatic DOM, and instead mineralized photo-degraded OM into CO₂. The two lakes differed in community composition and the higher concentration of dissolved organic carbon in the clear lake was associated with the community assemblages in the clear lake. Furthermore, functional genes relating to t-OM degradation were relatively higher in the dark lake. Our results suggest that future changes in t-OM inputs to lake sediments will have different effects on carbon cycling depending on the potential for photo-degradation of OM and composition of resident bacterial communities.

Fitch, A., Orland, C., **Willer, D.**, Emilson, E. J. S. & Tanentzap, A. J. (2018). Feasting on terrestrial organic matter: Dining in a dark lake changes microbial decomposition. *Global Change Biology* 24, 5110–5122. doi: 10.1111/gcb.14391.2017

Chapter 2: Microencapsulated diets to improve bivalve shellfish aquaculture for global food security

Willer, D. F. & Aldridge, D. C. (2019). Microencapsulated diets to improve bivalve shellfish aquaculture for global food security. *Global Food Security* 23, 64–73. doi: 10.1016/j.gfs.2019.04.007

There is a global need to sustainably increase aquaculture production to meet the needs of a growing population. Bivalve shellfish aquaculture is highly attractive from a human nutrition, economic, environmental and ecosystem standpoint. However, bivalve industry growth is falling behind fish aquaculture due to critical problems in the production process. Feed defects, disease, and quality issues are limiting production. New advances in microencapsulation technology have great potential to tackle these problems. Microencapsulated diets could efficiently deliver high-quality nutrients, disease control agents, and quality enhancers to bivalves. Microencapsulation has the potential to drive improvements in bivalve production, reduce production costs, enhance human nutrition and minimise impacts on the environment.

2.1. The global importance of bivalve shellfish aquaculture

2.1.1. Bivalves a strategic food source to sustainably feed a growing global population

Over 800 million people worldwide are hungry, one billion have inadequate protein intake, and an even greater number suffer from nutrient deficiencies³⁹. By 2050 these and 2.5 billion additional people will need access to nutritious food⁴⁰. Consequently by 2050 both total food demand and animal protein demand are expected to double⁴¹. Terrestrial meat production has tripled over the last 40 years to keep pace with demand⁴². But this growth is unsustainable; terrestrial meat production is a major driver behind humanity exceeding safe biophysical thresholds for the planet, and already uses 70% of agricultural land and 30% of freshwater, and causes 18% of greenhouse gas emissions and 30% of biodiversity loss^{42,43}. Expanding aquaculture is seen as a possible solution and has been identified as a critical component in securing food for 9.8 billion people by 2050^{19,44,45}.

Globally over 3 billion people depend on aquaculture for at least 20% of their dietary protein⁴⁴. Over the last decade animal aquaculture production has grown at 5.6% per year to 78 million tonnes, was worth US\$ 160 billion in 2015, and is the world's fastest growing food sector^{17,44,46,47}. However, like terrestrial meat production aquaculture is currently expanding in an unsustainable way^{45,46}. Production quantity of carnivorous species such as salmon, catfish, and shrimp has ballooned, growing 84% over the last decade, and today salmon is the largest single commodity in aquaculture^{47,48}. Production of these species is reliant upon fish meal and fish oil from wild-caught fish, with 5 kg of wild-caught fish required to produce 1 kg of farmed salmon²⁴. Wild fish stocks are suffering; 31% of stocks are overfished and a further 60% fished to their biological limit⁴⁸. Total capture of wild fish has been static since the 1980s despite increased fishing effort⁴⁸.

To sustainably provide food for a growing global population, there is a great need for aquaculture to focus on species lower in the food web that require little or no fish as feed^{45,46}. Bivalve molluscs offer one of the most attractive options for meeting this sustainability need. In 2015 14.8 million tonnes of bivalves were produced globally, and if bivalve aquaculture was to grow at the same rate as predicted for carnivorous fish aquaculture over the next decade, an extra 13.1 million tonnes of bivalves would be produced per year, feeding nearly twice as many people as bivalves do today⁴⁷.

2.1.2. Nutritional, economic and environmental benefits of bivalve aquaculture

Bivalve shellfish aquaculture is highly attractive from a human nutrition, economic, environmental and ecosystem standpoint and deserves a concerted research-led industry focus to increase production. Bivalves have a higher protein content than beef (140 vs 85 mg protein kcal⁻¹), and are a rich source of essential omega-3 fatty acids including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), needed for infant development, cognitive function and cardiovascular and neural disease prevention^{17,49}. DHA and EPA levels in bivalves (1.76 and 2.12 mg g⁻¹ respectively) are comparable with oily fish (2.61 and 2.27 mg g⁻¹) and far exceed that of terrestrial meats (0.02 and 0.03 mg g⁻¹)¹⁷. Bivalves are highly affordable, with a global average farm gate price of \$1.10 kg⁻¹, compared to \$4.70 kg⁻¹ for salmon and \$2.10 kg⁻¹ for aquaculture in general¹⁹.

The environmental footprint of bivalve aquaculture is also far lower than all other forms of meat or fish production and many arable crops, in terms of greenhouse gas emissions, land use, freshwater use, and eutrophication potential per unit protein (Figure

2.1.). To help appreciate just how significant this is, if just 25% of carnivorous fish aquaculture was replaced with an equivalent quantity of protein from bivalve shellfish aquaculture, 16.3 million tonnes of CO₂ emissions could be saved annually, equivalent to half the annual emissions of New Zealand^{19,50}. An area of land larger than Wales (2.7 million ha) could be spared from conversion to farmland, 11.8 billion litres of freshwater could be saved each year, and a net 21.1 million kg of P could be removed from eutricated waters globally¹⁹. Bivalve farming also provides a wide array of marine ecosystem benefits, including the provision of nursery habitats for fish, filtration of the water column, enhanced denitrification rates, coastal protection, buffering against harmful phytoplankton blooms, and even restoration of coastal and estuary ecosystems^{51,52}. Caution should of course be taken when expanding bivalve aquaculture to avoid some of the potentially deleterious environmental effects; species native to a region are best selected for aquaculture to avoid ecosystem damage caused by the introduction of non-indigenous species, and the potential risks of increased sedimentation from bivalve excrement should be assessed case by case^{53,54}. With careful planning, a concerted effort to increase bivalve production could make up an invaluable component of global goals to provide nutritious and sustainable food to people over the coming decades.

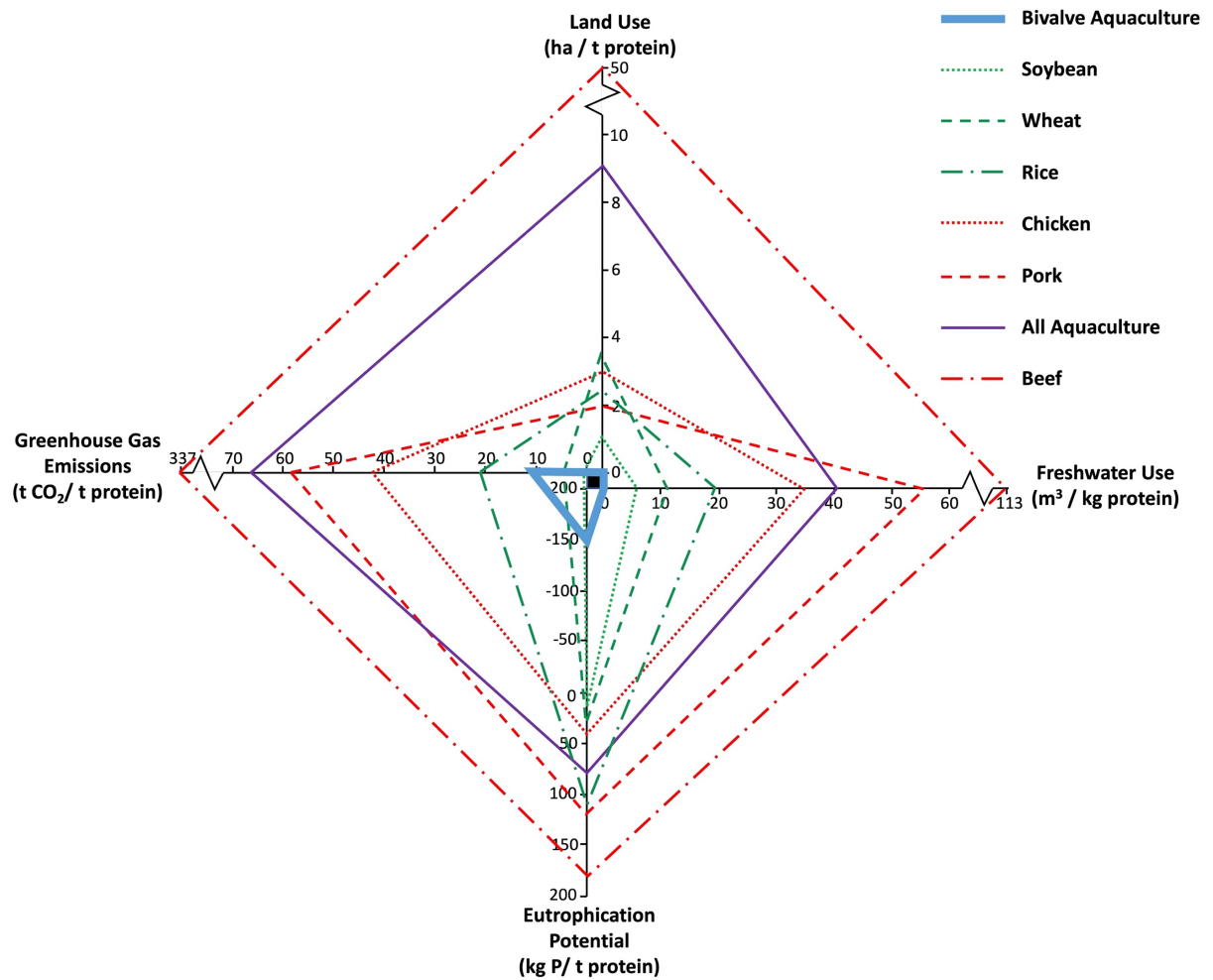


Figure 2.1. The lower environmental footprint of bivalve aquaculture compared to other plant and meat food sources. Raw data for animal meats from ¹⁹. Freshwater consumption data for plant crops from ²⁰ and converted from tonnes food to tonnes protein using ¹³. Greenhouse gas emissions, land use, and eutrophication potential data for plant crops from ⁴. Note the broken axes due to high values for beef.

2.1.3. Global bivalve aquaculture production, management, distribution and consumption

The bivalve shellfish sector is an important growing global industry. Production quantity has grown at 2.7% per year over the last decade and in 2015 14.8 million tonnes of the major bivalve species (oysters, clams, mussels and scallops) were produced with a farm gate value of US\$17 billion (Figure 2.2) ⁴⁷. Over 93% of bivalve production occurs in Asia, and over 90% of Asia's production is in China, with the remainder in coastal areas of Europe, the Americas, and Oceania (Figure 2.2) ⁴⁷. Production *per capita* varies significantly between countries and is highest in New Zealand at 16.8 kg *per capita* ^{47,55}. Historical farming culture and species site preferences strongly influence the range of species farmed within a given

region, for example in Asia clams are the primary farmed species and in Europe mussels^{47,56}. In China Shandong Province in particular is a highly productive region and produces 68% of the world's bivalves⁴⁷. A case study on shellfish production in China, where bivalve consumption has increased nearly 7-fold over the last 25 years, is provided in Figure 2.3⁴⁷.

Worldwide the bivalve shellfish industry has a distinctive structure with fragmented hatcheries and grow-out operators, overseen by regional and national governing bodies covering the value chain. The production process starts with bivalve juveniles ('spat', 'seed') which are hatched, reared and fed in hatcheries on land before being grown out in the sea⁵⁷. Hatcheries are small-scale, and continue to operate mostly by 'feel' rather than by 'science'⁵⁷. On a local level strong relationships exist between hatcheries and grow-out operators. Worldwide the producer industry remains fragmented and grow-out operators commonly farm bivalves as a sideline to other aquaculture species, in China represented by 20000 privately operated farms and 1200 state run farms⁵⁸ (Figure 2.3). In China the state-run Chinese National Fisheries Corporation oversees the entire value chain from production to retail. In Europe and Oceania the European Commission on Fisheries, the Australian Fisheries Management Authority and the New Zealand Ministry for Primary Industries oversee production, and in the United States and Canada large vertically integrated shellfish businesses and governmental departments link hatchery and grow-out operations.

Bivalves are mainly consumed domestically providing an inexpensive food for millions of people⁵⁹. Less than 5% of world production is traded. Currently suboptimal transportation pathways and a desire for bivalves to be consumed fresh are a technical constraint, but long distance distribution is possible, and typically used for the small proportion of higher value bivalves sold in quality restaurants^{47,56,59}. The market price varies widely, averaging \$1.10 kg⁻¹ across all bivalve species in China, whilst in Europe the average is \$3.36 kg⁻¹ (2017). This is significantly less than fish such as salmon at \$8.00 kg⁻¹, although in Europe some oyster species can fetch \$6 - 22 kg⁻¹^{47,60}. In Asia bivalves provide millions of people with an inexpensive source of food rich in amino acids, essential fatty acids, essential minerals, and vitamins⁶¹. In the developed world where over 2 billion consume too many calories but do not get the nutrients they need, bivalves provide an affordable healthy food^{61,62}.

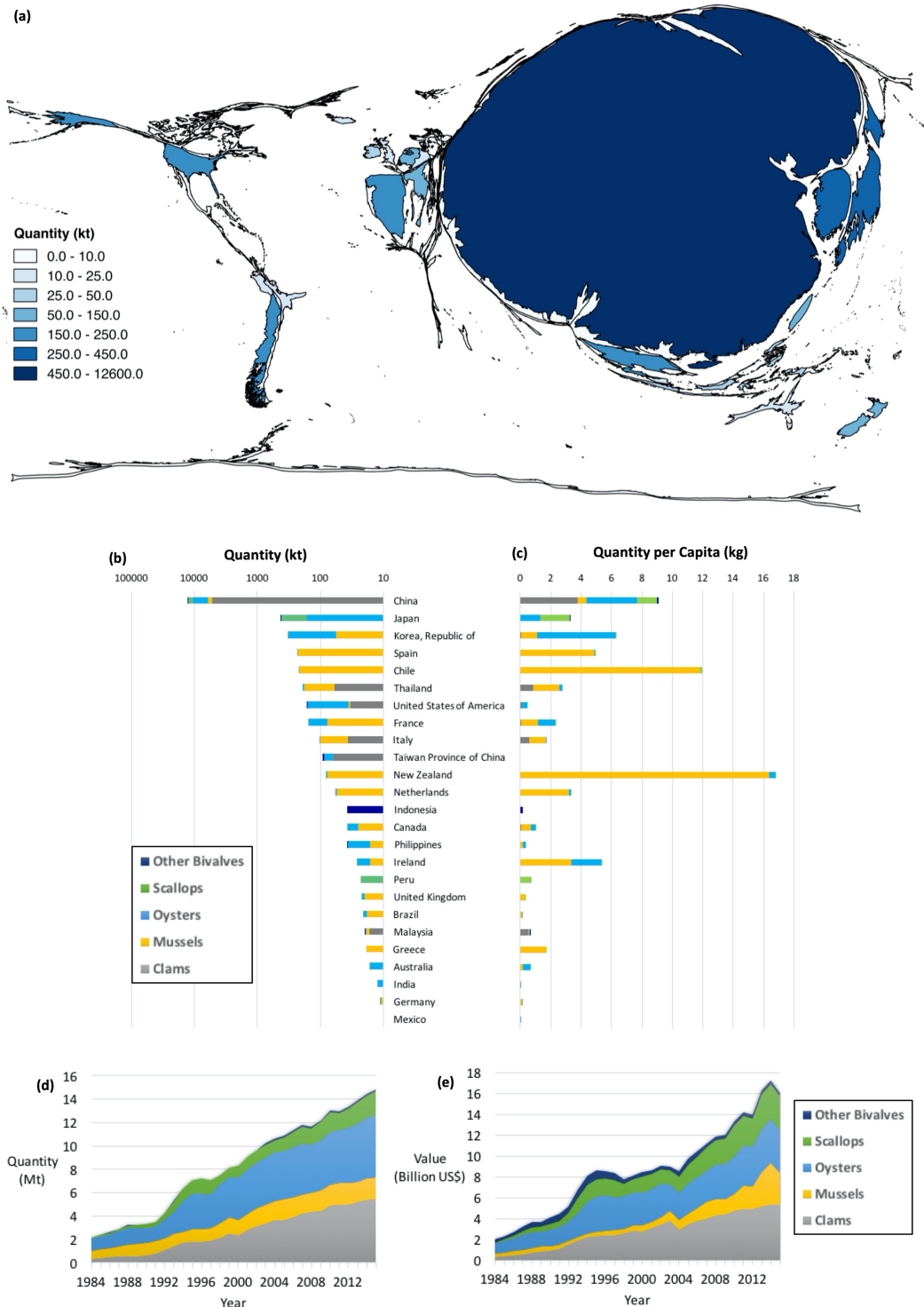


Figure 2.2. A synthesis of the global production of bivalves, highlighting the importance of Pacific and western European nations. The total quantity of bivalves produced in 2015 by country is shown on the cartogram in (a); the relative size and shading of countries is scaled according to production quantity⁶³. A breakdown of bivalve production quantity by species for the top 25 countries is shown on a logarithmic scale in (b), and the production quantity *per capita* for these countries in (c). The global growth in bivalve production quantity and value is displayed in (d) and (e) respectively. Raw data from^{47,55}.

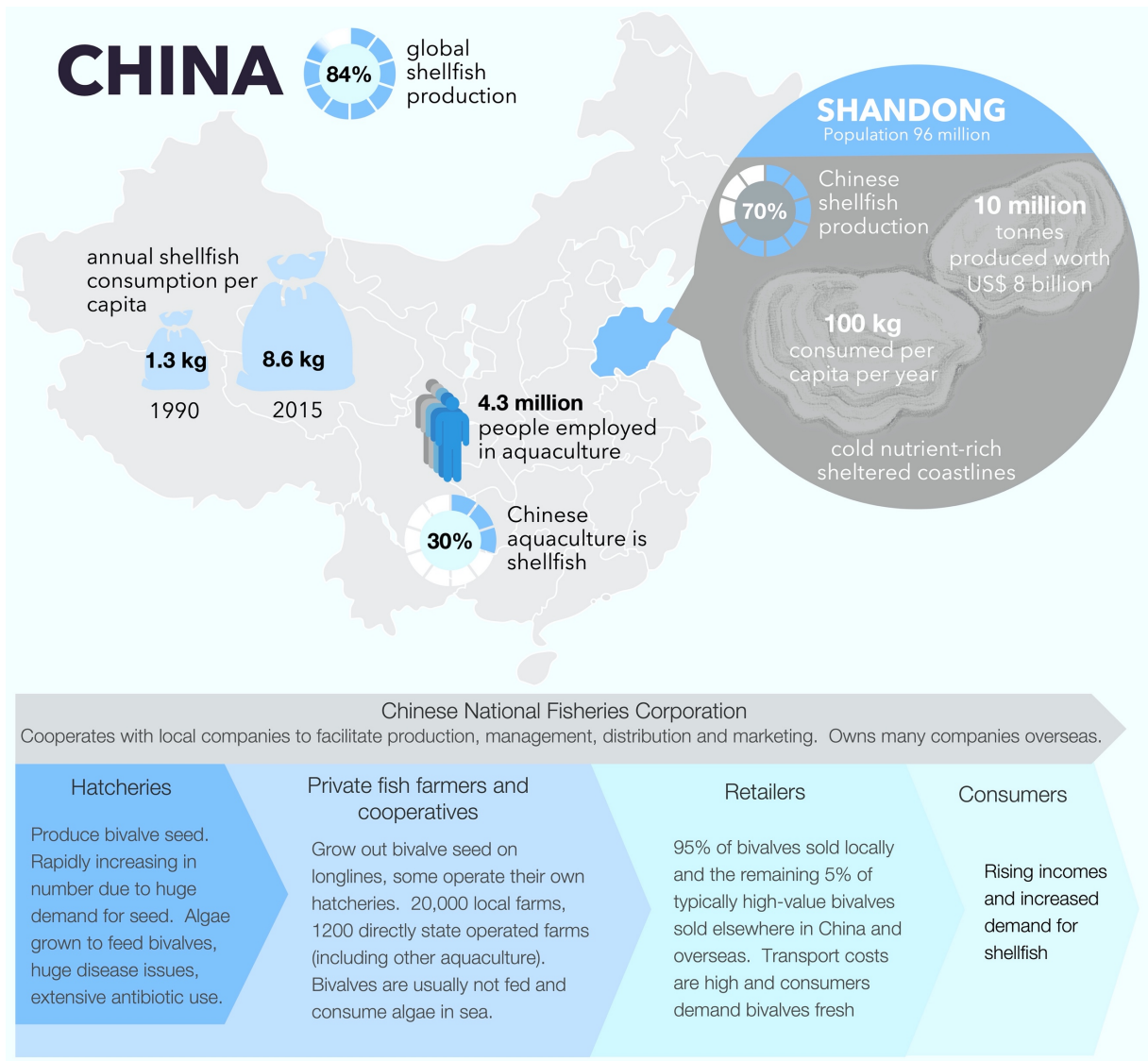


Figure 2.3. Shandong: a major shellfish production centre in China. The flow diagram provides an overview of the production value chain in China. Raw statistics from ^{47,58,64,65}.

2.2. Factors limiting industry growth in bivalve aquaculture

2.2.1. Production problems are a major driver to the falling share of bivalves in global aquaculture

Growth in the bivalve industry growth is falling behind the rest of aquaculture. Of the 78 million tonnes of animal aquaculture produced globally in 2015, bivalve shellfish made up 19%, down from 25% in 1990, and production has increased just 2.7% per year over the last decade, compared to 8.4% for carnivorous fish aquaculture ⁴⁷. Lower growth rates have a number of probable drivers, in which consumer taste, consumer access and marketing, food

processing, distribution, supplier fragmentation, expertise and research and development investment all have a part to play^{19,57}. However, it is clear that today several major problems specifically in the bivalve production process are contributing to low industry growth, outlined in Figure 2.4. and explained below⁵⁷.

2.2.2. Microalgae feed problems

Multiple defects in live microalgae feed increase costs and reduce bivalve growth and maturation rates yet so far no solutions or satisfactory alternatives have been found. Growing live microalgae to feed bivalves in hatcheries and nurseries takes up 50% of production costs at \$160-400 per kg biomass^{66,67}. The microalgae produced are of highly variable and often poor quality, and susceptible to frequent contamination and population crashes^{25,66}. Even the highest quality microalgae do not have the optimal nutrient composition for all stages of bivalve development, so multiple genera and cultures have to be grown on each site (e.g. *Isochrysis*, *Tetraselmis*, *Chaetoceros*, *Thalassiosira*, *Nannochloropsis*)^{49,68}. To ensure reasonably consistent culture quality, microalgae have to be grown in controlled indoor environments, creating an expensive and major bottleneck limiting bivalve production⁶⁷. Since 1990 commercial and research bivalve hatcheries worldwide have repeatedly identified a strong need for alternative diets to replace live microalgae, but to date no satisfactory product has been developed^{67,69,70}.

2.2.3. Disease losses in the hatchery

Disease causes significant losses, prevention is costly and often it generates environmental damage and human health risk. Complete bivalve batches including juveniles and breeding adults are often lost, leading to hatchery closure⁷¹. *Vibrio sp.* bacteria are the major disease problem; mortality of infected bivalves can be 90-100% within 24 hours and industry losses are typically 60%⁷¹⁻⁷³. Contaminated algal feeds are a major vector of *Vibrio sp* and current disease management is primarily dependent upon chemotherapy in the form of antibiotics^{71,73,74}. Streptomycin, penicillin, florfenicol, erythromycin, and chloramphenicol are routinely applied to and circulated around hatchery water supplies to prevent and treat disease^{71,73}. This is inefficient, and the economic costs can be equal to the cost of bivalve stock⁷¹. More importantly, far from optimising the success of larval and juvenile bivalve cultures, this level of widespread antibiotic use is driving the proliferation of antibiotic resistant *Vibrio* in hatcheries⁷³. Furthermore, water effluents and bivalve exports then act as

a delivery mechanism for resistant bacteria to different geographical locations or aquatic environments⁷³. There is a need for alternative solutions to manage disease, or at the bare minimum a more efficient delivery mechanism of antibiotics to bivalves to reduce overall antibiotic usage^{71,73,74}.

2.2.4. Genetic bottlenecks

Genetic bottlenecks and inbreeding depressions are occurring in adult bivalve broodstock due to the selective breeding of 'high quality' adults in hatcheries, reducing yield. Declines in performance characteristics including yield, growth rate, and survival have been documented across bivalve species, alongside reduced disease resistance and adaptability to environmental changes^{56,75,76}. Dietary intervention can improve gonadal nutrient reserves and gamete quality, quantity and viability in bivalve broodstock without a need for selective breeding⁷⁷⁻⁸⁰. Such intervention is needed in combination with improved breeding protocols that draw from a more diverse pool of individuals to improve broodstock quality without reducing genetic variation^{56,75}.

2.2.5. Meat quality and taste

There is a need to improve the palatability and quality of adult bivalves for human consumption. There remain consumer perceptions that bivalves do not taste as good as fish, and this has a negative impact on market value and demand¹⁹. For some farmed bivalve species, highly nutritious flesh is even discarded while the shells are used for industrial applications and road surfacing⁸¹. Dietary supplementation to improve the nutritional quality and taste of bivalves is needed to increase consumer uptake of bivalves and reduce food waste.

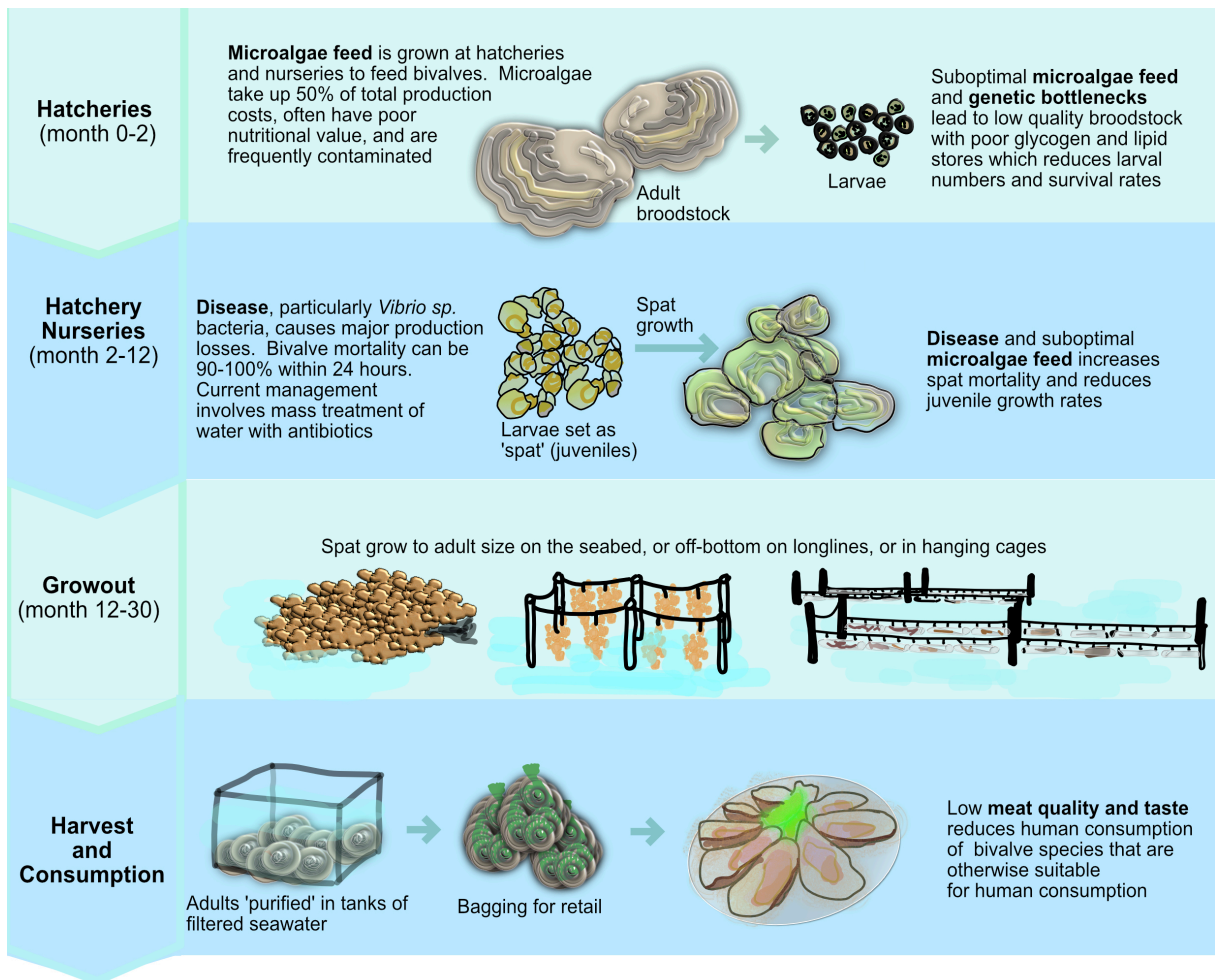


Figure 2.4. The four key stages in bivalve production, illustrating the primary factors limiting yields.

2.3. Harnessing microencapsulated diets to solve bivalve production problems

2.3.1. An opportunity for industry growth

There is major potential for bivalve industry growth from solutions which tackle current problems in the production process. To date, bivalve aquaculture has not received the same levels of research or investment as fish aquaculture, and there is a great need for science to complement traditional knowledge and help the industry become more efficient^{19,67,69}. Even modest increases in production could have a large impact; for example if just the United Kingdom were to increase its production per capita to the level of New Zealand, an extra 1.1 million tonnes of bivalves could be produced annually^{47,55}. There is an opportunity to realise such increases in production by developing microencapsulated diets.

2.3.2. Potential from new advances in microencapsulation technology

New advances in microencapsulation technology offer great potential to tackle key problems in bivalve shellfish production (Table 2.1.). A microencapsulated diet consists of a formulation of nutrients and agents surrounded by a digestible capsule. The concept of delivering microencapsulated artificial diets to aquatic filter feeders has been present since the 1970s⁶⁷. Since then a very limited number of studies have trialled the use of microencapsulated feeds including 'MySpat' (INVE Technologies, Dendermonde, Belgium) and 'Frippak' (Frippak Feeds, Basingstoke, Great Britain) to feed bivalves^{66,70,77,82}. These feeds are not directly representative of a natural bivalve diet; MySpat contains lipids originating from fish oils and protein from land vegetable origin, and Frippak is designed for shrimp and fish feeding^{82,83}. Microencapsulated feeds are still yet to be adopted for large scale commercial use in the bivalve shellfish industry.

However new advances in microencapsulation technology could change this situation. A novel form of microparticles have recently been developed for the targeted delivery of chemical control agents to invasive bivalve species, and are known as BioBullets (BioBullets Ltd, Cambridge, UK)²⁹. To manufacture the particles, a slurry of lipid encapsulant and powdered diet is pumped into an ultrasonic atomising nozzle, before the particles form perfect spheres in specialised cooling chambers and are then coated in a proprietary surfactant to aid dispersion in water²⁹. A comparable encapsulation system is already being used at scale and is highly cost effective in other food sectors. It was recently demonstrated that the blue mussel *Mytilus edulis* could ingest BioBullets particles containing a formulated diet including *Schizochytrium* algae, opening a new direction for this emerging technology in feeding desirable products to bivalves³². New microencapsulated diets offer many critical advantages over alternative strategies that could be used to tackle production limitations, and offer a solution to tackle problems with bivalve feed, disease, and quality (Figure 2.5.).

Characteristic of Microencapsulated Diet	Problems Addressed				Potential Outcomes		
	Microalgae Feed Problems	Disease	Genetic Bottlenecks	Meat Quality and Taste	Increased Growth	Reduced Costs	Improved Bivalve Quality
Physical characteristics optimised for maximum uptake and cost efficiency (3.3)	✓	✓			✓	✓	
Optimal formulation of high quality cost effective nutrients (3.4)	✓		✓	✓	✓	✓	✓
Targeted delivery of disease control agents including antibiotics and probiotics (3.5)		✓	✓		✓	✓	✓
Quality enhancers including flavourings (3.6)				✓			✓

Table 2.1. How new microencapsulated diets could tackle bivalve production problems.

Numbers represent relevant sections in the main text.

2.3.3. Beneficial physical characteristics of microencapsulated feeds

The physical characteristics of microencapsulated feeds can be optimised for maximum ingestion by bivalves and cost advantage relative to algal cultures. Microparticle size can be tailored to bivalve species or life stage preferences to maximise feeding efficiency, and buoyancy can be optimised to ensure particles remain within reach of filter feeding bivalves²⁵. This is a key advantage over nutrient delivery systems such as freeze-dried algal powders, which tend to float on the water surface, and can clump into particles too large to be accessed by bivalves. Pre-ingestive nutrient loss can be minimised by using an encapsulant that allows particles to remain stable and retain nutrients in seawater, but also be rapidly digested on entry to the bivalve gut⁶⁷. Lipid coatings allow delivery of low molecular weight, water soluble compounds with minimal leaching to the surrounding water⁸³. This is a significant advantage over non-encapsulated artificial diets, which tend to leach nutrients to the surrounding water, and could further reduce the small eutrophication risk that bivalve hatcheries can pose to marine ecosystems^{53,67}. The stability of the microparticles in air also enables cost efficient mass production and long term storage of feeds, for example in distribution centres or on hatchery sites²⁵. This is a major advantage over simply growing higher quality algal cultures; stored microparticles are more resistant to bacterial contamination than live algal cultures, and the costly process of synchronising microalgal feedstock with bivalve production is avoided^{25,67}. This could make small scale farms more economic, and reduce the need for skilled labour to grow algae.

2.3.4. Nutrient delivery

A single microencapsulated feed particle can contain an optimal formulation of high quality, cost effective nutrients to increase yield and growth rates. Powdering and encapsulation processes enable high quality natural food sources not traditionally used in bivalve aquaculture to be delivered to bivalves. This includes strains of algae that can be grown rapidly and efficiently under optimised mass production systems and that are incredibly rich in DHA and EPA. DHA can make up 50% of the algal lipid content of these strains, compared to <10% for microalgae used in hatcheries today^{49,68,84}. This offers a far more cost efficient method to improve bivalve nutrition than trying to grow higher quality microalgae on each individual hatchery site^{49,67}. Other nutrients can be added to the microparticles to tailor diets to specific bivalve species, or specific geographies where key nutrients are lacking, and even the lipid encapsulant itself can be of high nutritional value⁶⁷. Such microencapsulated feeds could improve bivalve broodstock quality; broodstock quality is influenced heavily by dietary DHA intake, and DHA supplementation increases broodstock glycogen and lipid reserves, egg size, egg quantity, larval growth rate, and larval survivorship⁸⁵⁻⁸⁷. Juvenile growth could be improved; juvenile bivalves will grow more rapidly on diets high in DHA and EPA^{88,89}. Combining these improvements promises to result in greater total production volume in hatcheries.

2.3.5. Disease control

Encapsulation enables targeted delivery of disease control agents such as antibiotics or probiotics to filter feeding bivalves, to improve disease control, and reduce costs, environmental damage, and human health risk²⁵. Antibiotic encapsulation could improve effectiveness, reduce overall antibiotic usage, and reduce proliferation of antibiotic resistant bacteria; a considerable advantage over simply applying more antibiotics to hatchery water supplies to solve the disease problem. However, disease management in aquaculture using antibiotics in any form remains an environmental and human health concern⁹⁰. Probiotics such as *Phaeobacter inhibens* and *Bacillus pumilus*, or antimicrobial peptides such as Tachyplesin are known to protect bivalves against bacterial infection and are a more sustainable disease management option than antibiotics⁹¹⁻⁹³. Microencapsulation provides a more direct and protected mechanism to deliver probiotics to the bivalve gut compared to liquid treatment systems⁹⁴. Microparticle components aside from probiotics can also be made sterile during production, mitigating the risk of introducing disease into bivalve

cultures through feed⁶⁷. Recent studies demonstrate that mortality in juvenile *Ostrea edulis* oysters reared on microencapsulated sterile algal powder is up to 60% lower than in juveniles reared on live algae or liquid algal concentrate, and reduced disease is identified as the likely cause³³. With further research and development microencapsulated diets could enable reduced disease incidence in bivalve broodstock, larvae, and juveniles, increase production output and improve bivalve quality, while minimising risk to the environment and human health.

2.3.6. Quality

Quality enhancers such as flavourings can be incorporated into microparticles fed to bivalves, which could strengthen consumer demand for bivalves and encourage a diet-shift towards more sustainable seafood. Flavourings fed during the purging stage (when bivalves are kept in purification tanks for a few days before retail) (Figure 2.4.) would persist within the gut tissue once the bivalves are harvested. This could improve the taste of some bivalves, and be a more effective method than flavouring the exterior of the tissue after harvest⁸¹. More importantly, flavouring could be a highly effective way of disguising a diet-shift away from high trophic level fish and towards more sustainable seafood. We know when marketing low trophic farmed seafood species to consumers, it is most effective to highlight attributes such as taste, affordability, and health benefits, rather than environmental sustainability¹⁹. A resounding result could be improved consumer uptake of bivalves, at the expense of less sustainable forms of meat production.

2.3.7. Challenges for the future

Clearly new microencapsulated diets present an opportunity to improve bivalve aquaculture, but several challenges need to be overcome in order to make them a commercially viable option. The scalability of these new diets must be formally assessed; we know the microencapsulation technology is cost effective at scale in other food sectors, but not yet for bivalve aquaculture⁹⁵. The physical characteristics of diets need to be optimised for long term storage in distribution networks and in hatcheries. The palatability, digestibility and composition of microencapsulated feeds needs to be tailored to specific bivalve species and growth stages³². The environmental impact of using microencapsulated diets in bivalve aquaculture needs to be formally assessed. Researchers will be required to work collaboratively with industry partners to bring technological innovation into practice.

Researchers also need to work with policy makers, retailers and the media to stimulate demand and change food preferences towards bivalves in place of less sustainable meat and fish products ¹⁹.

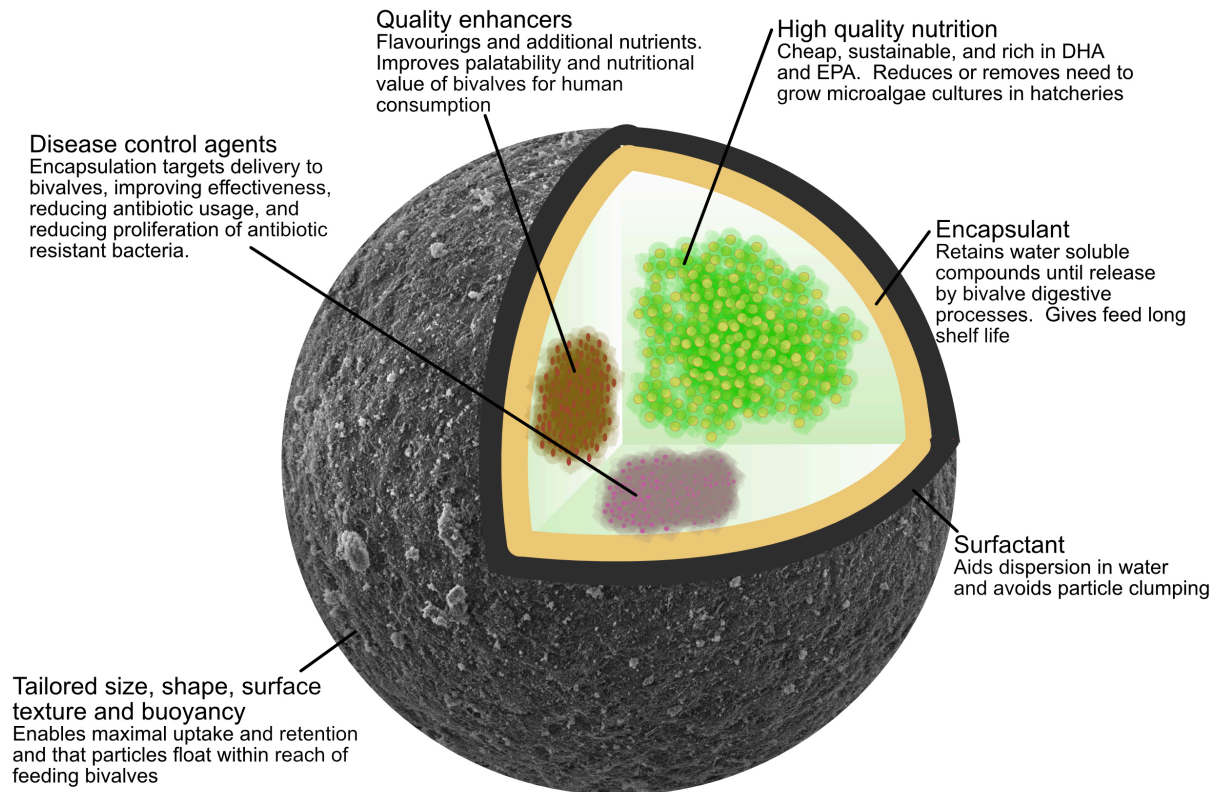


Figure 2.5. Schematic of a microencapsulated food particle demonstrating the desirable characteristics that could result in enhanced commerciality of bivalves.

2.4. Conclusion

Increasing production of bivalves through aquaculture offers an important opportunity in meeting global food security. There is great potential for new advances in microencapsulated feeds to offer an efficient way to deliver replacement or supplementary diets to bivalves that can tackle problems with bivalve feed, disease, and quality, enabling increased quality production output, increased industry growth and reduced costs. New microencapsulated diets could be deployed in major areas of potential production growth including Asia, as well as high-value markets such as Europe. Resulting growth improvements in the bivalve shellfish sector could dramatically improve food supply, and within the next decade twice as many people could be fed by bivalves as today if we could

enable bivalve aquaculture to grow at the same rate as predicted for fish aquaculture ⁴⁷. If 25% of the protein we currently obtain from fish aquaculture was obtained from bivalves, we could spare an area of land larger than Wales, annual CO₂ emissions equal to half New Zealand's emissions, and annually 11.8 billion litres of freshwater ^{19,50}. There is now an open opportunity for research and industry to overcome remaining hurdles in microencapsulated feed development and to realise the great benefit improved growth in bivalve aquaculture can have for the global population and our planet.

2.5. Footnotes

Data availability: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.1016/j.gfs.2019.04.007>.

Author contributions: D.W. and D.C.A. wrote the manuscript. Both authors gave final approval for publication.

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Chapter 3: Microencapsulated diets to improve bivalve shellfish aquaculture

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Aquaculture is the fastest growing food sector and feeds over 3 billion people. Bivalve shellfish aquaculture makes up 25% of global aquaculture production and is worth annually US \$19 billion, but continued growth is currently limited by suboptimal diets and limited tools for disease control. New advances in microencapsulation technology could provide an effective way to overcome these biological limitations. This study demonstrated that a new formulation of microencapsulated diet known as BioBullets could be ingested by a commercially farmed bivalve; the blue mussel *Mytilus edulis*. Microparticles could be captured by mussels with similar efficiency to natural foods. Microparticles too large for ingestion were rejected in pseudofaeces. Microparticles were successfully ingested and broken down by the gut. Further work is needed to assess the impact of BioBullets diets on bivalve growth. There is now an exciting opportunity to tailor the composition of microencapsulated diets for specific applications to improve production output and efficiency in the commercial bivalve shellfish industry.

3.1. Introduction

Aquaculture is the fastest growing food sector and continues to expand alongside terrestrial crop and livestock production ⁴⁴. Globally over 3 billion people depend on aquaculture for at least 20% of their dietary protein ⁴⁴. Expanding aquaculture has been identified as a critical component in securing food for 9 billion people by 2050 ⁴⁵. Bivalve shellfish aquaculture makes up nearly 25% of global production, and over the last decade has grown at 10% per year to a value of US\$ 19 billion in 2014 ⁴⁸. However, the continued growth of bivalve aquaculture is limited by suboptimal feeding protocols and disease control methods. Currently, lipid-rich cultures of microalgae are grown to feed bivalves whilst they are in the hatcheries. However, growing these cultures is expensive, equating to over 30-

50% of total production costs ⁶⁶, and cultures are highly subject to contamination and have variable nutritional value ²⁵. Bivalve aquaculture suffers also high losses due to frequent outbreaks of infectious disease ⁹⁶. New advances in microencapsulation technology through chemical engineering can provide a way to overcome current biological limitations in bivalve shellfish production. A novel form of microparticles have recently been developed by BioBullets (BioBullets Ltd, Cambridge, UK) for the targeted delivery of chemical control agents to invasive bivalve species ²⁹. The BioBullets delivery system is highly effective and cheap to manufacture, opening a new direction for this emerging technology in feeding desirable products to bivalves.

Microencapsulation technology offers many critical advantages over alternative strategies that could be used to tackle production limitations. First, a single microencapsulated feed particle can contain an optimal formulation of key nutrients for bivalve growth, specifically high levels of protein and polyunsaturated fatty acids including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), alongside disease control agents. The microencapsulated diet could be tailored to specific species, or specific geographies where certain nutrients are lacking. Even the highest quality live microalgae will rarely have an optimal nutrient composition ⁶⁸, and so currently multiple cultures of different microalgal species are grown, which increases production costs and commercial risk in the case of contamination. Second, maximal particle capture can be ensured by customising microparticle size and buoyancy. Size can be tailored to bivalve preferences for maximum retention efficiency, and buoyancy can be optimised to ensure particles remain within reach of feeding bivalves ²⁵. This is a key advantage over nutrient delivery systems such as powders, which tend to float on the water surface, and can clump into particles too large to be retained by bivalves. Third, pre-ingestive nutrient loss can be minimised by using an encapsulant that retains particle nutrients until they are released by the digestive processes of the bivalve. Lipid coatings allow delivery of low molecular weight, water soluble compounds with minimal leaching to the surrounding water ⁸³. Fourth, storage of high quality feeds for long time periods is made possible by the stable nature of microparticles in air ²⁵. This is a major advantage over simply growing higher quality algal cultures; stored microparticles are less likely to become contaminated over time by bacteria than live algal cultures, and the costly process of synchronising microalgal feedstock with bivalve production is avoided ²⁵. Therefore, new microencapsulation technology can offer an efficient way to deliver replacement or supplementary diets that could improve bivalve

nutrition, growth, and production output, whilst reducing bivalve mortality, production costs, and financial risks.

The capture and ingestion of food particles by bivalves in aquaculture or the wild is highly influenced by particle characteristics. Food particles are collected through the inhalant siphon and sorted on the labial palps and gills using size, shape and other physical attributes. Unwanted particles, particularly sharp edged or inorganic particles such as SiO₂, are preferentially rejected in pseudofaeces. Larger bivalves including oysters ingest organic particles up to 400 µm diameter. Blue mussels (*Mytilus edulis*) will select diatoms and dinoflagellates up to 200 µm⁹⁷. Particle size selectivity increases when food is more plentiful; blue mussels will preferentially select particles of between 20 – 40 µm, and reject a greater proportion of particles larger than 40 µm as food availability increases^{97 98}. Once ingested, food particles are swept along ciliated sorting areas in the stomach towards the digestive glands. Particles too large for digestion are rejected down deep grooves in the stomach to the intestine, although a small number to remain aid food grinding in the stomach^{97 56}. Any microencapsulated diet fed to bivalves will be subjected to these same sorting processes, and therefore the development of effective microencapsulated diets requires a sound understanding of microparticle ingestion by bivalves.

This study aimed to demonstrate that a new form of BioBullets microparticles containing a microalgal food could be successfully captured and ingested by a commercially farmed bivalve; the blue mussel *Mytilus edulis*. Blue mussels have great commercial importance, and with a value of US\$ 3.6 billion make up 20% of the global bivalve aquaculture market¹². In 2015 2.3 million tonnes were produced, primarily in coastal Western Europe and Canada. Three components of microparticle digestion were investigated as outlined in Figure 3.1. Firstly, particle capture, to assess whether and how rapidly microparticles could be cleared from water by mussels. Secondly, preingestive particle processing, to assess which sizes of microparticles were preferentially ingested or alternatively rejected in pseudofaeces. Thirdly, postingestive particle processing, to confirm successful ingestion and assess how well microparticles were broken down by the gut. By demonstrating successful ingestion of BioBullets microparticles, this study opens a gateway to further optimise the composition of microparticles for specific applications in bivalve aquaculture.

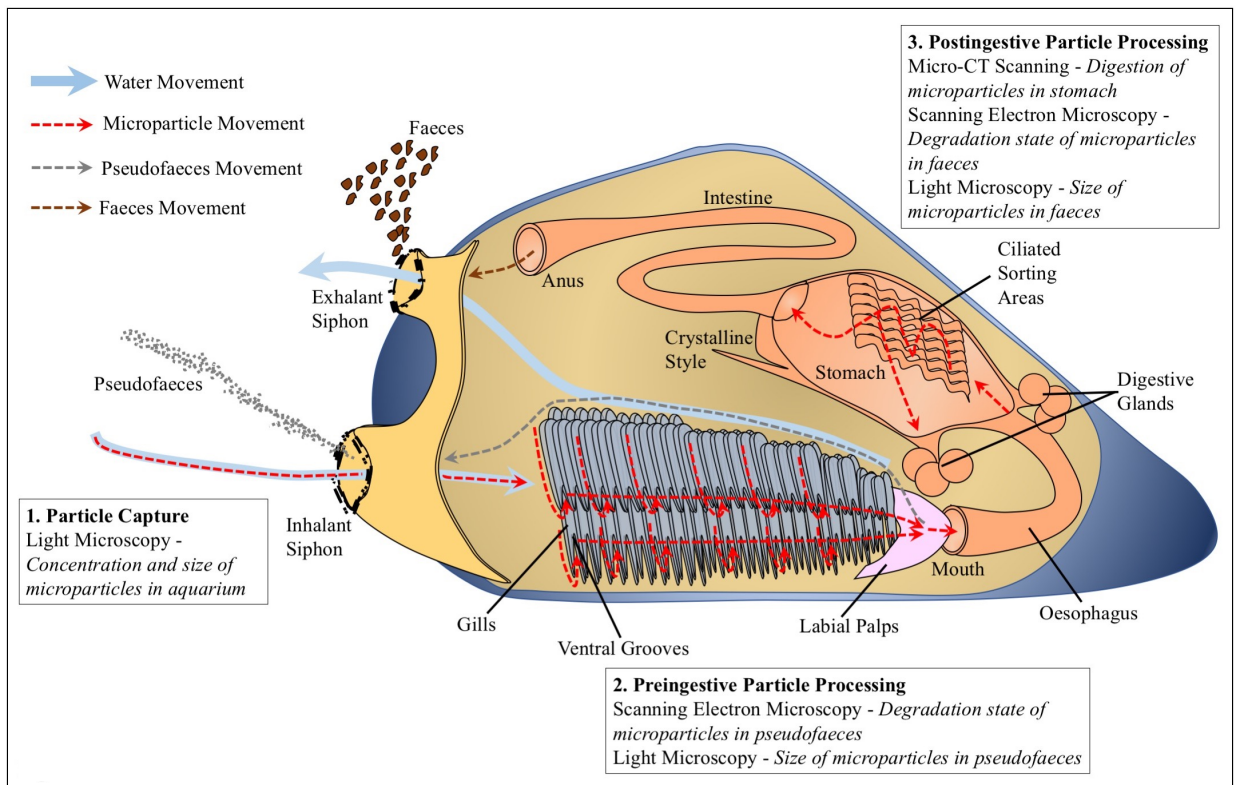


Figure 3.1. Schematic diagram of microparticle digestion in the blue mussel *Mytilus edulis*.

The boxes outline the analytical techniques used to characterise each digestion stage investigated during the study; particle capture, preingestive, and postingestive particle processing.

3.2. Methods

3.2.1. Microparticle Production

Lipid-walled microparticles containing *Schizochytrium* microalgae were manufactured by BioBullets Ltd. (Cambridge, UK). A premix slurry was prepared containing the encapsulant and powdered microalgae under conditions of controlled shear. The slurry was pumped into an ultrasonic atomising nozzle at the top of a cooling chamber. The atomized particles formed perfect spheres as they cooled and fell to the chamber base. Further particle cooling was achieved with an air-conveying system before discharge via cyclone to a fluid bed processor. The encapsulated particles were then coated with a proprietary non-ionic surfactant to aid dispersion in water. Further cooling in the fluid bed removed all heat of crystallization from the microparticles before packaging.

3.2.2. Mussel Husbandry

The blue mussel *Mytilus edulis* was selected as the model bivalve species to establish whether BioBullets microparticles could be successfully digested. Mussels were collected at low tide from the shore at Old Hunstanton, Norfolk, England (52°56'56 N, 0°29'27 E) on 1st February and 1st March 2017. Mussels were kept in constantly aerated aquaria at 10 °C in seawater from Old Hunstanton with a salinity of 32 ppt.

3.2.3. Particle Clearance

To investigate particle clearance, five aquaria aerated at a constant rate were set up with 200ml seawater from the collection site. A single mussel was measured in length and wet mass, and added to each aquarium, then left for 5 days to allow acclimation and byssus thread attachment. The mussels were large adults with a length of 35 – 40 mm. To provide a starting concentration of 50 mg litre⁻¹, 10 mg dry weight microparticles (90,000 microparticles) were then added to each aquarium. Next, 1 ml water samples were collected every 5 minutes, using a pipette with a modified 5 mm aperture tip to allow particle entry. Each water sample was mixed and placed on a 1 ml Sedgewick Rafter counting cell, and the number of microparticles in the central 25 mm² grid counted under a dissecting microscope, providing a microparticle concentration per 25 µl. The sampling process was repeated for three control aquaria, containing a starting concentration of 50 mg litre⁻¹ microparticles, but no mussels.

3.2.4. Particle Size Selectivity

To assess particle size selectivity, eight aerated aquaria were set up with 200ml seawater. A single mussel was added to each aquarium, along with 50 mg microparticles to provide a starting concentration of 250 mg litre⁻¹. The mussels were large adults with a length of 35 – 40 mm. Mussels were left for 4 days to allow pseudofaeces and faeces production. Pseudofaeces thread samples were then collected from outside the inhalant siphon of each mussel using tweezers, and faecal pellet samples from near the exhalant siphon, in sufficient quantities to fill a 9 mm² grid area for each sample. For each sample the diameter of every microparticle within the 9 mm² area was measured to the nearest 5 µm under light microscopy using a slide graticule and eyepiece micrometer. The measuring process was repeated with 3 control samples of microparticles in seawater in the absence of mussels.

3.2.5. Particle Imaging

Particles were imaged using scanning electron microscopy (SEM) to look for and assess the degradation state of microparticles in faeces and pseudofaeces. Micro Computed Tomography (Micro-CT) scanning was used to examine microparticle digestion within the mussel gut cavity.

To obtain samples for SEM imaging, two aquaria were set up with 1 litre seawater and 10 mussels each, left for seven days to allow any existing food to be purged from the mussel digestive tract, then 500mg microparticles were added to one aquarium. Mussel pseudofaeces and faecal pellets were collected from both aquaria after three days. The mussels were placed in fresh water at the point of pseudofaecal and faecal sampling to wash the salt from the pseudofaeces. The salt was removed from faecal pellets using a centrifuge at 1000 g for 30 seconds and three washes of deionized water. Pseudofaeces and faeces samples were freeze-dried overnight, mounted on SEM stubs with silver paint, and sputter-coated with 30 nm carbon. To allow for an assessment of the degradation of particles that had been processed by the mussels, a sample of unfed microparticles was also freeze dried, mounted and carbon-coated. SEM images were then taken using an FEI Verios 460 (Thermo Fisher Scientific, USA).

To prepare for the Micro-CT scanning, a 1 litre seawater aquarium with 10 mussels of length 40 mm was treated with 500 mg microparticles for 16 hours. Four mussels which had been observed feeding regularly were then selected, and after removing one shell half these were fixed in 4% formaldehyde overnight. The fixative was removed with three 10 minute washes in water and the mussels were then sequentially dehydrated in 25, 50, 75 and 100% methanol for 15 minutes each. The mussels were then immersed in a 1% phosphotungstic acid (PTA) in methanol stain for 10 days, with the staining solution changed after 5 days. This process was then repeated with two mussels that had not been fed microparticles as a negative control. Mussels were mounted and then scanned at the Cambridge Biotomography Centre using a Nikon XT 225 ST Micro-CT scanner; the settings were 120 kV and 130 μ A. The images were constructed from 1080 projections each with 1000 ms exposure. ImageJ⁹⁹ was used to process the images and search for microparticles within individual scan slices of the mussel gut.

3.2.6 Data analysis

The rate of particle clearance was normally distributed. A linear model and subsequent ANCOVA was used to compare the change in \log_{10} microparticle concentration over time between mussels and control samples. Clearance rates of microparticles were calculated per g wet mussel mass per minute, and the background decline in microparticle concentration was accounted for by subtracting the mean decline in microparticle concentration in control samples from each clearance rate. ANOVA was used to assess the change in clearance rate as microparticle concentration declined over time.

To investigate particle selectivity, pairwise comparisons among least-squares means were used to test for differences in particle size distribution between microparticles from faeces, pseudofaeces and unfed samples. Differences in mean microparticle diameter between all three sample types were assessed using Kruskal-Wallis with *post-hoc* Dunn's tests.

Data were analysed using the statistical package R¹⁰⁰.

3.3. Results

3.3.1. Particle Capture

Microparticles were cleared rapidly from seawater by mussels. The mean microparticle concentration in the aquaria declined by $83.6 \pm 6.4\%$ over a 60-minute period, from 440.0 ± 28.3 (standard error) to 72.0 ± 15.0 microparticles ml^{-1} (Figure 3.2.). This decline was significantly greater than in control aquaria without mussels (Figure 3.2.), where microparticle concentration declined slowly from 466.7 ± 26.7 to 306.7 ± 13.3 microparticles ml^{-1} (ANCOVA, $F_{3,100} = 222$, $p < 0.001$). Accounting for this background settlement, the mean decline in microparticle concentration in aquaria with mussels was $52.7 \pm 6.4\%$. The mean initial clearance rate over the first 5 minutes was 0.970 ± 0.326 microparticles $\text{ml}^{-1} \text{g}^{-1}$ wet mussel mass minute^{-1} . Clearance rates fell significantly over time to a final value at 60 minutes of 0.051 ± 0.278 microparticles $\text{ml}^{-1} \text{g}^{-1}$ wet mussel mass minute^{-1} as microparticle concentration in the water declined (ANOVA, $F_{1,58} = 13.9$, $p < 0.001$, comparison between 5 and 60 minutes with correction for settling).

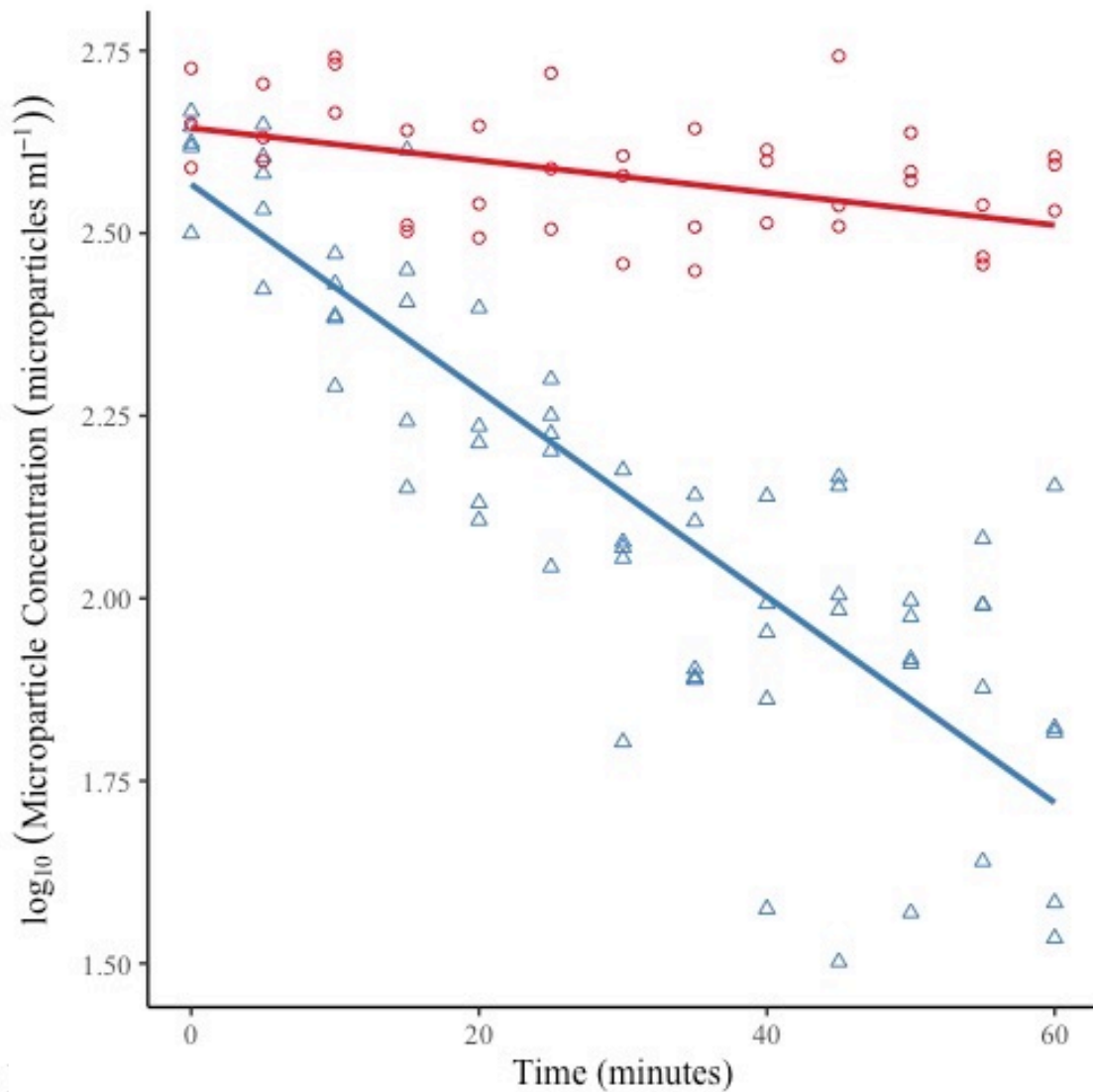


Figure 3.2. Comparison of microparticle concentration over time between mussel (blue triangles) and control (red circles) aquaria. Ten mg of microparticles were added to 200ml aquaria containing one mussel or no mussel for the controls, and the microparticle concentration was recorded every 5 minutes for 60 minutes. The decline in microparticle concentration in mussel aquaria was significantly greater than the decline in control aquaria (ANCOVA, $p < 0.001$). Sample sizes: 5 mussels of length 35 – 40 mm, 3 controls. Regression lines are linear models of \log_{10} microparticle concentration on mussels or controls. For mussels $y = 2.57 - 0.014x$, $r^2 = 0.78$, $p < 0.001$; for controls $y = 2.64 - 0.0022x$, $r^2 = 0.38$, $p < 0.001$.

3.3.2. Preingestive Particle Processing

A greater proportion of large microparticles was present in pseudofaeces than in unfed microparticle samples (Figure 3.3.; least-square means, $z = 23.7$, $p < 0.001$). At $92.85 \pm$

SE 0.86 μm , the mean diameter of microparticles in pseudofaeces was significantly greater than the $76.15 \pm 1.61 \mu\text{m}$ in samples of unfed microparticles (Kruskal-Wallis, $\chi^2_2 = 112$, $p < 0.001$, *post-hoc* Dunn's test $p < 0.001$).

The SEM images showed microparticles embedded within pseudofaecal threads (Figure 3.4.). These microparticles were mostly undamaged and similar in morphology to unfed microparticles.

3.3.3. Postingestive Particle Processing

Micro-CT scanning revealed microparticles within the stomach cavity of mussels fed microparticles (Figure 3.5.). The microparticles were of approximately 100 μm in diameter, and present across multiple sections of the mussel stomach.

Analyses of faecal samples revealed the presence of microparticles within faeces. Of the complete microparticles found in faeces samples, a greater proportion were of large diameters compared to unfed microparticle samples. Figure 3.3. demonstrates how the distribution of microparticle sizes differed significantly between faeces and unfed microparticles, with a much greater proportion of large diameter microparticles in faeces (least-square means, $z = 26.5$, $p < 0.001$). In addition, the mean diameter of microparticles in faeces ($97.60 \pm 1.33 \mu\text{m}$) was significantly greater than in samples of unfed microparticles ($76.15 \pm 1.61 \mu\text{m}$) (Kruskal-Wallis, $\chi^2_2 = 112$, $p < 0.001$, *post-hoc* Dunn's test $p < 0.001$). When comparing faeces with pseudofaeces, the mean microparticle diameter was large in both cases, but significantly greater in faeces (Dunn's test, $p < 0.01$).

The SEM images further confirmed the presence of microparticles within faeces (Figure 3.4.). These microparticles were far more degraded in morphology than unfed microparticles or microparticles found in pseudofaeces. Completely fragmented microparticles were also seen in the faecal samples.

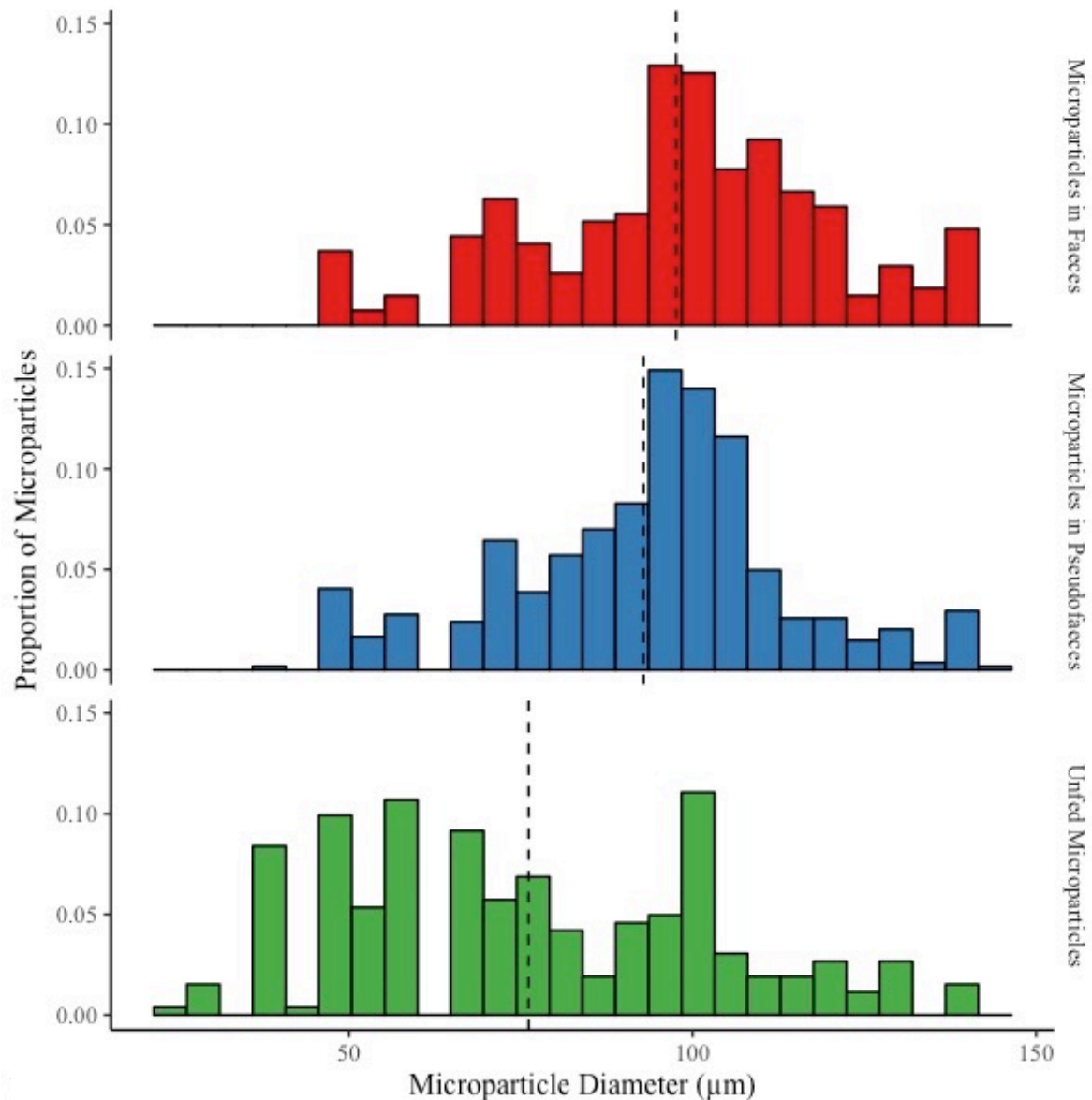


Figure 3.3. Histograms showing a greater proportion of large microparticles in faeces (red bars) and pseudofaeces (blue bars) than in unfed microparticle samples (green bars). The diameter of microparticles within faeces and pseudofaeces of eight mussels and three unfed microparticle samples was measured. Microparticle size distribution differed significantly between all sample types (least-square means, $p < 0.001$). Mean particle diameter (dotted black lines), was greatest in faeces, then pseudofaeces, and lowest in unfed microparticles (Kruskal-Wallis with post-hoc Dunn's test, $p < 0.001$). Microparticles in each sample type: faeces $n = 271$, pseudofaeces $n = 543$, unfed $n = 262$.

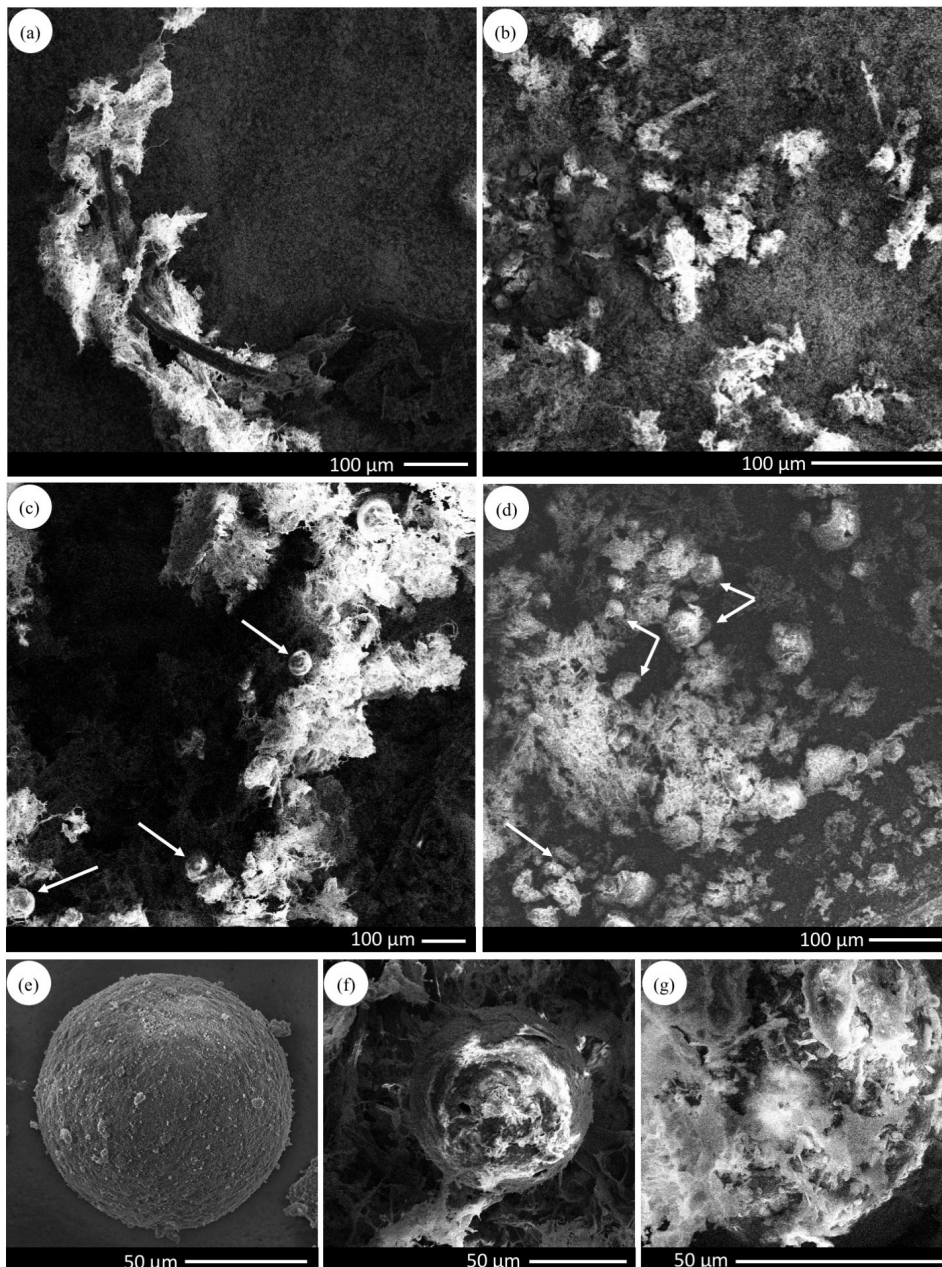


Figure 3.4. SEM images showing microparticles in mussel pseudofaeces and faeces. The following samples were collected: unfed microparticles, mussel pseudofaeces and faeces, and pseudofaeces and faeces from mussels fed microparticles. Samples were freeze-dried, carbon coated, and visualised under SEM. (a) Pseudofaeces and (b) faeces from mussels not fed microparticles. (c) Pseudofaeces and (d) faeces from mussels fed microparticles. Microparticles are indicated by white arrows. In (c) microparticles are morphologically similar to unfed microparticles. In (d) microparticles are present in a variety of degradation states; at the bottom left there is a particle which appears to be split into four pieces, and near the centre there are several particle fragments alongside multiple complete but degraded particles. (e) Single unfed microparticle, (f) single microparticle in pseudofaeces and (g) single microparticle in faeces.

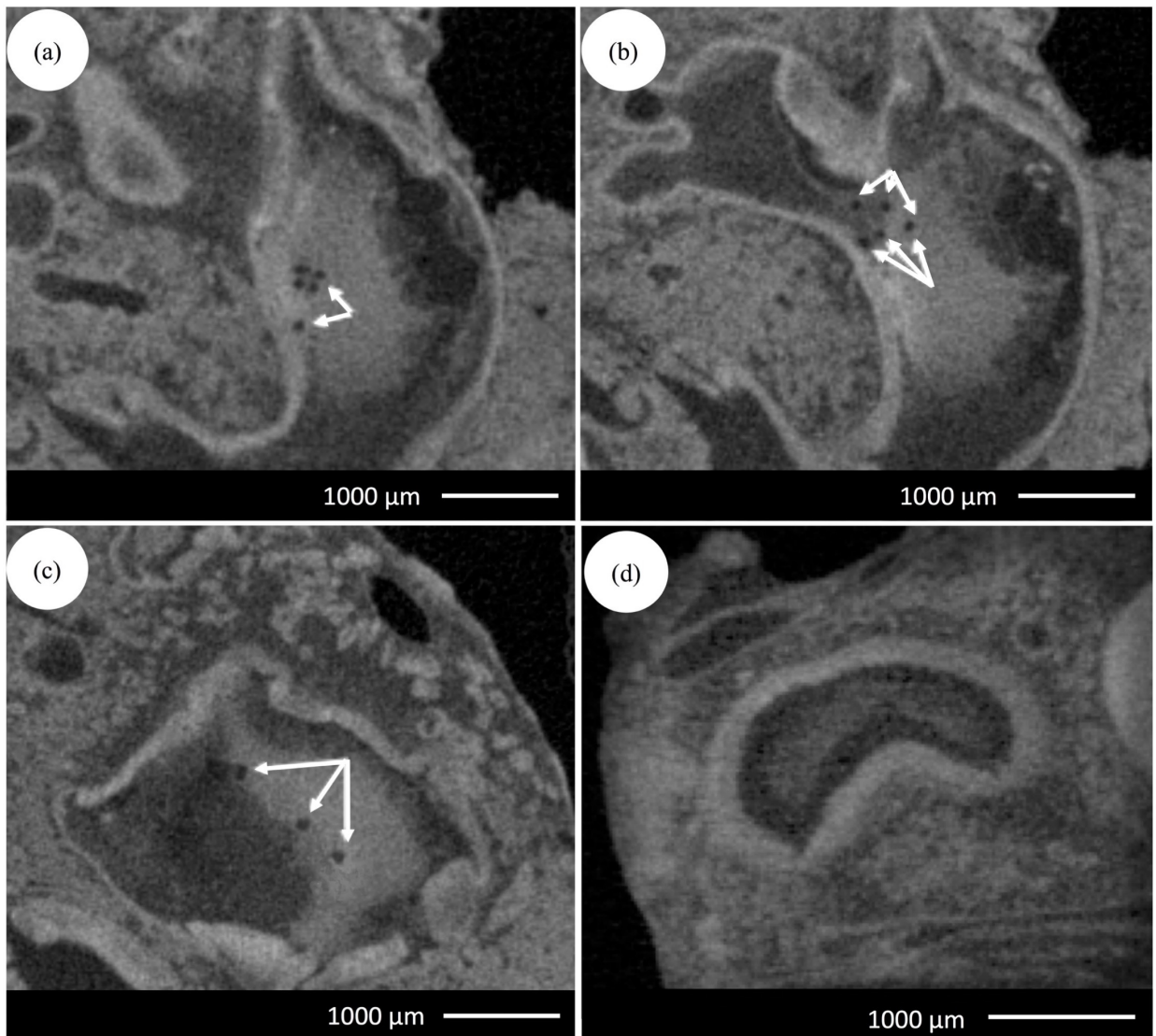


Figure 3.5. Micro-CT images of microparticles within the mussel stomach. Mussels were fed microparticles, fixed in formaldehyde, stained in phosphotungstic acid (PTA), scanned using Micro-CT, then analysed using ImageJ⁹⁹. This process was repeated for mussels not fed microparticles. The white arrows in (a), (b) and (c) point to microparticles of approximately 100 µm in stomach sections from mussels fed microparticles. (d) Stomach section from a mussel not fed microparticles.

3.4. Discussion

3.4.1. Particle Capture

The particle capture experiments demonstrated that BioBullets microparticles containing a microalgal food can be cleared from water by bivalves. This is supported by the

52.7% decline in microparticle concentration in aquaria with mussels (after accounting for background settlement), and suggests that microparticles are successfully processed during the initial stages of digestion; specifically the movement of food particles along gill filaments, into the ventral grooves and onto the labial palps (Figure 3.1.). The decline in clearance rate with falling aquaria microparticle concentration indicates that the rate of microparticle capture by mussels is proportional to the abundance of microparticles in the water. The gradual and small decay in microparticle concentration in control aquaria can be explained by the slow sinking of microparticles to the aquaria base. The particles were specifically engineered to possess slightly negative buoyancy in order to increase the likelihood of uptake by bivalve molluscs, which are found at the bottom of aquaria in commercial hatcheries.

Our investigation also indicates that BioBullets microparticles can be cleared with similar efficiency to other food sources. Firstly, natural food sources; studies on feeding greenshell mussels *Perna canaliculus* have shown that the typical decline in concentration of a standard microalgal food over 60-minutes, without accounting for background settlement, is 90%, very comparable to the 83.6% decline in BioBullets microparticles in our investigation (without accounting for background settlement)⁷⁰. Secondly, microencapsulated foods; there are a very limited number of studies which have trialled the use of older lines of microencapsulated feeds including 'MySpat' (INVE Technologies, Dendermonde, Belgium) and 'Frippak' (Frippak Feeds, Basingstoke, Great Britain) to feed bivalves. These studies assessed bivalve growth rates, and did not assess stages of digestive processing beyond particle clearance and rejection in pseudofaeces^{66 82 77}. The 80% decline in MySpat concentration over 60-minutes caused by feeding greenshell mussel spat in these studies is again comparable to the rate of BioBullets microparticle clearance in our investigation⁷⁰. However, whilst MySpat is specifically designed for juvenile mussels of around 1 mm shell length, BioBullets is more broadscale and can be used to feed larger mussels. This could be highly desirable for improving broodstock, delivering therapeutics, or for other final 'polishing' such as adding desirable nutrients or flavours. Additionally, an artificial diet suitable for larger individuals may be of particular benefit to other bivalve species, such as oysters, that are held in hatcheries for many years before open water grow-out.

3.4.2. Preingestive Particle Processing

Preingestive particle processing experiments led to two key findings. Firstly, BioBullets microparticles can be processed and rejected by the labial palps of mussels, indicated by the presence of microparticles in the SEM images. Secondly, mussels preferentially rejected larger diameter microparticles, demonstrated by the larger mean diameter of microparticles in pseudofaeces than in unfed microparticle samples. The greater proportion of large microparticles in pseudofaeces suggests the presence of a size threshold above which microparticles have a greater chance of rejection on the labial palps. Microparticles below threshold size should be ingested through the mouth (Figure 3.1.), although the efficiency of this may have been reduced in our investigations if the gills and labial palps became partially blocked by large microparticles during feeding. Both the preferential ingestion of smaller particles and the potential for larger particles to block the gills and labial palps has been shown for other artificial diets fed to *Perna canaliculus* mussels⁷⁰. Previous studies have also shown that particle size preference of natural algal foods differs across bivalve growth stages, with mussel larvae preferring smaller particles of around 25 µm, and juveniles preferring 40 µm particles⁹⁷. Therefore, when formulating microencapsulated foods for specific applications in the bivalve industry, it will be important to tailor particle diameter to the bivalve growth stage to avoid high rejection rates and wastage.

3.4.3. Postingestive Particle Processing

The postingestive particle processing experiments demonstrated that BioBullets microparticles were ingested by mussels, and that microparticles could be broken down by the mussel gut. Ingestion was demonstrated by the presence of microparticles in Micro-CT scans of the mussel stomach, and successful microparticle breakdown was indicated by the presence of degraded and fragmented microparticles in faeces. The finding that most microparticles present in faeces were of large diameters compared to unfed microparticle samples again suggests the presence of a threshold particle size, above which digestion in the stomach becomes problematic. Any large microparticles that are accepted through the labial palps are likely being channeled from the ciliated sorting areas to deep rejection grooves in the stomach, as also occurs with large natural food particles⁵⁶ (Figure 3.1.). They then pass into the intestines and are excreted, leading to a high proportion of large microparticles in faeces. In comparison, smaller microparticles are successfully broken down

by digestive enzymes released due to the rotating action of the crystalline style, kept in suspension, and swept towards to digestive gland ducts for absorption.

3.5. Conclusion

Our study has demonstrated that a new form of microencapsulated diet known as BioBullets can successfully be ingested by a commercially farmed bivalve, the blue mussel. With further work, this could open up numerous opportunities for the application of novel microencapsulation technologies in the bivalve shellfish industry. There is a need for future investigations to demonstrate that the contents of ingested BioBullets can be assimilated by bivalves and used to fuel anabolic processes, by comparing the growth responses of bivalves fed BioBullets to those fed standard diets. This study highlighted the importance of developing and feeding microencapsulated diets of an optimal size to bivalves, to avoid high levels of rejection in pseudofaeces or faeces. The chemical engineering approach can allow us to tailor the size of particles to the feeding preferences of specific bivalve species or growth stages. There is also a need to understand the optimal formulation of nutrients to encapsulate within microencapsulated diets. This would enable us to enhance the growth and conditioning of specific bivalve species or growth stages, or improve bivalve growth in geographies where key nutrients are lacking.

There is therefore considerable opportunity in developing the BioBullets system as a method to deliver highly nutritious microencapsulated diets to bivalve shellfish. Microencapsulated diets have potential to significantly reduce bivalve production costs, increase production output, and contribute to the continued growth of bivalve aquaculture.

3.6. Footnotes

Data availability: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.1098/rsos.171142>

Author contributions: D.W. carried out all the experimental work and data analysis. D.C.A. developed the concept, designed the formulation, and oversaw the manufacture of the microencapsulated particles. Both authors participated in the design of the study and wrote the manuscript. Both authors gave final approval for publication.

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Chapter 4: Microencapsulated diets to improve growth and survivorship in juvenile European flat oysters (*Ostrea edulis*)

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Sustainable expansion of aquaculture is critical to global food security, and bivalve shellfish aquaculture represents a sustainable method to provide people with affordable nutritious food. Oysters represent 54% of the global bivalve market by value, with propagation of juveniles within hatcheries critical to allow the industry to grow. Growth and survival of juvenile oysters in hatchery systems is constrained by suboptimal feed. The live algal feed currently used is expensive, of variable quality, contamination prone, and the high level of skill and equipment required limits where hatcheries can be located. We demonstrate how a novel microencapsulated diet can increase the growth and survivorship of *Ostrea edulis* (European flat oyster) juveniles in both the laboratory and hatchery setting. The microcapsules are easily produced in large quantities, stable for long term storage, and can be customised to have exceptionally high levels of nutrients key for oyster growth. *O. edulis* larvae fed a combined diet of microcapsules and algae for 8 days had a 46% greater increase in maximum size, 171% greater increase in minimum size, and 5% higher survival than larvae fed algae alone. *O. edulis* spat of 4 mm fed the combined diet for 7 weeks also had significantly greater survivorship (16% greater in hatchery, 58% greater in laboratory) and growth comparable (hatchery) or better (laboratory experiments) than algae alone. Further tailoring of the nutritional composition of microcapsules to specific bivalve species or growth stages could allow microcapsules to replace a greater proportion of or even completely replace algal diets. There is potential for these diets to revolutionise bivalve shellfish farming globally.

4.1. Introduction

Sustainable expansion of aquaculture is a critical component in securing food for 10 billion people by 2050 ^{19,44}. To meet increasing demand, bivalve shellfish aquaculture has been identified as a highly attractive solution, offering human nutrition at a low economic and environmental cost ³¹. For every new tonne of protein that is produced from bivalve instead of fish aquaculture, we spare 9 ha land, 67 tonnes CO₂ emissions, and 40,000 litres of freshwater ³¹. Oysters make up the biggest proportion of bivalve production (22% by weight), and oyster beds provide key ecosystem services including flood protection, pollution filtration, and fish nurseries ^{47,101}.

In many areas worldwide however oyster stocks are in a poor state. Natural oyster beds have been in extensive decline; in the United States alone oyster biomass has declined 88% and spatial habitat extent has fallen by 64% across 90 estuaries over the past century ¹⁰¹. Disease outbreaks of *Marteilia refringens* and *Bonamia ostreae* throughout Europe since the 1970s have led to the collapse of European flat oyster, *Ostrea edulis*, production. Over this period production of the Pacific oyster, *Crassostrea gigas*, has increased in an attempt to compensate for losses in native oysters, but *C. gigas* is now too being struck by disease ⁸⁰. The global community is encouraging diversification in the oyster species we farm, and in particular a reinvigoration in the production of *O. edulis*, which remains an emblematic species in Europe ^{80,102}.

Demand for juvenile oysters is currently increasing rapidly, in response to schemes aimed at restoring natural beds and through increasing aquaculture production ¹⁰². The hatcheries which rear juveniles are the critical rate-limiting step in market growth ¹⁰³. There is a need to improve juvenile rearing in hatcheries, in a way which is both sustainable and scalable.

There are multiple factors which are currently constraining the production of juvenile oysters in hatcheries. These include limited consumer access, poor marketing, supplier fragmentation, low investment into research and development, disease in the hatchery and suboptimal diets ^{19,57}. Juvenile oysters in hatcheries today are fed on microalgal diets, a system which brings many challenges. First, growing live microalgae in hatcheries accounts for 50% of production costs at US \$220 per kg biomass in 2016 ^{66,67}. Second, the microalgae produced are of highly variable and often poor nutritional quality for the oysters. Third, algal

cultures are susceptible to frequent contamination by bacteria which can result in sudden and dramatic population crashes^{25,66}. Fourth, feeds can be a major vector of disease, and complete batches of oysters are often lost, sometimes leading to hatchery closure⁷¹. To ensure reasonably consistent culture quality, microalgae must be grown in controlled indoor environments, creating an expensive bottleneck that often limits production⁶⁷. Since the 1990s commercial and research hatcheries worldwide have repeatedly identified a strong need for alternative diets to replace live microalgae, but to date no satisfactory product has been developed^{67,69,70}.

New advances in microencapsulation technology offer strong promise to improve bivalve feeding systems³¹. A microencapsulated diet consists of a formulation of nutrients and agents surrounded by a digestible capsule. Since the 1970s a small number of studies have tried using microencapsulated feeds including 'MySpat' (INVE Technologies, Dendermonde, Belgium) and 'Frippak' (Frippak Feeds, Basingstoke, Great Britain) to feed bivalves^{66,70,77,82}. These feeds are not directly representative of a natural bivalve diet; MySpat contains lipids originating from fish oils and protein from terrestrial vegetable origin, and Frippak is designed for shrimp and fish feeding^{82,83}.

Recently it was shown that a novel form of microcapsules known as BioBullets (BioBullets Ltd, Cambridge, UK) could be digested by the blue mussel *Mytilus edulis*³². The microcapsules can be produced in large quantities, are stable for long term storage, and have highly customisable physical characteristics and contents^{29,104}. They can be designed to carry exceptionally high levels of nutrients key to juvenile growth, such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid)^{88,89}, with the nutrients sourced from marine algae³¹.

Juvenile growth may be limited in traditional hatchery systems due to an insufficiency of essential nutrients; these nutrients could be delivered by microcapsules and therefore dramatically improve production success. While Willer & Aldridge (2017) demonstrated a proof-of-concept for microencapsulated diets they did not quantify the effect of BioBullets diets on bivalve growth. Our study here aimed to assess the impact of microencapsulated feed on juvenile growth in *O. edulis*. We hypothesised that juveniles would grow and survive least well in the absence of any diet, that algae and microcapsules might offer different nutritional benefits that would improve growth and survival, and that a combination of microcapsules and algae are likely to deliver the most nutritionally complete diet and might therefore be predicted to yield the fastest growth and highest survival.

4.2. Materials and Methods

4.2.1. Diets

This study had two major components: laboratory experiments on *O. edulis* spat (4 mm juveniles) in the Department of Zoology, University of Cambridge, England; and hatchery trials on *O. edulis* larvae (160 – 200 µm) and spat at SeaSalter Shellfish (Whistable) Ltd, Kent, England. Six different types of diet were fed to spat and larvae during the study, which comprised a series of four in the laboratory (1a, 2, 3a, 4) and a series of four in the hatchery (1b, 2, 3b, 4). Diet 1; algae only, which was algal concentrate in laboratory trials (Diet 1a) (Shellfish Diet 1800, Reed Mariculture, California, USA) and live algae in the hatchery trials (Diet 1b) (SeaSalter's formulation). For both algal diets spat were fed at 0.057 g dry weight feed g mean live weight bivalve⁻¹ day⁻¹. This is the ration recommended by Reed Mariculture and additional algal ration above this value has little effect on *O. edulis* growth⁶⁹, ensuring that any differences in growth when microcapsules were added would likely be driven by improved nutritional value rather than increased ration. Diet 2; microcapsules only, these were lipid-walled microcapsules containing 50% powdered *Schizochytrium* algae by weight and manufactured by BioBullets (BioBullets Ltd, Cambridge, UK), fed at 0.057 g dw feed g mean lw bivalve⁻¹ day⁻¹. To manufacture the particles, a slurry of lipid encapsulant and powdered diet is pumped into an ultrasonic atomising nozzle, before the particles form perfect spheres in specialised cooling chambers and are then coated in a proprietary surfactant to aid dispersion in water (more details on manufacture can be found in Aldridge et al., 2006). Diet 3; algal concentrate + microcapsules (Diet 3a) or live algae + microcapsules (Diet 3b), which comprised microcapsules and algae in a 1:1 ratio, both components were fed at 0.057 g dry weight feed g mean live weight bivalve⁻¹ day⁻¹. Diet 4; no food, a seawater only diet. The nutritional breakdown of each food type was obtained via a literature review (Table 4.1.).

4.2.2. Laboratory experimental design

Laboratory experiments on spat took place over seven weeks between 31/01/2018 - 21/03/2018. Experiments were carried out in a controlled temperature room held at 10 °C, in constantly aerated tanks each containing 1 L of artificial seawater at salinity 30 ‰

(H2Ocean Aquarium Salt, D-D The Aquarium Solution Ltd, UK), simulating typical sea conditions at the time of year. Tanks received a 100% water change every seven days. Each tank contained 15 spat with an initial mean length of 3.91 ± 0.64 mm, approximating to the typical size they would be moved into nurseries at hatcheries⁶⁹. There were eight tanks for each of the four diets; 1a, 2, 3a and 4, so thirty-two tanks in total. Each diet was fed daily and mixed into the water.

4.2.3. Hatchery trial design

Hatchery trials on larvae took place over eight days between 17/04/2018 - 25/04/2018. Aerated 250 ml tanks of *O. edulis* larvae at 1000 larvae L⁻¹ were kept at 21 °C and 28 ‰ salinity, and water changed every two days. There were seven tanks for each of the four diets; 1b, 2, 3b and 4, so twenty-eight tanks in total. Each diet was delivered in seawater at a continuous flow rate of 10 ml minute⁻¹ using a system of hypodermic capillaries connected to header tanks containing daily feed.

Spat trials took place over seven weeks between 29/03/2018 - 16/05/2018. These were carried out in four 25 L aerated flow-through tanks kept at ambient hatchery temperatures (18 – 21 °C) and salinities (26 – 28 ‰). Each tank contained 100 *O. edulis* spat, and received one of the four diets; 1b, 2, 3b and 4. Feed was again delivered using a continuous system with a flow rate of 10 ml minute⁻¹, with the feed lines discharging halfway to the base of each tank. In the hatchery it was not possible to have replicate tanks for each diet, therefore, a greater number of spat per tank (100) was used compared to the 15 spat used in the laboratory.

4.2.4. Survivorship and growth tracking

Larval survivorship, maximum and minimum size was measured every two days in the hatchery. A 25 ml sample was taken from each tank following stirring, and the number of live and dead larvae in the entire sample was recorded using a microscope. The maximum and minimum size of live larvae along the longest axis was recorded after washing them through grading sieves using a microscope with a calibrated eyepiece graticule. Maximum and minimum larval size in each sample was measured rather than the average size of many larvae in each sample to reduce the amount of time larvae were exposed to the harsher environment outside of the tank. All larvae were returned to the tank after counting and measuring.

Spat survivorship was measured weekly at the laboratory and hatchery using counting sieves, and any dead spat were removed from the tanks. To track growth using fluorescence imaging, spat at both the hatchery and laboratory were treated with calcein (C0875, Sigma-Aldrich, UK) at 100 mg litre⁻¹ for 24 hours on day zero, and then thoroughly rinsed in seawater^{105,106}. Unfortunately, the calcein label did not persist over the course of the experiments, so the following alternative measures were used to track growth. In the laboratory the live weight of individual spat was measured weekly from day seven onwards. For the hatchery trials the individual live weights of 30 spat were recorded at day zero, and the final live weights of all individual spat were recorded at the end of week seven; there was not capacity to weigh spat weekly in the hatchery. Calibrated Mettler Toledo AB54-S laboratory scales were used for all weighing to a precision of ±1 mg.

4.2.5. Statistical analyses

Power analyses were performed using G*Power (2018, Heinrich-Heine-Universität Düsseldorf, Germany) before experiments started, to ensure experimental designs and sample sizes were appropriate. Statistical analyses of the data were performed using R Statistics¹⁰⁰. For larvae and spat survivorship, linear models with subsequent ANCOVA and least-square means were used to compare the number of live larvae or spat against time (the covariate) between diets. For hatchery larvae and laboratory spat growth, linear models with subsequent ANCOVA and least-square means were used to test for differences in size measurements or weight across time between diets. For spat growth in the hatchery Kruskal-Wallis and *post-hoc* Wilcoxon tests were used to test for differences between diets and initial spat length.

Table 4.1. Nutritional composition of live algae, algal concentrate, and microencapsulated feed. All values are in g per 100 g dry weight (dw). For SeaSalter live algae, overall nutritional composition is an average of the component species, and assumes an even mix of flagellate and diatom algae ⁶⁹. For microcapsules (BioBullets Ltd.) and algal concentrate (Shellfish Diet 1800), overall nutritional data is sourced direct from the manufacturer. Superscript letters refer to source of individual data: a ⁶⁸, b ¹⁰⁷, c ¹⁰⁸, d ¹⁰⁹, e ¹¹⁰, f ¹¹¹, g ¹¹², h ¹¹³.

		Protein	Carbohydrate	Lipid	Ash and fibre	20:5n-3 (EPA)	22:6n-3 (DHA)
Diet 1a: algal concentrate		52 ^g	16 ^g	22 ^g	10	1.61	0.78
Components	<i>Isochrysis galbana</i>	29 ^a	13 ^a	23 ^a	35	0.21 ^a	2.32 ^a
	<i>Pavlova lutheri</i>	29 ^a	13 ^a	23 ^a	35	4.53 ^b	2.16 ^b
	<i>Tetraselmis suecica</i>	31 ^a	12 ^a	10 ^a	47	0.54 ^a	0.01 ^a
	<i>Chaetoceros calcitrans</i>	34 ^a	6 ^a	16 ^a	44	2.84 ^a	0.14 ^a
	<i>Thalassiosira weissflogii</i>	28 ^h	23 ^h	22 ^h	27	1.10 ^h	0.00 ^h
	<i>Thalassiosira pseudonona</i>	34 ^a	9 ^a	19 ^a	38	0.44 ^c	0.06 ^c
Diet 1b: live algae		31	12	13	44	2.31	0.70
Components	<i>Pavlova lutheri</i>	29 ^a	13 ^a	23 ^a	35	4.53 ^b	2.16 ^b
	<i>Tetraselmis suecica</i>	31 ^a	12 ^a	10 ^a	47	0.54 ^a	0.01 ^a
	<i>Isochrysis galbana</i>	29 ^a	13 ^a	23 ^a	35	0.21 ^a	2.32 ^a
	<i>Chaetoceros muelleri</i>	37 ^a	17 ^a	6 ^a	40	2.84 ^a	0.14 ^a
	<i>Chaetoceros ceratosporum</i>	37 ^a	17 ^a	6 ^a	40	2.84 ^a	0.14 ^a
	<i>Skeletonema costatum</i>	25 ^a	5 ^a	10 ^a	60	0.29 ^c	0.06 ^c
	<i>Rhinomonas sp.</i>	47 ^a	22 ^a	4 ^a	27	2.64 ^a	1.45 ^a
	<i>Chaetoceros gracilis</i>	12 ^a	5 ^a	7 ^a	76	2.84 ^a	0.14 ^a
	<i>Chaetoceros calcitrans</i>	34 ^a	6 ^a	16 ^a	44	2.84 ^a	0.14 ^a
	<i>Pyramimonas virginica</i>	21 ^d	16 ^d	14 ^d	49	0.00 ^e	0.47 ^e
	<i>Nannochloropsis oculata</i>	36 ^a	8 ^a	18 ^a	38	6.53 ^f	0.07 ^f
Diet 2: microcapsules		6	16	58	20	0.25	9
Diet 3a: algal concentrate + microcapsules		29	16	40	15	0.93	4.89
Diet 3b: live algae + microcapsules		18	14	36	32	1.28	4.85

4.3. Results

4.3.1. Larval survivorship and growth in the hatchery

O. edulis larvae fed a combination of live algae and microcapsules experienced the greatest survivorship and most rapid growth. After an 8-day growth period, all sampled larvae from tanks containing live algae + microcapsules were alive, compared to 95.0 ± 5.0 (SE) % for live algae only, 70.0 ± 20.0 % for no food tanks, and 25.3 ± 12.6 % for microcapsules only (Figure 4.1a.). The overall decline in percentage larvae alive was significantly lower in algae + microcapsules and algae only tanks than in the other tanks (ANCOVA, $F_{7,89} = 8.99$, $r^2 = 0.37$, $p < 0.001$, post-hoc least-square means (LSM) $p < 0.05$), and after 6 days larval survivorship was significantly higher in algae + microcapsule tanks than in all other tanks (post-hoc LSM $p < 0.05$). Both maximum and minimum size was greater for larvae fed live algae + microcapsules compared to all other diets (for maximum size ANCOVA, $F_{7,85} = 90.54$, $r^2 = 0.87$, $p < 0.001$, LSM $p < 0.001$; for minimum size ANCOVA, $F_{7,85} = 33.51$, $r^2 = 0.71$, $p < 0.001$, LSM $p < 0.05$) (Figure 4.1b, c.). At day-8, the respective maximum

and minimum size of larvae fed live algae + microcapsules was 284 ± 12 and $238 \pm 11 \mu\text{m}$, compared to 255 ± 5 and $202 \pm 4 \mu\text{m}$ for live algae, 177 ± 3 and $173 \pm 3 \mu\text{m}$ for microcapsules, and 175 ± 3 and $175 \pm 3 \mu\text{m}$ for no food.

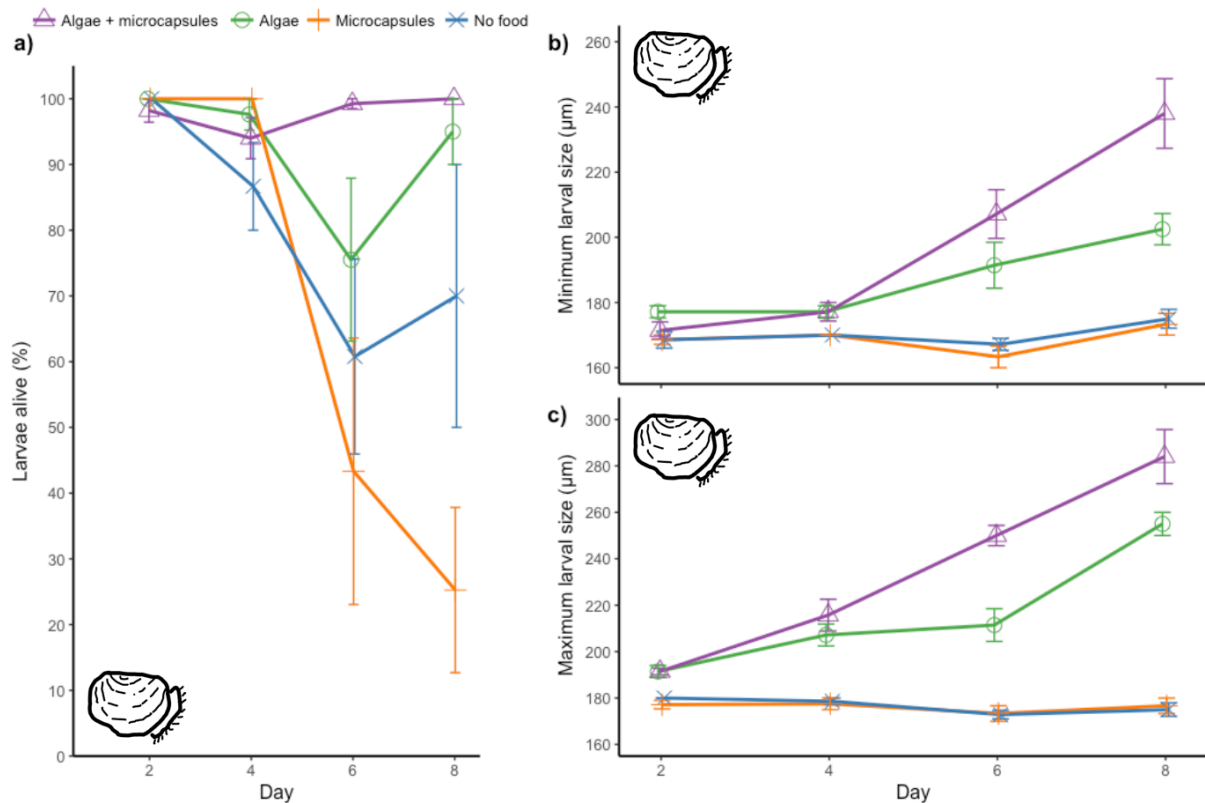


Figure 4.1. Survivorship and growth of *O.edulis* larvae (160 – 300 μm) grown on microencapsulated and algal diets in the hatchery. *O.edulis* larvae were fed algae and microcapsules (purple Δ), live algae (green o), microcapsules (orange +), or no food (blue x) over 8 days in the hatchery. Survivorship (a) and maximum and minimum larval size (b and c) were recorded. Data are from 25 ml samples, n = 7 tanks, error bars represent Standard Error.

4.3.2. Spat survivorship and growth in the laboratory

In the laboratory *O. edulis* spat fed a diet solely of microcapsules experienced the greatest survivorship and growth. Survivorship was significantly greater in spat fed on only microcapsules compared to all other diets (ANCOVA, $F_{7,248} = 99.12$, $r^2 = 0.73$, $p < 0.001$, LSM $p < 0.001$) (Figure 4.2a.). From a starting point of 15 live spat per replicate, after 7 weeks the mean number of live spat in tanks supplied solely with microcapsules was 6.88 ± 0.95 ,

compared to 3.75 ± 1.04 in microcapsules + algal concentrate, 2.38 ± 1.18 in algal concentrate only, and 2.13 ± 0.55 in no food tanks.

Spat fed on only microcapsules in the laboratory underwent the greatest increase in mean weight of all diets, significantly greater than spat fed algal concentrate only or no food (ANCOVA, $F_{4,219} = 4.57$, $r^2 = 0.06$, $p < 0.01$, LSM $p < 0.05$) (fig. 3a). Between weeks 1 and 7 the mean weight of spat fed only microcapsules increased by 40.6 ± 4.6 % from 24.55 ± 1.13 to 34.42 ± 2.31 mg. In comparison the mean weight of spat fed only algal concentrate or no food decreased, with declines of 33.0 ± 0.1 % and 11.4 ± 0.1 % respectively.

4.3.3. Spat survivorship and growth in the hatchery

In the hatchery microcapsules only was the food type that resulted in the greatest survivorship, but algae only and algae + microcapsules led to better growth. Survivorship of spat fed only microcapsules was significantly greater than spat fed only live algae or live algae + microcapsules (ANCOVA, $F_{7,24} = 32.97$, $r^2 = 0.88$, $p < 0.001$, LSM $p < 0.05$). Notably, survivorship of spat fed microcapsules was not significantly different from unfed spat (LSM, $p > 0.05$) (Figure 4.2b.).

Spat fed only live algae or live algae + microcapsules had a significantly greater increase in shell length than spat fed only microcapsules or no food (Kruskal-Wallis, $X^2_4 = 136.13$, $p < 0.001$, post-hoc Wilcoxon rank sum $p < 0.001$) (Figure 4.3b.). From an initial length of 3.91 ± 0.12 mm, spat fed only live algae grew by 67.9 ± 0.1 % to 6.56 ± 0.23 mm, and spat fed live algae + microcapsules grew by 68.1 ± 0.1 % to 6.57 ± 0.13 mm. There was no significant difference in length increase between spat fed only algae or live algae + microcapsules (Wilcoxon rank sum $p > 0.05$). The length increase in spat fed only microcapsules was 38.5 ± 0.1 %, to 5.41 ± 0.12 mm.

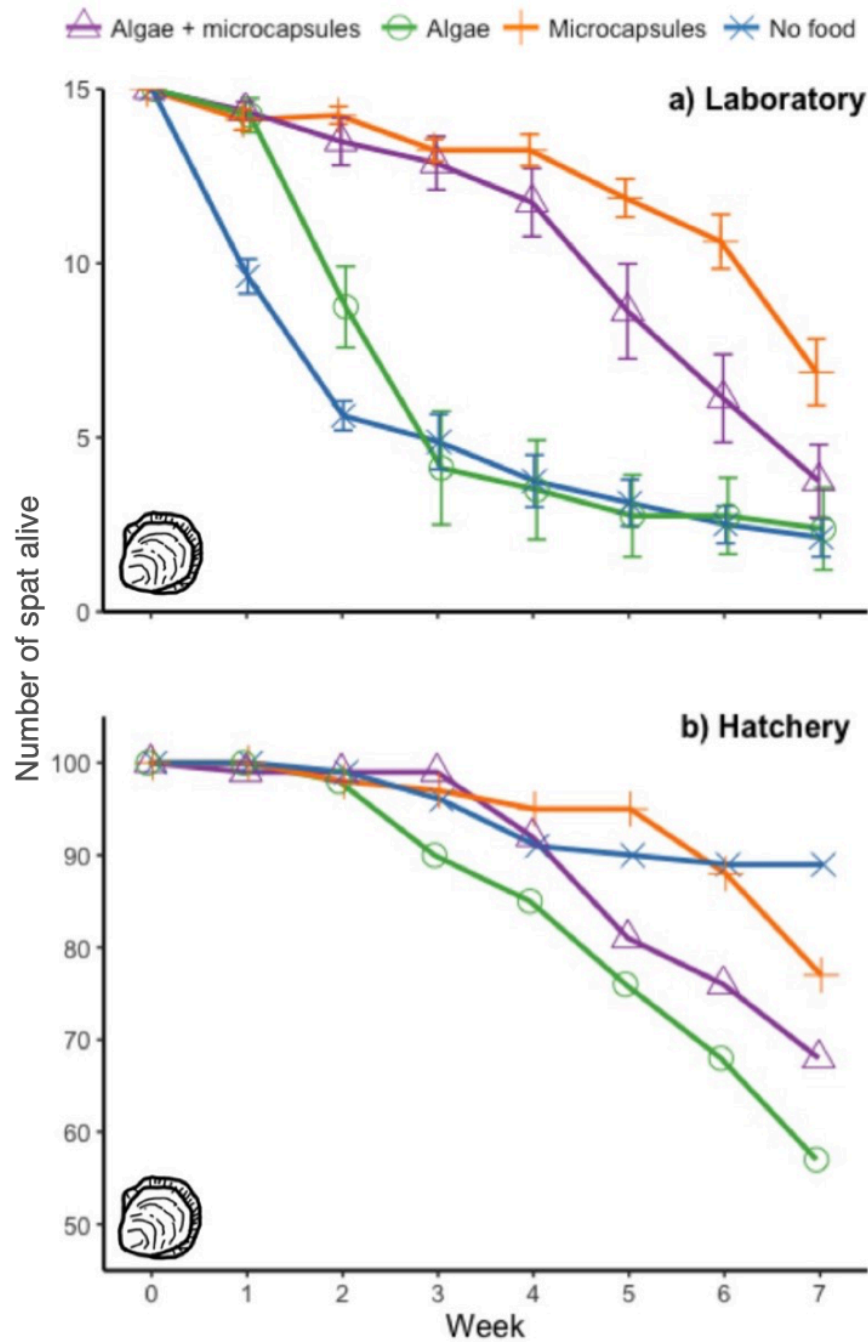


Figure 4.2. Survivorship of *O. edulis* spat (> 3 mm) grown on microencapsulated and algal diets. *O. edulis* spat were fed microcapsules (orange +), algae (green o), a combination of both (purple Δ), or no food (blue x) over 7 weeks, and survivorship was recorded. In the laboratory (a) algal concentrate and in the hatchery (b) live algae were fed. For (a) n = 8 tanks, for (b) n = 1 tank, error bars represent standard error. No error bars in b) because the same tank was used for all spat on a given diet at the hatchery.

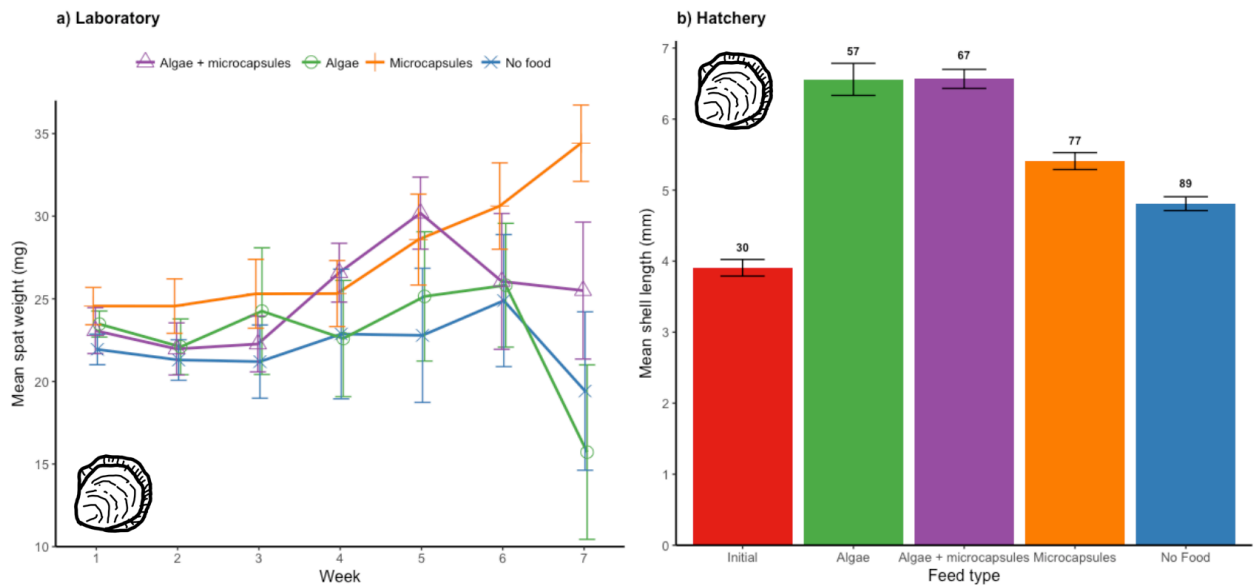


Figure 4.3. Growth of *O. edulis* spat (> 3 mm) grown on microencapsulated and algal diets.

O. edulis spat were fed microcapsules (orange), algae (green), a combination of both (purple), or no food (blue) over 7 weeks. In the laboratory (a) spat were weighed weekly and algal concentrate was fed. In the hatchery (b) shell length was measured at week 0 (initial, red) and after 7 weeks growth, and live algae was fed. For (a) n = 8 tanks of initially 15 spat. For (b) n = 1 tank and number of spat are shown above bars. Error bars represent standard error.

4.4. Discussion

Our investigation indicates that use of microencapsulated feeds can lead to significant improvements in the survivorship and growth of juvenile oysters relative to traditional algal diets. In *O. edulis* larvae a diet containing both microcapsules and live algae resulted in greater survivorship, a 46% greater increase in maximum size and a 171% greater increase in minimum size over 8 days compared to a diet of live algae alone. *O. edulis* spat fed microcapsules alone had greater survivorship than those fed any other diet. In the laboratory a diet of only microcapsules also resulted in the most spat growth, while in the hatchery combining microcapsules with algae led to better spat growth than microcapsules alone.

The improved growth of larvae fed on the combined diet of microcapsules and algae may be driven by the exceptionally high 22:6n-3 fatty acid (DHA) levels in the microcapsules. DHA is a nutrient known to increase juvenile growth, and at 9 g per 100g dw levels in

microcapsules were nearly 13 times that in live algal feed (Table 4.1.)^{88,89}. The relatively poor performance of microcapsules alone for survivorship and growth of larvae may be driven by deficiencies of nutrients important to larval proliferation⁶⁹. Protein levels were five times lower in the microcapsules versus live algal feed (6 vs 31 g per 100g dw), and 20:5n-3 fatty acid (EPA) levels were also markedly lower (Table 4.1.).

The physical attributes of the microcapsules are a probable driver for the greater survivorship of *O. edulis* spat fed microcapsules relative to spat fed other diets. The microencapsulated feed is not live hence reducing risk of introducing disease through feed, and the waxy encapsulant avoids microcapsules degrading before they enter the gut, resulting in less risk of bacteria growing on microencapsulated food compared to conventional algal diets³². The components of the proprietary encapsulant also have antibacterial properties which may explain the lower mortality when microcapsules were given in combination with algae compared to algae alone.

The quality difference between live algal feed and algal concentrate may offer an explanation as to why in the hatchery live algae alone led to the same improvements in spat growth as combining live algae and microcapsules, whereas in the laboratory combining algal concentrate with microcapsules led to better growth than algal concentrate alone. The live algal mix used at SeaSalter is renowned for its world-leading quality, with the system supplied to 16 countries worldwide (SeaSalter Shellfish (Whistable) Ltd). Appropriate quantities of a wide multitude of key amino acids and lipids are needed for optimal bivalve growth and larval survival¹¹⁴. The microencapsulated feed may not have been able to provide any additional nutritional benefit when given in combination with the high quality SeaSalter live algae, whereas microcapsules may have greatly enhanced dietary nutritional value when given in combination with algal concentrate. However, the survival improvements provided when microcapsules were given in addition to live algae in the hatchery demonstrate there is great potential value for microencapsulated feeds in even the best spat rearing systems of today.

There remains capacity to further refine the formulations of microencapsulated feeds. The current low protein levels in microcapsules (Table 4.1.) may explain why when given alone they provided less larval and spat growth in the hatchery compared to combined live algae and microcapsules. In order for microencapsulated feeds to replace a greater proportion of or completely replace live algal diets, the nutritional content of the capsules will need to be carefully tailored to specific bivalve species and growth stages.

Overall this study demonstrates that the use of microencapsulated feed can lead to major improvements in survivorship and growth in juvenile oysters, an outcome of great importance in aquaculture as a field. Existing hatchery processes represent an expensive bottleneck in oyster production. Using microencapsulated diets to drive more rapid progression of juvenile oysters through the hatchery and onto open water grow-out could increase overall production output and reduce costs. Microencapsulated feeds could also enable hatcheries to be set up where there is a lack of expertise or space to rear live algae, increasing the number of potential locations that bivalves can be farmed. Increased availability of a sustainably produced micronutrient and protein-rich food in the form of bivalves would be of great value in both developing and developed nations; worldwide 795 million people don't get access to the calories they need while at least 2 billion consume too many calories but don't get access to the nutrients they need ^{19,39,44}.

We have demonstrated that diets containing microcapsules can reduce mortality in spat and larvae. There is also the opportunity for expanding the technology for tackling the management of disease in the hatchery. Microcapsules have already been identified as an optimal delivery mechanism for immunostimulants and probiotics to tackle *Bonamia ostreae* disease in *O. edulis* ¹¹⁵. There is a need for future investigations to assess whether microcapsule delivery of disease control agents can effectively reduce disease in bivalve shellfish, and the BioBullets technology used in this study offers considerable promise.

Improvements made in the bivalve shellfish industry, in part driven by the use of new microencapsulated feed, could play a key role in creating sustainably produced and reliable food across wide geographies. Bivalves have a higher nutritional value, are cheaper to farm, and have a lower environmental footprint than most other animal foods ^{19,31}. If we could to continue replace just 25% of salmon aquaculture with bivalves, CO2 emissions equal to those of New Zealand, an area of larger than Wales, and 11.8 billion litres of freshwater could be spared annually ³¹. There is considerable opportunity for research and industry continue to test and implement microencapsulated diets to realise the great benefit improved growth in bivalve aquaculture can have for meeting the needs of global food security.

4.5. Footnotes

Data availability: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.1016/j.aquaculture.2019.02.072>.

Author contributions: D.W. and D.C.A. wrote the manuscript. Both authors gave final approval for publication.

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Chapter 5: Microencapsulated algal feeds as a sustainable replacement diet for broodstock in commercial bivalve aquaculture

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The global bivalve shellfish industry makes up 25% of aquaculture, is worth USD \$17.2 billion yr⁻¹, and relies upon a supply of juvenile bivalves produced by adult broodstock in hatcheries. Today large quantities of live algae are grown to feed broodstock at \$220 kg⁻¹, driving highly unsustainable energy and resource use. New advances in algal and microencapsulation technology provide solutions. We developed microencapsulated *Schizochytrium* algae diets, which can be produced sustainably at < \$2 kg⁻¹ from organic side-streams, and are shelf-stable to minimise waste. Physiological, histological, and cutting-edge metabolomic analyses demonstrate that in commercial settings sustainable microencapsulated diets facilitate improved sexual development and 12x greater omega-3 levels in oysters relative to conventional live algal diets. Every tonne bivalve protein produced instead of fish spares 9 ha, 67 tonnes CO₂, and 40,000 litres freshwater. Further research into microencapsulated diets could support bivalve industry expansion, and contribute towards a step-change in sustainable global food production through improved aquaculture practices.

5.1. Introduction

The USD \$17.2 billion global bivalve shellfish industry relies upon a supply of juvenile bivalves produced by adult broodstock in hatcheries⁴⁷. Current estimates suggest 220 million broodstock bivalves are held in hatcheries worldwide^{31,47,116–119}. At present broodstock must be fed live algae, production of which drives unsustainable land, energy and antibiotic use³¹.

Algal production in hatcheries makes highly inefficient use of land and energy ^{31,32,67}. Typical hatcheries require 400 m² of algal tanks to maintain just 400 broodstock, equating to 220 million m² worldwide, an area larger than Washington, D.C. ¹¹⁸. High-intensity artificial lighting, temperature, and air control systems are needed to support algal growth ⁶⁹. Furthermore, algal stocks are difficult to maintain and frequently lost due to contamination and disease, so greater quantities must be produced, to the extent that each unit of viable hatchery-grown algae is 20-fold more expensive than units grown in contamination-free commercial photobioreactor facilities ^{27,31,33,120}, accounting for 50% of bivalve production costs at USD \$220 kg⁻¹ algal biomass in 2016 ^{66,70}.

Algal production presents a major disease control issue. Live algal feeds are the primary vector of bivalve disease, which is controlled with antibiotics ³¹. Antibiotics cause severe damage to marine ecosystems; 80% of aquaculture antibiotics are not metabolised by stock, persist in the open sea, and drive proliferation of antibiotic resistant bacteria ^{25,90}. In the world's largest bivalve producers including China no veterinary prescriptions are required for antibiotics with use essentially unregulated ⁹⁰. In Europe twice the number of antimicrobial substances were sold for animal versus human use in 2014 ⁹⁰. Non-live diets are more sterile, and hence since the 1990s the bivalve aquaculture industry has been seeking non-live alternative feeds to reduce antibiotic needs ^{25,31,33,67}. FAO and EU sustainable aquaculture policies identify urgent and immediate needs to reduce land, energy, and antibiotic use in aquaculture ^{19,90,121,122}.

New advances in algal production and microencapsulation technology offer a groundbreaking solution to reduce the environmental footprint of bivalve aquaculture. *Schizochytrium* algae can be grown heterotrophically on industrial scales at USD \$1.50 kg⁻¹, using low-cost food waste and agricultural side-streams as inputs ^{120,123–125}. For bivalve nutrition *Schizochytrium* has advantages, with levels of key nutrients such as docosahexaenoic acid (DHA) exceeding 20% dry-weight; greater than twice the abundance of DHA in hatchery-grown algae ^{68,125}. Novel microcapsules are an ideal vehicle for delivering sustainable *Schizochytrium*-based diets to bivalve broodstock ^{29,31–33}. Mass production is simple and cost-effective ^{29,32}, and the microcapsules dry and shelf-stable thus circumventing conventional feed wastage costs ²⁵. Capsule characteristics can be tailored to maximise feeding efficiency ²⁵ and minimize nutrient leaching to water ^{53,67,83}, whilst also being sterile and not a disease vector ³¹. The nutritional profile of microencapsulated feed compared to conventional algal feed is shown in Table 5.1.

There are major sustainability advantages of replacing live algal feeds with microencapsulated feeds in bivalve aquaculture, including 20-fold reductions in energy use, carbon emissions and production costs (Figure 5.1.). However, for replacement to be commercially viable it is critical to assess whether microencapsulated feeds provide comparable sexual development in bivalve broodstock compared to conventional algal feeds.

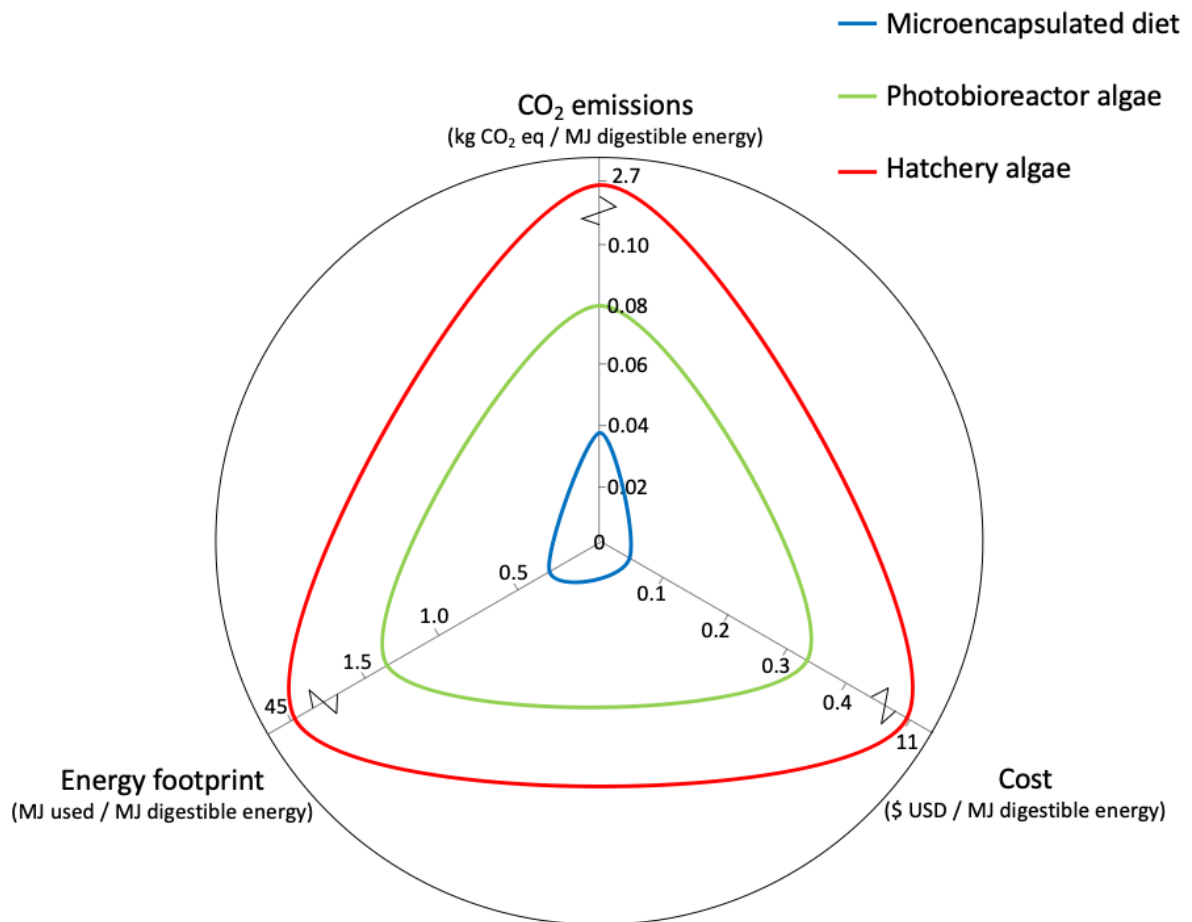


Figure 5.1. The sustainability advantage of new microencapsulated diets. The radar-plot demonstrates lower CO₂ emissions, reduced energy usage, and more efficient use of economic resources in new microencapsulated diets. Comparison is relative to the most efficient to produce form of autotrophic live algae grown on an industrial scale (photobioreactor algae), and algae grown in a relatively efficient bivalve hatchery today. Note the broken axes for hatchery algae. We also note that a 100% replacement of live algae will require further tailoring of the microcapsule formulation for increased protein content. Data sources and figure methodology: Supplementary Data S1, ^{29,32,33,66,70,120,123,126–129}.

5.2. Results

5.2.1. Sexual maturation: Gonad weight

We first tested the impact of replacing live algal diets with microencapsulated *Scizochytrium* diets on oyster gonad weight. Both microencapsulated and live algal diets resulted in greater gonad weight European oysters (*O. edulis*) following a six-week broodstock conditioning period in a commercial hatchery, relative to pre-conditioning control (t = 0) oysters. Mean wet gonad weight was significantly greater in oysters fed algae (3.51 ± 1.24 g (Standard Error)), microcapsules (4.40 ± 1.10 g), or algae + microcapsules (4.15 ± 0.75 g) compared to oysters pre-conditioning (1.59 ± 0.46 g) (ANOVA, $F_{3,32} = 16.58$, $p < 0.001$). Figure 5.2. demonstrates how the difference between sample and control gonad weight was greatest in oysters fed microcapsules (2.81 ± 1.10 g), although this value was not significantly different from oysters fed algae (1.92 ± 1.24 g) (ANOVA, $F_{2,24} = 1.73$, $p = 0.19$). The observed greater gonad weight in conditioned relative to pre-conditioned oysters is expected as oysters build up energy reserves for gametogenesis, and the greater values can be assumed to represent an increase in weight over time relative to the controls¹³⁰.

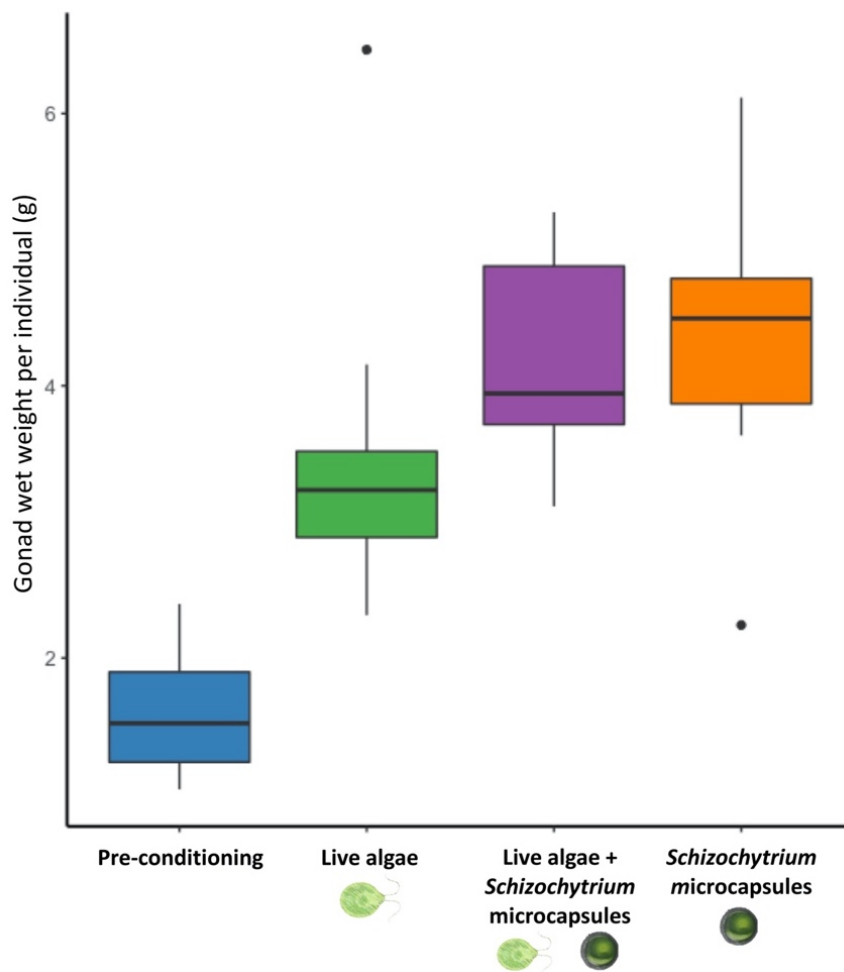


Figure 5.2. Increased oyster broodstock gonad weight following conditioning. The boxplot shows the total wet gonad weight of oysters fed algae (green), microcapsules (orange), or algae + microcapsules (purple) over a six-week conditioning period, relative to oysters pre-conditioning (blue). $n = 9$ oysters per diet, gonad weight was significantly greater in oysters fed a diet relative to oysters pre-conditioning.

5.2.2. Sexual maturation: Fatty acid and lipid abundance.

Demonstration that microencapsulated *Schizochytrium* diets could facilitate comparable or greater increases in gonad mass compared to live algal diets provided macroscopic evidence that microencapsulated diets could be an effective replacement for live algae in sexual maturation. We hence embarked upon a molecular investigation of gonad lipids and gametogenesis to provide further explanation.

Mass spectrometry was used to determine abundance and profile of fatty acids. These data showed that fatty acid mass in *O. edulis* gonads was greater post-conditioning compared to pre-conditioning. The greatest differences in abundance (over 400 %) were present in 16:0, 18:0, 18:1, 20:5 (EPA), and 22:6 (DHA) fatty acids (Figure 5.3a.). There was a

significant difference between diets in the magnitude of the abundance difference for 42 of the 45 fatty acids analysed (see Supplementary Information Table S1a.).

The greatest difference in fatty acid abundance relative to the pre-conditioning controls was seen in oysters fed only microcapsules. For 40 of the 45 fatty acids the difference was significantly greater for microcapsule fed oysters compared to algae fed oysters (Table S1a.). In particular, the difference in 20:5 and 22:6 fatty acids was 12 times greater in microcapsule compared to algae fed oysters (microcapsule fed: 20:5 = 553.3 ± 254.8 ‰, 22:6 = 398.5 ± 158.6 ‰; algae fed: 20:5 = 34.9 ± 86.0 ‰, 22:6 = 33.4 ± 62.6 ‰). The post-conditioning difference in fatty acid abundance was also significantly greater in oysters fed only microcapsules compared to oysters fed algae + microcapsules in 41 of 45 cases (Table S1a.). Whilst algae + microcapsule fed oysters did in general have greater post-conditioning differences in fatty acid abundance than algae fed oysters, this difference was only statistically significant in 3 cases (Table S1a.).

The abundance of other lipids in *O. edulis* gonads was also greater post-conditioning compared to pre-conditioning, although there was only a significant difference between diets for 30 of the 792 lipids assessed. Table S1b. presents lipids where there was a significant difference between diets; the remainder of the lipids can be found in Table S2. For the following four lipids the difference in abundance was particularly large (over 100 ‰) and also significantly greater in oysters fed microcapsules or algae + microcapsules compared to oysters fed algae alone: LPE(18:1)_{[M-H]1-}, LPE(20:1)_{[M-H]1-}, LPE(22:4)_{[M-H]1-}, PG(20:0) (Figure 5.3b.) (Table S1b.).

The significantly greater post-conditioning abundance of fatty acids and lipids in oysters fed microcapsules provide a biochemical explanation to our initial finding of strong increases in gonad mass in oysters fed microcapsules. We can again assume that the greater abundance represents an increase over time relative to the pre-conditioning controls¹³⁰.

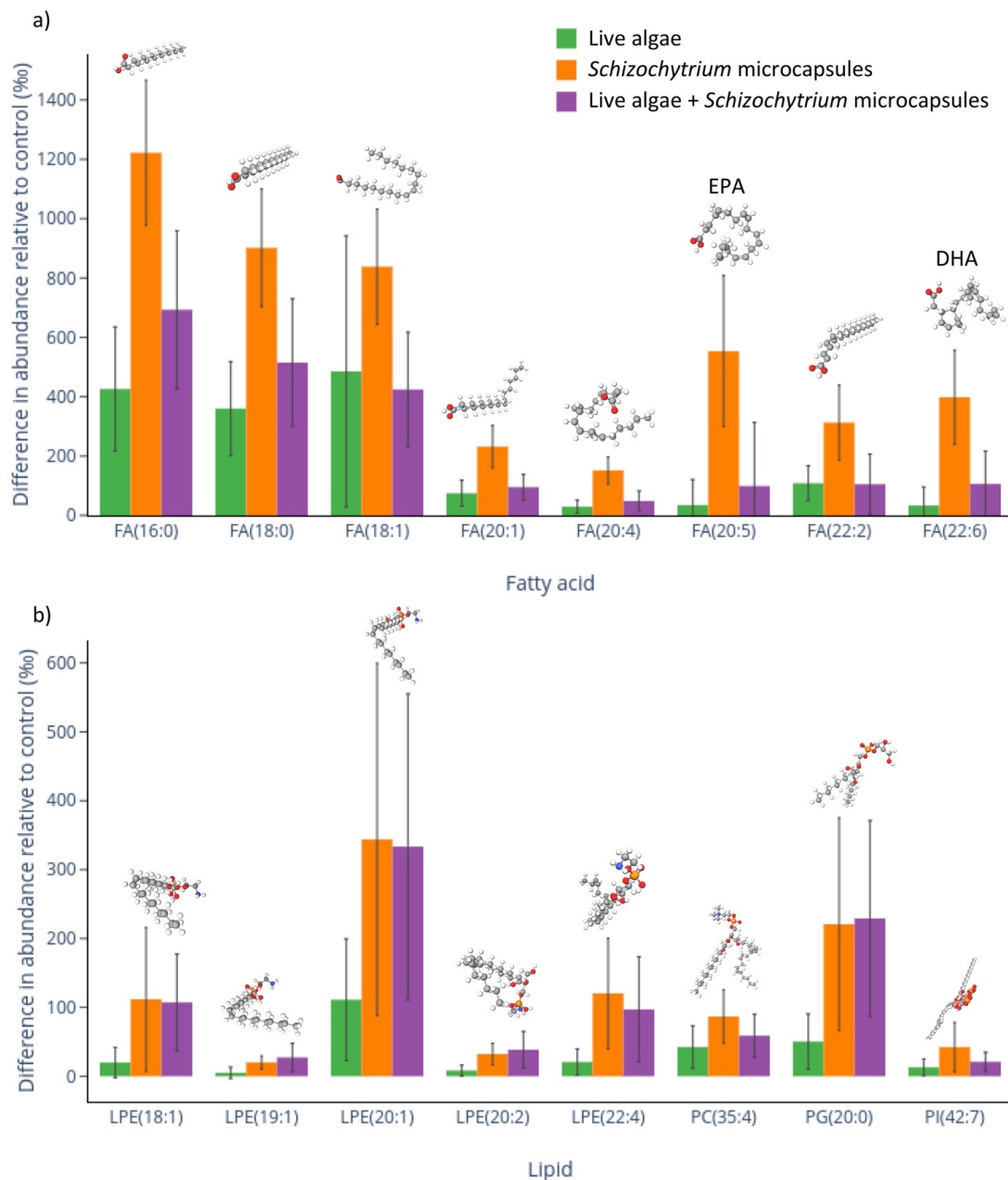


Figure 5.3. Increase in abundance of fatty acids (a) and other lipids (b) over conditioning period. The increase shown in the bar plots represents the difference in fatty acid and lipid abundance in the gonads of oysters fed algae (green), microcapsules (orange), or algae + microcapsules (purple) over a six-week conditioning period, relative to oysters pre-conditioning. Abundance was calculated from mass spectrometry data using total wet gonad weight as a scaling factor. $n = 9$ oysters per diet, error bars represent standard error. The eight fatty acids (a) and lipids (b) with the greatest change in abundance across all diets are presented in the bar plots, and for these all diets differ significantly ($p < 0.05$ with Holm-Bonferroni). For (b) lipid names have been abbreviated for clarity, full names are shown in Table S2.

5.2.3. Sexual maturation: Histological imaging

Histological imaging of the oyster gonads revealed that oysters fed either microcapsules or algae + microcapsules were at a more advanced stage of sexual maturation after six-weeks of conditioning than oysters fed algae alone (Figure 5.4.). Oysters fed algae alone had progressed from having largely inactive gonads (Figure 5.4a, b.) to advanced spermatogenesis, with follicles filled with spermatogonia and spermatocytes (Figure 5.4c, d.). In contrast, oysters fed microcapsules in addition to or in replacement of algae had reached full maturity and had dense follicles with many spermatids (Figure 5.4e – h.).

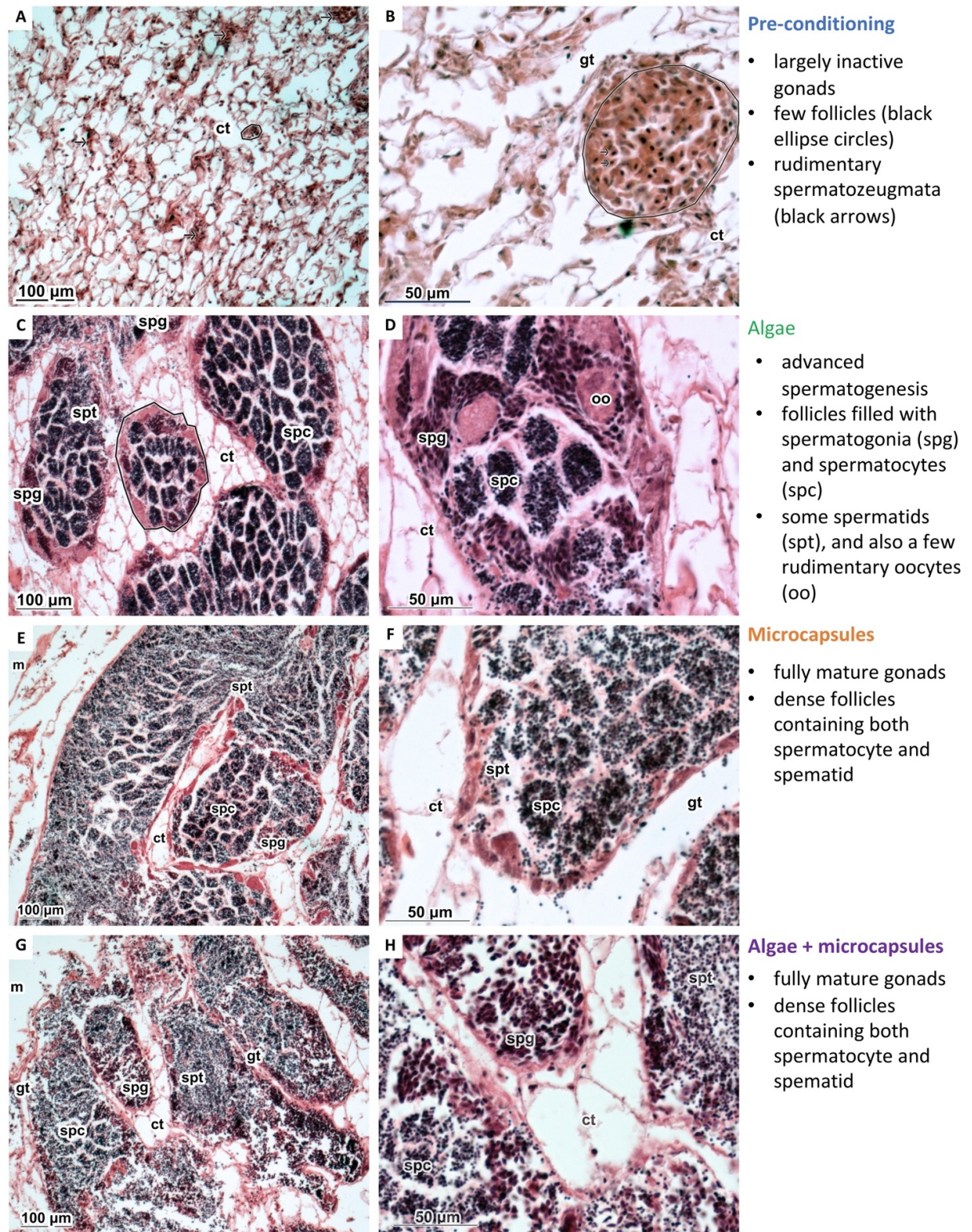


Figure 5.4. Greater sexual maturation in oysters fed *Schizochytrium* microcapsules.

Histological sections of *O. edulis* gonads from predominantly male individuals fed one of three diets compared to pre-conditioning controls: pre-conditioning (a - b), algae (c - d), microcapsules (e - f), algae + microcapsules (g - h). Selected images are haematoxylin eosin stained and represent the typical gonadal state of five oysters from each treatment type after a six-week feeding period. ct: connective tissues; gt: gonadal tubule; m: mantle.

5.3. Discussion

5.3.1. Microencapsulated diets enable improved sexual maturation in oysters

Our investigations demonstrate that *Schizochytrium*-based microencapsulated diets enable not only comparable but improved sexual maturation in oyster broodstock compared with conventional live algal diets. The gonads of oysters fed microencapsulated diets were of greater weight, contained higher levels of omega-3 fatty acids crucial for sexual maturation, and underwent accelerated spermatogenesis.

Of particular importance are the large increases in EPA and DHA in microcapsule relative to algae fed oysters. EPA is the primary energy source for gamete maturation, with higher levels directly increasing gamete quantity and development rate¹³¹. DHA is pivotal to the structure and function of gamete cell membranes, with higher levels increasing gamete quality and egg survival rates^{85–87,130,131}. High levels of EPA and DHA in the *Schizochytrium*-based microcapsules are likely driving this increase^{68,125}. To date there is no clear evidence that either fatty acid can be synthesised *de-novo* by oysters from shorter chain precursors^{131,132}. The more rapid advance of spermatogenesis in oysters fed a microencapsulated diet is highly likely being driven by the greater levels of EPA and DHA in these animals; a causal relationship demonstrated by several previous studies^{69,80,133}. This offers strong support for the use of microcapsules as a broodstock conditioning feed.

It is important to consider that for future application the nutritional formulation of the microcapsules would need to be tailored further for increased protein content or fed alongside a quantity of live algae. The current protein content of the microcapsules is lower than that of live algae (6 vs 31g protein per 100g dry weight, Table 5.1.). Protein is important in bivalve larval development and for shell formation, and if insufficient juvenile growth can be suppressed³³. There would be significant value in performing additional studies investigating the effectiveness of a higher protein formulation of microcapsules on bivalve broodstock conditioning and juvenile development.

Regarding the newly developed microcapsules; the size is tailored to maximise bivalve feeding efficiency (20 – 140 µm diameter), and buoyancy neutral to ensure particles remain within reach of the filter feeders²⁵. This is an improvement over a basic freeze-dried algal powder delivery system; powders tend to float on the water surface, and can clump

into particles too large to be accessed by bivalves⁶⁷. The waxy encapsulant minimises pre-ingestive nutrient loss by allowing particles to remain stable and retain nutrients in seawater, yet still be rapidly digested on entry to the bivalve gut⁶⁷. The specialised coating allows delivery of low molecular weight, water soluble compounds, alongside fatty acids, with minimal leaching to the surrounding water⁸³, reducing eutrophication risks^{53,67}. The encapsulant also has strong antibacterial properties, and contents are sterile, which reduces disease incidence in aquaculture relative to live feeds^{33,134}, and enables reduced antibiotic usage¹³⁴. There remains scope for further experiments allow greater optimization of the physical characteristics of the microcapsules. For example, whilst we know that capsules of 20 - 140 µm can be ingested by oysters⁹⁷, it would be useful to perform a study to investigate the filtration and ingestion of different sizes of microcapsule within this range to identify the preferred specific capsule diameters for both bivalve broodstock and juveniles.

5.3.2. Sustainability and Commercial Implications

The use of live algae is driving excessive and unsustainable energy and resource use in bivalve production. This investigation demonstrated that sustainable *Schizochytrium*-based microencapsulated diets can help support more effective sexual maturation in oyster broodstock. Further research could help open up the opportunity initially outlined; to upscale production and associated infrastructure to allow microencapsulated diets to partially or fully replace live algae in hatcheries, and reduce the environmental footprint of bivalve aquaculture.

However, given that microencapsulated diets enabled not only comparable but improved sexual development in bivalve broodstock, there is potential to reap even further commercial and sustainability benefits. Higher quality broodstock with greater lipid stores directly translates into higher quality seed with a greater inherent survival rate¹³¹. More rapid sexual maturation enables seed production earlier in the season, giving the seed a greater growing period prior to their first overwintering, with the corresponding greater size and cold-tolerance again increasing survival^{69,135}. Increased sexual maturation rates also mean shorter conditioning cycles and greater larvae production for a given hatchery each year, increasing the total output of bivalve seed⁶⁹. As the supply of bivalve seed is one of the biggest factors limiting the growth of the bivalve industry, with demand far outstripping supply, microencapsulated feeds could play an important role in enabling the bivalve industry to expand^{33,69,135}. Key next steps towards reaching this goal will include further

tailoring of the microcapsule formation for increased protein content and use-specific size profile to maximise ingestion efficiency and bivalve growth.

Bivalve aquaculture is far more environmentally sustainable than other forms of aquaculture and meat production, and even some cereal crops^{19,31}. For every new tonne of protein that is produced from bivalve instead of fish aquaculture, we spare 9 ha land, 67 tonnes CO₂ emissions, and 40,000 litres freshwater³¹. Any technology, such as microencapsulated diets, that might enable bivalve aquaculture to grow instead of other aquaculture should be viewed as of great benefit and a worthwhile recipient of further research and industry attention. More widely, live algae remains a key component in the USD \$99.3 billion fish aquaculture industry, where application of microencapsulated diets would also be appropriate^{25,48,83}. New microencapsulated diets derived from aquacultural waste streams could contribute towards a step change in sustainable global food production through improved industry practices.

5.4. Methods

5.4.1. Microcapsule manufacture

Lipid-walled microcapsules containing 50% powdered *Schizochytrium* algae by weight were manufactured under patent by BioBullets (BioBullets Ltd, Cambridge, UK). To manufacture the particles a premix slurry containing a waxy encapsulant with antibacterial properties and powdered algae were prepared under conditions of controlled shear. The slurry was pumped into an ultrasonic atomizing nozzle at the top of a cooling chamber. The atomized particles formed near-perfect spheres as they cooled and fell to the chamber base. Further particle cooling was achieved with an air-conveying system before discharge via cyclone to a fluid bed processor. The encapsulated particles were then coated with a proprietary non-ionic surfactant to aid dispersion in water. Further cooling in the fluid bed removed all heat of crystallization from the microparticles before packaging³². All components of the formulation were food grade. The final microcapsules had a diameter between 20-140 µm, spherical shape, and near neutral buoyancy.

5.4.2. Broodstock conditioning

Conditioning experiments on *O. edulis* broodstock took place under commercial production conditions at SeaSalter Shellfish (Whistable) Ltd, Kent, England. Experiments took place over six-weeks between 29/03/2018 - 10/05/2018. These were carried out in three 25 L aerated flow-through tanks kept at ambient hatchery temperatures (18 - 24 °C) and salinities (26 – 28 ‰) – offering further commercial context to our experiments although presenting a limitation in the form of the necessity for pseudoreplication. Each tank contained 15 *O. edulis* broodstock and received one of the following three diets: live algae (SeaSalter's formulation), microcapsules (BioBullets), or algae + microcapsules. The nutritional profiles of each diet were obtained from the manufacturer and are shown in Table 5.1. Each feed was fed at 3% g dw feed g mean dw broodstock⁻¹ day⁻¹, meaning oysters on the algae + microcapsules diet received twice as much food as oysters on the single food diets. The 3% ration is recommended by SeaSalter and additional algal ration above this value has been shown to have little effect on *O. edulis* nutrient uptake or growth⁶⁹, ensuring that any differences in growth when microcapsules were added would likely be driven by improved nutritional value rather than increased ration. Feed was delivered using a continuous system with a flow-through rate of 10 ml minute⁻¹, with the feed lines discharging halfway to the base of each tank. At the end of the six-week conditioning period all broodstock were frozen and transported in cool boxes to the Department of Zoology, University of Cambridge, England, where they were then frozen at -80 °C. Before the conditioning period began an additional sample of 15 broodstock was also transported to Cambridge and frozen at -80 °C for use as pre-conditioning controls.

5.4.3. Gonad weight analysis

The entire gonad mass was dissected from nine oysters from each diet and the control sample, keeping the samples below 0 °C on dry ice. Gonad wet weight for each oyster was measured using calibrated Mettler Toledo AB54-S laboratory scales to a precision of ±1 mg. Gonad tissue was stored at -80 °C.

5.4.4. Fatty acid and lipid analyses

Reagents: Solvents were purchased from Sigma-Aldrich Ltd (Dorset, UK) of at least HPLC grade and were not purified further. Lipid standards were purchased from Avanti Polar lipids (Alabaster, AL; via InstruChemie, Delfzijl, NL) and used without purification.

Consumables were purchased from Sarstedt AG & Co (Leicester, UK) or Wolf Labs (Wolverhampton, UK).

Preparation of oyster gonads for extraction of the lipid fraction: The method used was newly developed for the oyster samples. Frozen adipose tissue was dispersed in an aqueous solution of guanidine and thiourea (6M/1.5M) ; 20× w/v) and diluted with methanol (30%) and TBME (10%). The dispersions were freeze-thawed and agitated before extraction of the lipid and glyceride fraction.

Extraction of the lipid fraction: The method used for extracting the lipid fraction was described recently ¹³⁶. Briefly, the solution of adipose (20 μ L, prepared as above) was injected into a well (96w plate, Esslab Plate+™, 2.4 mL/well, glass-coated) followed by internal standards (150 μ L, Mixture of Internal Standards in methanol (see table S3), water (500 μ L) and DMT (500 μ L, Dichloromethane, methanol and triethylammonium chloride, 3:1:0.005). The mixture was agitated (96 channel pipette) before being centrifuged ($3.2 \times g$, 2 min). A portion of the organic solution (20 μ L) was transferred to an analytical plate (96w, glass-coated, Esslab Plate+™) before being dried (N_2 (g)). The dried films were re-dissolved (TBME, 30 μ L/well) and diluted with a stock mixture of alcohols and ammonium acetate (100 μ L/well; propan-2-ol: methanol, 2:1; $CH_3COO.NH_4$ 7.5 mM). The analytical plate was heat-sealed and run immediately.

Mass spectrometry: In order to survey the profiles of glycerides, phospholipids and the fatty acids within phospholipids, we used a direct infusion mass spectrometry method developed recently ^{136,137} that was based on one that has been used in several studies of samples (infant formula ¹³⁶, human dried blood spots ^{138–141}, plasma ^{142,143} and serum ¹⁴⁴). The method consists of positive ion mode, negative ion mode and a negative ion mode with collision-induced dissociation. Positive mode processing used a deviations threshold of 9 ppm and a signal strength threshold of 2. Abundance/signal intensity was plotted using 25, 50, 100 % quality control (QC) samples and a correlation threshold of 0.75 was used. Variables with 0 % values across all samples were removed before the intensities were signal-corrected. Negative mode processing used a deviations threshold of 9 ppm. QC samples and a correlation threshold of 0.75 was used. A deviations threshold of 12.5 ppm was used for processing of the negative ionization mode with CID, on a list of fatty acids of chain length 14 to 36 with up to six olefin bonds and/or one hydroxyl group. All signals stronger than noise were carried forward. Signals consistent with fatty acids were found in 3/3 samples checked. QC samples consisted of a pooled mixture of aliquots of six samples

from across the study. QC concentrations of 0.25, 0.5 and 1.0 were used to assess the correlation between sample concentration and signal intensity.

5.4.5. Sexual maturation analyses

To assess sexual maturation all gonadal tissue was dissected from 5 oysters from each diet and the control. Tissue was fixed, sectioned, stained using hematoxylin and eosin, and imaged under light microscopy following a standard protocol ¹⁴⁵.

5.4.6. Data processing and statistical analyses

Power analyses were performed using G*Power (2018, Heinrich-Heine-Universität Düsseldorf, Germany) before experiments started, to ensure experimental designs and sample sizes were appropriate. For the lipid analyses Principal Component Analyses (PCAs) were carried out using Metaboanalyst 4.0 ¹⁴⁶ to identify which samples grouped together in a data-driven manner. To account for the difference in gonad mass between samples, abundance values from mass spectrometry data for fatty acids and lipids were scaled by gonad wet weight (Table S4). Statistical analyses of the data were then performed using R Statistics ¹⁰⁰. For the gonad weight analysis ANOVA with post-hoc Tukey's tests were used to compare gonad weight between the diets and control. For the fatty acid and lipid analyses ANOVA with post-hoc Tukey's test and a Holm-Bonferroni correction for multiple comparisons were used to assess differences in the abundance of each fatty acid or lipid between diets, relative to the control ¹⁴⁷. Molview (2015, Herman Bergwerf) was used to generate molecular images for Figure 5.3.

Table 5.1. Nutritional composition of broodstock diets: live algae, microcapsules, or live algae + microcapsules. All values are in g per 100 g dry weight (dw). Nutritional data is sourced directly from the manufacturer – for live algae SeaSalter Shellfish (Whistable) Ltd, for microcapsules BioBullets Ltd, and for the combined diet a mean is used.

Diet	Protein	Carbohydrate	Lipid	Ash and fibre	20:5n-3 (EPA)	22:6n-3 (DHA)
Live algae	31	12	13	44	2.31	0.70
Microcapsules	6	16	58	20	3.25	9.01
Live algae + microcapsules	18	14	36	32	2.78	4.85

5.5. Footnotes

Data availability: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.1038/s41598-020-69645-0>.

Author contributions: D.F.W. led the project and wrote the manuscript. D.C.A. contributed to literature search, study design, and interpretation. S.F. led the lipid analyses and interpretation. All authors gave final approval for publication.

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Chapter 6: Vitamin Bullets. Microencapsulated Feeds To Fortify Shellfish And Tackle Human Nutrient Deficiencies

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Over two billion people worldwide are micronutrient deficient, with regionally specific deficiencies. Fortification of food with micronutrients has become an industry standard for enhancing public health. Bivalve shellfish (e.g. oysters, clams and mussels) provide the most sustainable source of animal protein on the planet, and the market is rapidly growing – with production in China increasing 1000-fold since 1980 to an annual 36 kg capita⁻¹ consumption level. Bivalves are also unique in that micronutrients consumed at their end-life stage will be digested by humans, as humans consume the entire organism including the gut. We have developed a novel microencapsulated vehicle for delivering micronutrients to bivalves, tailored for optimal size, shape, buoyancy and palatability, demonstrating the potential of fortified bivalves to tackle human nutrient deficiencies. Oysters fed vitamin A and D microcapsules at a 3% initial dosage for just 8 hours had elevated tissue vitamin content. A serving of just two such bivalves provides enough vitamin A and D to meet human dietary RDAs. Scale-up of this technology and application to other bivalve species including clams and mussels could provide a low-cost and highly sustainable mechanism to contribute towards tackling nutrient deficiencies globally.

6.1. Introduction

The World Health Organisation (WHO) estimates over two billion people worldwide are micronutrient deficient¹⁴⁸. Vitamin A and D deficiencies are of particular concern¹⁴⁹, with 33 % of children and 1 in 6 pregnant women lacking sufficient vitamin A^{148,150}. Regional deficiencies can be especially pronounced. In Ghana more than 76 % of children are vitamin A deficient, causing widespread mortality and blindness^{148,149}. In India 85 % of citizens are vitamin D deficient, causing cardiovascular diseases, osteoporosis and rickets^{151–153}. Even in

the US over 40% of the population is vitamin D deficient ¹⁵⁴. Here we demonstrate a cheap and effective way of integrating micronutrients into the food supply, thus representing a highly efficient and attractive way to help tackle a major human health challenge ¹⁵².

Delivering micronutrients to the human population through animal products offers major advantages. Nutrients important to human health are less bioavailable in plants than meat, and rising atmospheric CO₂ content is reducing the absolute concentration of these nutrients in plants ^{10,11}. Nutrients consumed alongside the muscle and fat of an animal are also more bioavailable to the human digestive system than nutrients in a supplemental pill ¹⁵⁵. Fat must be present in the digestive tract for essential fat-soluble vitamins such as A, D, E, K and carotenoids to be absorbed, and muscle protein breakdown enhances absorption of key micronutrients including iron concurrently present in the gut ^{156–158}. In addition, alternatives such as vitamin supplements or fortified food condiments are often expensive and seen as a luxury by the people who really need them ¹⁵². Given that the global regions where vitamin deficiencies are most prevalent also tend to be the poorest, targeted integration of nutrients directly into the food supply (e.g. in rice and milk) has become important and commonplace. Costs are comparable or lower than providing a supplemental pill, and compliance is easier; poor consumers will continue to buy their now marginally more expensive food whereas they are unlikely to make an additional purchase to buy supplements ^{149,152,159}. However, current animal meat production methods are causing catastrophic environmental damage, driving 15 % of greenhouse gas emissions and widespread biodiversity loss ¹². There is an urgent need for a sustainable alternative.

Bivalve shellfish, such as clams, oysters, mussels and scallops, are a highly attractive yet underutilised food source with the capacity to provide the global population with key nutrients. Bivalves have a higher protein content than beef, are a rich source of omega-3 fatty acids, and have some of the highest levels of key minerals of all animal foods ¹³. They are also very sustainable to farm, having a far lower environmental footprint than animal meat or fish, and lower even than many plant crops such as wheat, soya, and rice ³¹. Bivalves are a highly affordable food source in nations where they are produced at large scale, such as China ⁴⁷. There is great potential to sustainably expand bivalve aquaculture worldwide, with over 1,500,000 km² available for sustainable low-cost industry development, particularly around the west coast of Africa and India ¹⁸. In areas including the Malabar and Goa coasts of India bivalves such as the green mussel (*Perna viridis*) are already staple foods for poor populations ^{47,160}. However, whilst bivalves are nutrient rich the level of nutrients

they deliver naturally is unlikely to solve global nutrient deficiencies. Innovations in bivalve production can change this.

The 'depuration' stage of bivalve production, during which bivalves are held in cleansing tanks for 48 hours after harvest, represents a unique opportunity for integrating nutrients into the bivalve gut and surrounding tissue. As humans consume the entire organism including the gut when they eat a bivalve, these nutrients will be available to humans¹⁶¹. In other animals, supplemental nutrients can be included into the feed, but this method is inefficient because feeds must be fed to animals for a far longer period of the animals' lifetime in order to generate elevated nutrient levels in the animals' tissue^{162,163}. Micronutrient fortification during the depuration stage could allow the levels of a specific nutrient such as vitamin A or D to be increased in the food supply to meet specific regional needs. As bivalves also tend to be consumed locally⁴⁷, this would be a highly efficient and targeted method to tackle nutrient deficiencies. There is however a need for a method to deliver micronutrients to bivalves during depuration.

Novel microencapsulated feeds developed through recent chemical engineering innovations can provide a delivery vehicle for micronutrients to bivalves²⁹. It has already been demonstrated that this form of microcapsules are digestible by bivalves and can improve bivalve growth and sexual maturation³²⁻³⁴. Mass production is simple and cost-effective^{29,32}, and the dry microcapsules have shelf lives in excess of one year in any sealed dry container (e.g. mylar bags) thus circumventing conventional feed wastage costs²⁵. Capsule characteristics are designed to maximise feeding efficiency²⁵ and minimize nutrient leaching to water^{53,67,83}. The specific nutritional content of the microcapsules can easily be tailored. For depuration, this makes it possible to create microcapsules containing only the micronutrients required by the human population for fortification, without any other food, minimizing the overall quantity of microcapsules required.

This investigation aimed to formulate and characterise a new form of micronutrient microcapsules, find out whether bivalves would consume them, and whether this would lead to elevated micronutrient levels in bivalve tissue. We also aimed to determine the optimum concentration and timeframe for delivering microencapsulated micronutrients to bivalves, and how the resultant micronutrient levels in bivalve tissue would compare to human Recommended Daily Allowances (RDAs) and other foods. Microcapsules fortified with vitamins A or D were selected as a case study, due to the prevalence of vitamin A and D deficiencies worldwide. Pacific oysters (*Crassostrea gigas*) were used as a case bivalve

species, due to their widespread popularity as a food source, worth \$ USD 6.7 billion in 2017⁴⁷. The natural diet of these oysters is phytoplankton between 10 – 400 μm ⁹⁷. Our target size microcapsule to develop was around $\sim 100 \mu\text{m}$ – small enough to avoid excessive rejection in psuedofaeces but with enough mass to allow relatively long retention times in the stomach⁹⁷. The microcapsules also needed to have a rough surface texture to facilitate uptake and a neutral or slightly negative buoyancy to maximise uptake into the inhalant current^{25,29}.

6.2. Materials and Methods

6.2.1. Microcapsule manufacture

Lipid-walled microcapsules containing vitamin A at retinyl acetate at 200 mg g⁻¹ or vitamin D as cholecalciferol at 20mg g⁻¹ were manufactured under patent by BioBullets (BioBullets Ltd, Cambridge, UK)^{156,164}. The remainder of the weight consisted the vegetable oil-based encapsulant and lipid-based bulking agents. To manufacture the particles a premix slurry containing the waxy encapsulant, bulking agents, and the powdered vitamin were prepared under conditions of controlled shear. The slurry was pumped into an ultrasonic atomizing nozzle at the top of a cooling chamber. The atomized particles formed near-perfect spheres as they cooled and fell to the chamber base. Further particle cooling was achieved with an air-conveying system before discharge via cyclone to a fluid bed processor. The encapsulated particles were then coated with a proprietary non-ionic surfactant to aid dispersion in water. Further cooling in the fluid bed removed all heat of crystallization from the microparticles before packaging. All components of the formulation were food grade.

6.2.2. Microcapsule characterization

Scanning Electron Microscopy (SEM) was used to examine the morphology of complete vitamin A and D microcapsules, and microcapsules freeze-fractured using liquid nitrogen and a cold hammer. The entirety of a 1 g sample was mapped for each vitamin, and then a representative selection of SEM images were taken using an FEI Quanta 650F (Thermo Fisher Scientific, USA) under high-vacuum and 3kV. A Malvern Mastersizer 3000 (Malvern Panalytical, UK) was used to assess the particle size distribution of microcapsules. Five samples of both vitamin A and D microcapsules were analysed. The Mastersizer 3000

generated fitted size distribution curves for each microcapsule type, alongside mean particle size and residual standard deviation.

6.2.3. Bivalve nutritional fortification

Bivalve nutritional fortification experiments were undertaken at the University of Cambridge UK in December 2019, under conditions to simulate commercial depuration protocols. Experiments were carried out in a controlled temperature room held at 15 °C, in constantly aerated tanks each containing 1 L of artificial seawater at salinity 30‰ (H2Ocean Aquarium Salt, D-D The Aquarium Solution Ltd., UK) ^{69,161}. Each tank contained one adult *Crassostrea gigas* oyster, size grade AA, received directly from commercial depuration tanks at Colchester Oyster Fishery, UK. The mean dry weight (dw) of these grade AA oysters was obtained from 20 samples at 1.88 ± 0.11 g. Each oyster was fed a 50 : 50 blend of both vitamin A and vitamin D microcapsules at doses and timeframes feasible during the 48-hour depuration period. There were 105 individual tanks, allowing for 5 biological replicate oysters to be fed microcapsules at doses of 3, 6, and 9 % ⁶⁹ dw feed per dw oyster over 2, 4, 8, 16, and 32 hours ¹⁶¹, alongside 0 and 32 hour controls at doses of 0 %. Feed concentrations refer to the initial quantity of feed given at time = 0, no feed was added to the tanks during the remainder of the course of the experiments. At the end of each timeframe, each oyster was immediately removed from its tank. Oysters were then shucked and any water inside the shells was drained off. The entire soft tissue of each oyster was then removed and frozen at -80 °C ⁸⁰.

6.2.4. Bivalve vitamin A and D analysis

The total vitamin A and D content of entire oyster soft tissue samples was measured by a UKAS accredited analytical service (Premier Analytical Services (PAS), UK). PAS are also regulated by external quality performance testing (FAPAS and LGC schemes) to demonstrate the accuracy of their results. Samples were delivered to PAS from Cambridge within 4 hours under dry ice. All five biological replicates for each dose and timeframe sample type were pooled into a single compound sample during the analysis. Each sample run included a control sample with established control limits that had to be met for the run to be passed, alongside spiked samples for which the recovery of these also had to be within acceptable limits.

Vitamin A was determined as the sum contribution of retinol and carotenes, and the limit of detection (LOD) was $10 \mu\text{g } 100 \text{ g}^{-1}$. Measurement of retinol followed UKAS protocol C-TM-021; retinol was saponified with alcoholic KOH and extracted into hexane, then the cis and trans isomers were determined using High-performance Liquid Chromatography (HPLC) with UV detection at 325nm¹⁶⁵. Measurement of carotenes followed UKAS protocol C-TM-087; samples were saponified with alcoholic KOH and carotenes extracted into hexane, then the alpha- and β -carotenes were determined using reverse-phase HPLC with visible detection¹⁶⁶. Vitamin D was determined as the sum of vitamins D2 and D3 following UKAS protocol C-TM-273, and the limit of detection was $0.3 \mu\text{g } 100 \text{ g}^{-1}$. Vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) were saponified with alcoholic potassium hydroxide and extracted into hexane/diethyl ether, then the vitamin D2 and D3 were measured using HPLC with UV detection¹⁶⁷.

The output data consisted of a single compound measurement of vitamin A or D for each dose and timeframe sample type. Relative uncertainty in the measurements was calculated as $2 \times \text{standard deviation} / \text{mean value}$ from quality control tests run immediately before our sample set. The relative uncertainty (RU) for the vitamin A data points was 12.6 % and for the vitamin D data points 19.6 %. A statistical analysis was not appropriate as biological replicates were pooled for analysis to give the single compound measurement for each sample type. Pooling was necessary due to limits of detection and practical constraints, and followed a widely used approach for such analyses^{168–170}. Dose response curves were then plotted for both the vitamin A and D microcapsules (Figure 6.3.). The yield, or percentage of microcapsules in the oyster sample in relation to the total amount in the tank, was also calculated for each oyster sample (Supplementary Information; Figure 6.3. Raw Data).

6.3. Results

6.3.1. Characteristics of micronutrient microcapsules

Micronutrient microcapsules containing vitamin A or vitamin D were successfully produced and established to have generally homogenous morphology. Scanning Electron Microscopy (SEM) analyses revealed the microcapsules to be of a consistent spherical shape

(Figure 6.1a, b.). Closer examination of the particles showed a roughened surface to the spheres (Figure 6.1c, d.), and imaging following freeze-fracture confirmed the interior of the capsules to be solid without large air pockets (Figure 6.1e, f.). The particles were of neutral buoyancy in saltwater. Laser diffraction particle size analysis indicated that the majority of vitamin A and D microcapsules fell within a size range of 50 to 200 μm diameter. Vitamin A microcapsules had a mean diameter of 120 μm (Residual Standard Deviation (RSD) 0.4 μm) (Figure 6.2., blue line), and vitamin D slightly larger with a mean diameter of 134 μm (RSD 0.4 μm) (Figure 6.2., red line). For both vitamin A and D microcapsules, there were peaks in particle abundance around 0.5 and 10 μm , but these were very small compared to the main peaks of 50 – 200 μm microcapsules.

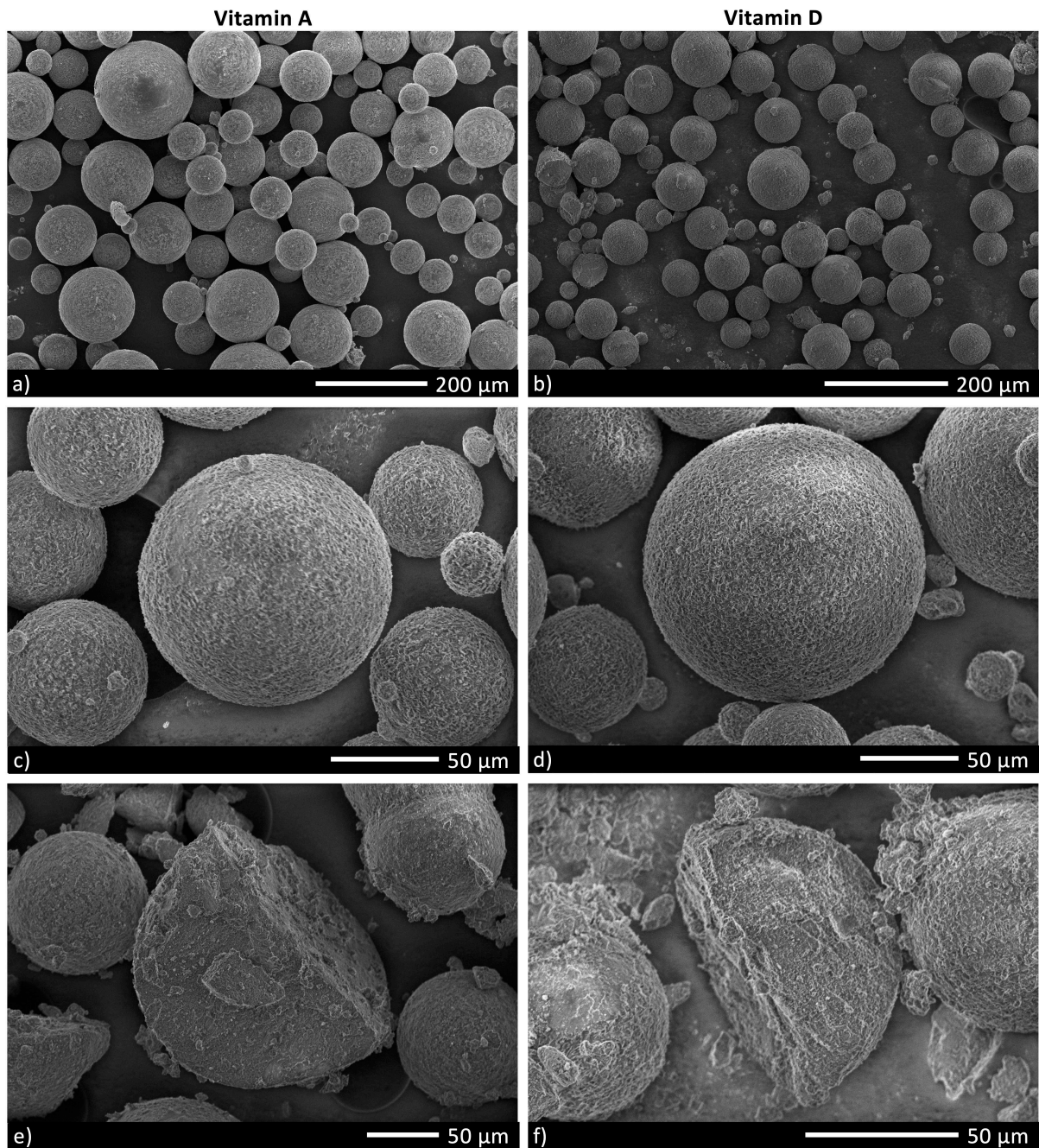


Figure 6.1. Scanning Electron Microscopy (SEM) images of Vitamin A and D microcapsules. (a) and (b) demonstrate the typical variation in morphology in a sample of microcapsules. (c) and (d) are close-up images of individual microcapsules. The microcapsules in (e) and (f) have been freeze-fractured to visualise internal structure.

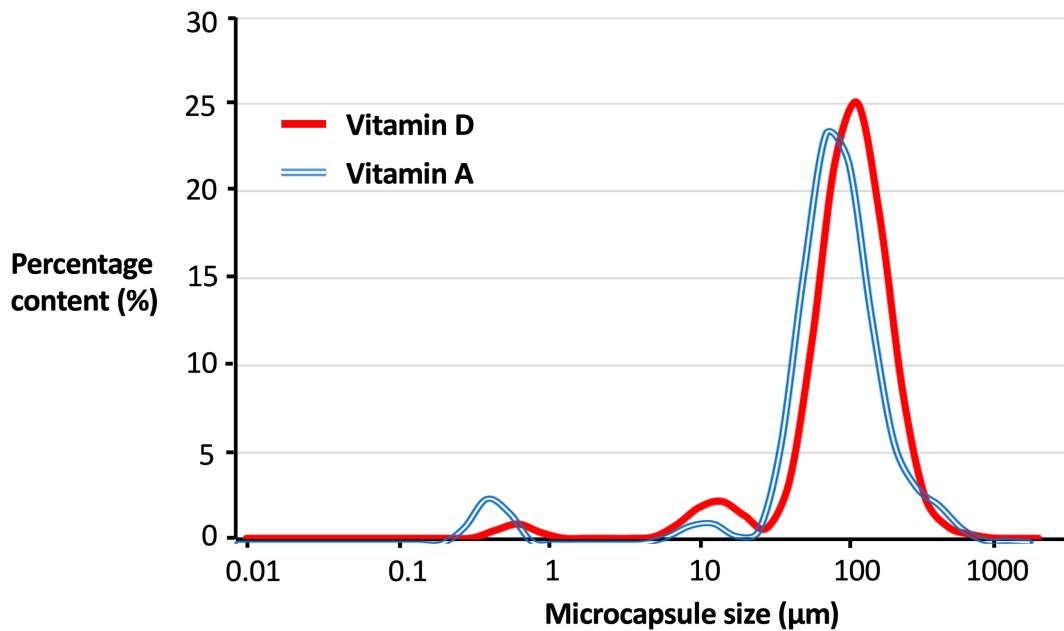


Figure 6.2. Particle size distribution of vitamin A and D microcapsules. Curves plotted are fitted regressions from a Malvern Mastersizer 3000 (Malvern Panalytical, UK) based off 5 individual samples. Percentage content (%) is by number of particles. For Vitamin A Residual Standard Deviation (RSD) = 0.4, mean microcapsule size = 120 μm . For Vitamin D RSD = 0.4, mean microcapsule size = 134 μm .

6.3.2. Nutrient fortification

Pacific oysters successfully consumed microcapsules and this resulted in elevated micronutrient levels in whole-organism tissue samples. In general, increasing the microcapsule concentration and feeding timeframe resulted in higher micronutrient levels in oyster tissue relative to 0 % feed concentration controls. This relationship was not completely linear, although the patterns for vitamin A and vitamin D microcapsules were the same (Figure 6.3.). The relative uncertainty (RU) for vitamin A data points was 12.6 % and for vitamin D 19.6 %.

For 3 % feed concentrations, oyster vitamin A and vitamin D levels after 2 hours were 81 and 8.1 $\mu\text{g } 100\text{g}^{-1}$ respectively. At longer timeframes micronutrient levels increased, with the greatest change in micronutrient levels occurring when moving from a 4 to 8-hour timeframe. Micronutrient levels peaked at 997 $\mu\text{g } 100\text{g}^{-1}$ for vitamin A after 8 hours and at 57 $\mu\text{g } 100\text{g}^{-1}$ for vitamin D after 16 hours. At these peaks the percentage of microcapsules in the oysters in relation to the amount added to the tanks (i.e. yield) was 89 % for vitamin A

and 51 % for D. After 32 hours levels of both vitamins were lower, at 389 $\mu\text{g } 100 \text{ g}^{-1}$ for vitamin A and 39 $\mu\text{g } 100 \text{ g}^{-1}$ for vitamin D.

Oyster micronutrient after 2 hours for the 6 % feed concentration were similar to the 3 % feed concentration, at 52 and 6 $\mu\text{g } 100\text{g}^{-1}$ for vitamins A and D respectively. However, by 8 hours vitamin levels in the oysters on the 6 % feed were less than half that of oysters on 3 %, at 375 $\mu\text{g } 100\text{g}^{-1}$ for vitamin A and 23 $\mu\text{g } 100\text{g}^{-1}$ for vitamin D. For the 6 % feed micronutrient levels did not reach their maximum until the 32-hour mark, at 560 and 79 $\mu\text{g } 100\text{g}^{-1}$ for vitamins A and D respectively. At this point the yield for Vitamin A was 25 % and for vitamin D 35 %.

The relationship between feeding timeframe and micronutrient levels was broadly similar for oysters on the 9 % feed compared to oysters on the 6 % feed. Again, micronutrient levels at 8 and 16 hours were lower on the 9 % feed than on the 3 % feed, and on the 9 % feed micronutrient levels did not peak until the 32-hour mark, with yields of 28 and 19 % for vitamin A and D respectively. The exception was at the 2-hour timeframe, where levels of vitamin A at 327 $\mu\text{g } 100\text{g}^{-1}$ and vitamin D at 25 $\mu\text{g } 100\text{g}^{-1}$ were markedly higher than levels on the 3 and 6 % feeds.

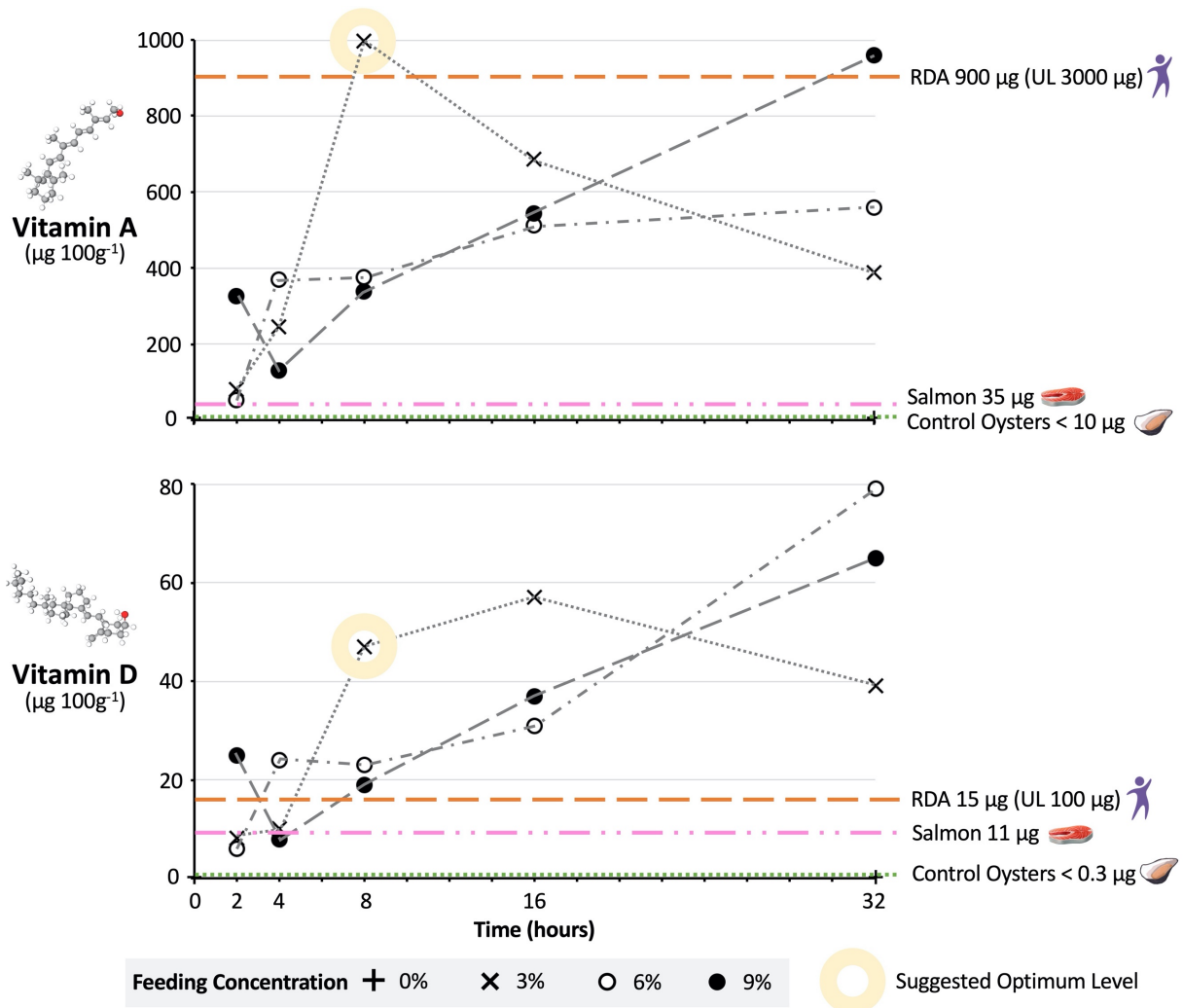


Figure 6.3. Nutritional uplift of Vitamin A and D in oysters fed fortified microcapsules.

Pacific oysters were fed vitamin A and D fortified microcapsules at 3, 6, and 9% dry weight feed per dry weight oyster feeding levels, over time periods of 2, 4, 8, 16, and 32 hours.

Individual data points are compound analysis values from 5 oysters individually fed in separate tanks. The relative uncertainty for vitamin A data points is 12.6 % and for vitamin D

19.6 %. Vitamin levels in μg are per 100g of wet oyster. RDA: Recommended Daily

Allowance. UL: Upper Daily Limit ¹⁷¹. RDA assumes 100g portion of oyster meat consumed.

Vitamin values for salmon and control oysters are per 100g wet tissue ¹³. UK and US

regulations respectively stipulate minimum 42- and 44-hour depuration periods for bivalves

6.4. Discussion

Microcapsules were developed with appropriate properties to achieve efficient capture and digestion by filter feeding bivalves. The consistent spherical morphology and size range of 50 to 200 μm , were of a shape and size that *C.gigas* could harvest from the water⁹⁷. For both vitamin A and D microcapsules, particles at the peaks around 0.5 and 10 μm likely represent ingredient fragments which can be seen on close inspection of the SEM images (Figure 6.1a, b.). The scarcity of these fragments confirms high purity in the microcapsule samples. The roughened surface structure of the microcapsules will likely have improved their palatability to bivalves²⁵, and the lack of air pockets helped ensure neutral buoyancy so that the particles remained at the appropriate position in the water column for filter feeders to access²⁹. These physical properties made the microcapsules an ideal delivery vehicle for the micronutrients in this study and the key component in allowing us to nutritionally fortify bivalves.

Feeding micronutrient microcapsules under depuration conditions led to successful fortification of bivalves, and we suggest that for vitamins A and D an optimum dose regarding feed concentration and timeframe might be 3 % for 8 hours. After an 8-hour timeframe, vitamin A and D levels in oysters were higher on the 3 % feed than on the 6 % or 9 % feed. This relationship is less surprising than first appears; when bivalves are exposed to too much food they will reduce their feeding rate to avoid overloading the filtering system on their gill stacks⁹⁷. The only other feed concentrations and timeframe that resulted in comparable vitamin levels to 3 % at 8 hours were 6 and 9 % at 32 hours. Feeding at this higher dosage would however not be optimal, representing a wasteful and excessive use of feed resources to achieve a very marginal further increase in oyster vitamin levels. This is demonstrated by the lower yields of the 6 and 9 % treatment at 32 hours relative to the yield of the 3 % treatment at 8 hours. We note that the drop-off in micronutrient levels after 32 hours for the 3 % feed is likely occurring as by this point the oysters have depleted the microcapsules in the tank, and are digesting and excreting the excess vitamin A and D they do not need⁵⁶. We therefore suggest that if an 8-hour fortification period is used it should be performed at the later stages of depuration to reduce the risk of bivalves excreting nutrients in faeces. Optimising concentration and timeframe are clearly important in ensuring efficient use of resources.

Oysters fortified with vitamins A and D at 3 % for 8 hours also performed well regarding nutritional value when compared to other foods and the RDAs, providing further support to our suggested optimum dose. In a small portion (100g, or 3 small or 2 large oysters) of oysters fortified at the 3 % 8-hour dosage, vitamin A and D levels were 997 and 47 $\mu\text{g } 100\text{g}^{-1}$ respectively. This exceeds the levels in natural oysters (< 10 and $< 0.3 \mu\text{g } 100\text{g}^{-1}$). More importantly, it far exceeds the levels found in one of the best natural sources of vitamin A and D; salmon (37 and 11 $\mu\text{g } 100\text{g}^{-1}$, Figure 6.3.). Given the highly unsustainable nature of salmon farming relative to bivalve farming and the destructive impact salmon production is having on the environment ²⁴, this offers promise for using bivalves as a planetary health food – good for people and good for the planet ¹. In addition, a 100g serving of oysters fortified at 3 % 8-hours meets US Department of Health RDAs for vitamin A and D (without exceeding Upper Daily Limits (UL)) ¹⁷¹. Based upon predicted manufacturing, distribution and implementation costs for the microcapsules, fortification would add just \$0.0056 to the cost of a single oyster, which could readily be recuperated through a small additional increase ($\sim 0.9\%$) in oyster retail price. This offers strong hope – for people in deficient populations just two fortified oysters a day could provide them with all their vitamin A and D needs in a highly bioavailable form ¹⁵⁵.

6.4.1. Future Prospects

Looking forwards, there are important steps that can be made by the research and industrial community in order to realise the potential of bivalves and microencapsulation innovations to help tackle micronutrient deficiencies worldwide. Researchers will need to carry out larger laboratory studies with a greater number of replicates to enable quantitative analysis of the individual variation in vitamin uptake by bivalves; such variation is often seen in the fortification of foods including eggs and meat via dietary intervention ¹⁷². There is also a need to assess the bioaccumulation of microencapsulated vitamins specifically into bivalve storage tissues, the impact of high-level vitamin accumulation on bivalve physiology, and whether the presence of microcapsules in the bivalve gut promotes the micellarisation and absorption of vitamins in the human gut. There is hence a need for proof of concept trials on humans. Future studies would need to feed fortified bivalves to human participants and assess the impact on physical health and blood markers, to establish the true bioavailability of the initially microencapsulated micronutrients to people.

At an international scale, there will be a requirement to tailor the selection of vitamins encapsulated and the microcapsule dosage given, in order to apply the technology to global regions with specific nutritional deficiencies or food consumption patterns. Despite the increased cost of fortified oysters relative to conventional oysters being small (0.9 %), and the falling price of oysters with new breeding innovations and the use of fast growing triploids, oysters remain one of the more expensive bivalves ¹². It will therefore also be crucial to apply the technology to other bivalve species including mussel and clam species such as *Perna viridis* and *Ruditapes phillipinarum* which are cheaper to farm in many developing regions ¹². Completion of these steps will help enable scale-up of micronutrient fortified microcapsules at the commercial level.

There are major economic, sustainability, and health wins that can be made from integrating micronutrient fortified bivalves into our global food system. The ability to use tiny doses of microcapsules to fortify a food organism at its final life stage has major cost advantages. It represents a cheaper option than attempting to fortify other terrestrial animals or fish, which need to be fed fortified feeds for a greater period of their lifespan. Bivalves are also the most sustainable animal food on the planet, with farming having important ecosystem benefits ³¹, so there are conservation gains that could be made from bivalve aquaculture expanding in place of other meat production. Most importantly, microencapsulated micronutrients combined with bivalve aquaculture can act as a next-level tool to target and tackle nutritional deficiencies worldwide. Just two fortified bivalves a day has the potential to contribute towards saving and improving the lives of over 2 billion people worldwide.

6.4.2. Conclusions

In summary, this study marks the first successful fortification of bivalves with micronutrients beneficial to human health, using a novel microencapsulated feed supplied at the depuration stage of production. The microcapsules were tailored for optimal size, shape, buoyancy and palatability to maximise uptake by bivalves. Pacific oysters were selected as a case species, due to their sustainable production and economic importance as the most widely cultivated bivalve globally. Our study found that oysters fed vitamin A or D microcapsules at a dose of 3% over 8 hours had increased vitamin content, to the extent that two such oysters would provide enough vitamin A and D to meet human dietary RDAs.

Fortification at this level would be highly cost effective and offset by a small (0.9 %) increase in retail price.

Further research studies and industry trials are warranted in order to realise the potential benefits of fortified bivalves to the global food system. These can allow us to gain a greater understanding of the inter-individual variation in micronutrient accumulation by bivalves, the bioavailability of delivered nutrients to humans, and the optimum combination of bivalve species, encapsulated nutrients, and fortification dose to help tackle nutrient deficiencies in specific global regions. Taking these steps can provide stakeholders in aquaculture to make an invaluable contribution towards improving the quality and sustainability of our global food system.

6.5. Footnotes

Data availability: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.3389/fnut.2020.00102>.

Author contributions: D.F.W. led the project and wrote the manuscript. D.C.A. contributed to study design, interpretation and reviewed the manuscript. Both authors gave final approval for publication.

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Chapter 7: Sustainable bivalve farming can deliver food security in the tropics

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Bivalve shellfish represent a nutritious and low-impact food source that is underutilized. New innovations in production in this sector could fulfil the protein needs of nearly one billion people in the most vulnerable global regions.

7.1. Introduction

Suboptimal global food production is directly related to poor diets, nutrition-related disease and environmental pressure. The tropical regions bear the brunt of this crisis, concentrating the fastest population growth ¹⁷³ and the greatest problems related to food production, distribution, and loss. Over-reliance on processed food imports is also driving a rapid increase in obesity in the developing tropics relative to the Global North ¹⁷⁴.

Livestock meat, though nutrient rich, has a limited potential to solve these issues. Current production methods are unsustainable, and consumption must be halved by 2050 to avoid a catastrophic overstep of planetary environmental boundaries ¹. Sustainable diets are primarily plant-based ¹, yet many nutrients vital to human health are far less bioavailable in plant crops than meat ¹ while rising CO₂ is dramatically reducing the absolute content of these nutrients in plant crops ¹⁰. Without new food sources and productivity increases, tropical regions may be forced to open up new land to unsustainable agricultural development, or face economic debt and public health problems through unsustainable food imports ¹⁷⁵.

Bivalve shellfish aquaculture represents a key opportunity for sustainable diets, and has been identified as an alternative to fill the gap left by livestock meat ^{1,19,31}. Bivalves, which include clams, oysters, mussels, and scallops, have a higher protein content than many meats and plant crops, high levels of essential omega 3 fatty acids, and micronutrients such as zinc, iron, vitamin A and vitamin B12 ³¹ (Table 7.1.). Bivalve farming also has a

smaller environmental footprint than most other foods, using up almost no land or freshwater, relying on seawater instead, having lower carbon emissions than many cereal crops, and helping to restore and protect coastal ecosystems ³¹ (Table 7.1.). Bivalve reefs (and bivalve farms, during the period between harvests) can buffer estuaries and coastal waters against phytoplankton blooms caused by anthropogenic nitrogen loading, increase water clarity, provide a nursery habitat for fish, provide coastal flood and storm protection, and shell production acts as a form of carbon capture ¹⁷⁶. The ecosystem services yielded from bivalve aquaculture are currently estimated at \$30.5 billion per year and only set to grow as the industry expands ¹⁷⁶. From a nutritional and environmental standpoint, bivalve shellfish represent a promising choice for farmers in the coastal tropics (Table 7.1.).

Table 7.1. Nutritional properties and environmental footprints of selected food items that can be farmed in the tropics. Bold values indicate that bivalves provide maximal nutritional value for minimal environmental footprint. Environmental footprints are based on today’s production methods and fresh consumption of bivalves. Production intensification and increased food processing are expected to increase footprint values, but sustainable development methods could minimize environmental costs. IU, international unit. Data on beef, pork, chicken, tilapia, bivalves and shrimp were obtained from ref. ¹⁹, data on rice, soya and wheat were obtained from refs. ^{4,20}, and ref. ¹³ was used for unit conversion.

	Beef	Pork	Chicken	Tilapia	Bivalves	Shrimp	Rice	Soya	Wheat
Nutrient									
Protein (mg kcal ⁻¹)	98	64	121	205	150	242	19	88	33
Omega 3 (mg kcal ⁻¹)	0.5	0.3	0.7	1.9	4.8	0.3	0.1	2.6	0.2
Iron (µg kcal ⁻¹)	10.1	3.3	5.7	5.4	34.3	5.2	11.8	24.1	9.9
Zinc (µg kcal ⁻¹)	23	8	10	3	61	17	3	7	8
Vitamin B12 (ng kcal ⁻¹)	10	3	4	15	126	15	0	0	0
Vitamin A (IU kcal ⁻¹)	0.1	0.1	0.1	0.1	2.30	0.51	0	1.2	0
Environmental footprint									
Land use (ha per t protein)	50	2	3	7.5	0	16.4	21.2	0.578	4.62
Greenhouse gas emissions (tCO ₂ per t protein)	337.2	57.6	42.3	40.7	11.1	161.7	2.36	1.04	3.54
Freshwater use (m ³ per kg protein)	112.5	56.5	34.3	15.9	0	4.4	19.81	5.76	11.84
Eutrophication potential (kg P per t protein)	180	120	40	82	-148	104	109	17.8	30

There is great potential to expand bivalve aquaculture in the tropics. Today, bivalve production in tropical regions is almost non-existent, providing just over 2 Mt of meat annually ^{13,47} (Figure 7.1.). Low levels of knowledge, funding, infrastructure, and consumer acceptance are key contributing factors. Yet, worldwide, over 1,500,000 km² of currently undeveloped coastlines are suitable for productive bivalve aquaculture, two-thirds of which

are in the tropics (Figure 7.1.)¹⁸. If just 1% of the area in the topical regions alone would be developed, over 120 Mt of bivalve meat could be produced annually – enough to satisfy the protein demands of approximately 715 million people (Figure 7.1.). Production costs are also relatively low, making bivalve farming an accessible venture for both large businesses and also small-scale farmers in the developing world^{116,177}. The economic viability of bivalve farming in a rapidly developing nation has been proven in China, which begun extensive bivalve farming in the 1950s and now yields over 85% of global production¹¹⁶. Mirroring this development in the rest of Asia, Africa, the Americas and Oceania could generate considerable human health and environmental benefits.

To meet and expand its potential, the bivalve industry in tropical regions requires development across the entire value chain. As discussed below, major challenges must be overcome across hatchery, grow-out and depuration stages of production, as well as in infrastructure and consumer marketing – but innovations and technologies can turn these challenges into exciting opportunities for success.

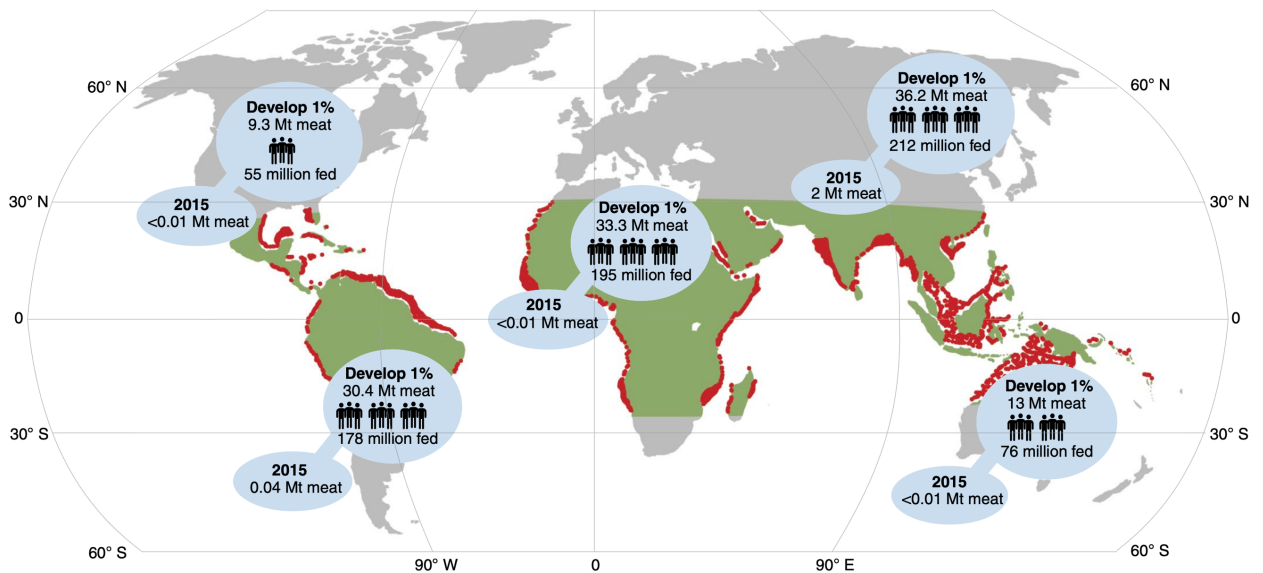


Figure 7.1. Potential for bivalve coastline production in the tropics. Areas of coastline suitable for the development of productive bivalve farming shown in red were determined based upon the satisfaction of physical factors – that is depth (< 200m), chlorophyll *a* concentration (annual mean > 2 mg m⁻³) and oxygen concentration (> 1.99 mg l⁻¹) – and the exclusion of areas dedicated to activities such as shipping and oil rigs. The tropical zone is shown in green. Blue circles show the quantity of bivalve meat produced in 2015 (in Mt), the potential additional quantity if 1% of the suitable coastline that is not yet in use was developed (in Mt), and the number of people (in millions) that could potentially be fed on bivalves as their only protein source for each of the following regions: Central America, South America, Africa, Asia, Oceania. Data on coastline suitability including physical factors and exclusion zones were obtained from refs. ^{18,178}, data on 2015 production were obtained from ref. ⁴⁷, and projection data were calculated using refs. ^{13,18,179}.

7.2. Hatcheries

The need for research and investment in hatchery systems represents a major challenge for establishing bivalve aquaculture in the tropics. Lack of seed (juvenile bivalves) is severely constraining industry expansion ¹¹⁶. Seed from natural reefs is in very limited supply and its collection has detrimental ecosystem impacts, making hatcheries crucial for seed provision ¹¹⁶. Research and breeding programmes akin to those performed in China for the Pacific oyster *Crassostrea gigas* are needed to produce broodstock with high reproductive output and resulting good quality seed (that is, triploid genetics for faster

growth and enhanced disease resistance¹⁷⁷ and other desirable characteristics). There is a particular need for greater knowledge and expertise in breeding species such as *Perna perna*, *Perna viridis*, *Crassostrea gasar* and *Ruditapes decussatus*; these are suitable species to farm in unexploited tropical areas and offer high potential productivity (Box 7.1.). Hatcheries also require affordable and sustainable feed for juveniles and broodstock. Current methodologies make inefficient use of natural and economic resources, and only minor development has occurred since the 1990s; the live microalgae used today is disease prone and of variable quality, energy-intensive to grow and accounts for 50% of hatchery costs^{25,31,33,67}. Investment in grow-out, distribution, and marketing of bivalves is all at risk without industry investment in hatcheries that underpins the production process⁵⁷.

Solutions that can provide investment, high quality broodstock, and feed for hatcheries are emerging. Overseas investment from private companies such as China's Tongwei Co, the nation's industry leader in aquaculture for 20 years, could play an important role in establishing hatcheries in the tropics. From 2005-2018, China invested US\$300 billion in Africa (primarily into the production of arable crops, cattle, poultry and enabling resources including power and infrastructure), and has indicated that additional investment could go towards bivalves^{19,180}. Better collaboration of bivalve hatcheries with the rest of the seafood industry can also increase resource use efficiency and reduce costs; a working example is the Chinese National Fisheries Corporation who implement legislation across the entire value-chain⁶⁹. Advances in DNA analysis and increasing affordability can enable accelerated selective breeding programmes in tropical areas in need of rapid aquaculture development, including sub-Saharan Africa¹⁹. Additionally, innovations in algal production and microencapsulation technology can provide sustainable low-cost feed for hatcheries. Recently developed microencapsulated diets containing *Schizochytrium* algae grown on food waste have facilitated accelerated bivalve growth and sexual development with a 100-fold reduction in energy usage and costs relative to conventional live algae hatchery feeds^{31,33}.

7.3. Grow-out

Productive bivalve farming in the tropics requires the identification and management of suitable grow-out areas. High levels of primary production or eutrophication from nutrient runoff are needed to support bivalve filter feeding and growth, yet many tropical

waters are relatively oligotrophic, thus making these resources less available ^{18,116}. In some areas, integrated multi-trophic aquaculture can provide additional but not always adequate resources, and the availability of these resources is also dependent on water exchange rates ¹⁸¹. Waste streams from urban and industrial areas can provide a further source of nutrients, but without adequate treatment hazardous substances such as cadmium, lead, and microplastics can accumulate in bivalves intended for food ^{182,183}. Careful consideration is also needed regarding species selection for grow-out, as production and conservation interests can conflict. For example, *C. gigas* has received investments in production efficiency and now dominates global oyster production, yet must be managed carefully so that it does not displace native species and modify natural ecosystems ¹⁸⁴.

Careful site selection and management can enable expansion of bivalve grow-out in the tropics. Regions of West Africa, South Asia and South America have particularly good potential (Box 7.1.), with mean annual Chlorophyll *a* concentrations above 2 mg m⁻³ to support bivalve grow-out, alongside additional nutrient sources from human activities ^{178,179}. Knowledge exchange with established grow-out operators in the United States, Western Europe, and China could help farmers with poor experience in these new grow-out regions improve the economic viability of their own farms ⁶⁹. Offshore production methods such as longlines can be chosen to reduce accumulation of hazardous pollutants by bivalves, as many pollutants are most concentrated nearshore to urban areas ^{179,183}. Regulatory approaches, such as the marine functional zoning already used in China, can further reduce food safety concerns and minimise conflict between bivalve aquaculture and other activities (such as the oil industry in Venezuela ¹¹⁶). Increased hatchery breeding and grow-out of native species could reduce risks of ecosystem modification from farming non-natives (Box 1) ⁵¹. In addition, to ensure that the ecological carrying capacity of a given region is not exceeded when developing 1% of the potential productive coastline for bivalves, it may be pertinent to distribute development across the entire tropics rather than focussing on one given region ^{18,51,116}. This would likely result in increased infrastructure costs, but may still be favourable for small-scale fisheries and in supporting local livelihoods.

Box 7.1. Opportunities for bivalves in the tropics

Each of the coastal regions presented has a mean annual Chlorophyll *a* concentration above 2 mg m⁻³, suitable to support highly productive bivalve grow-out. Production data in 2017 were obtained from ref. ⁴⁷. 'Additional nutrients' refer to sources of N and P that can support phytoplankton proliferation and bivalve growth, with data obtained from refs. ^{18,178,179}. Suitable species' are bivalve species that would be most economically feasible and environmentally appropriate to farm in the given region, with data obtained from ref. ¹⁷⁹. Considerations on food safety and socioeconomic factors were taken from refs. ^{116,173,177,183}, with 'GDP per capita' referring to the gross domestic product per person in 2015 ¹⁷³.

East Asia (China)

Production in 2017: 14.6 Mt (85% of global bivalve production)

Additional nutrients: integrated multi-trophic aquaculture.

Suitable species: *C. gigas*, *P. viridis*, *Ruditapes phillipinarum*, *Siliqua patula*.

Food safety: marine functional zoning), bivalve meat bacterial load regulations.

GDP per capita: US\$10,000.

Socioeconomic considerations: in 1960 GDP per capita was US\$100 (current equivalent US\$) and bivalve consumption 4.8 kg per capita. Heavy investment in hatcheries and breeding followed; by 2015, consumption reached 35.7 kg per capita.

West Africa (Sierra Leone, Senegal)

Production in 2017: 563 t

Additional nutrients: by 2050 agricultural N & P runoff will triple, and intervention is needed to avoid eutrophication.

Suitable species: *R. decussatus*, *C. gasar*, *Mytilus edulis*.

Food safety: Sewage runoff is increasing, and improved waste infrastructure and food safety regulations are needed.

GDP per capita: < US\$1,000.

Socioeconomic considerations: facilities and marketing investment is required and available – since 2005 China invested US\$300 billion in Africa in food and resource production with further investment planned.

South America (Venezuela)

Production in 2017: 8 t

Additional nutrients: industrial and urban discharge.

Suitable species: *P. perna*, *C. gigas*, *Crassostrea rhizophorae*.

Food safety: marine functional zoning and regulation on oil spill detection and food safety required due to active oil industry.

GDP per capita: US\$10,000.

Socioeconomic considerations: competing marine shrimp industry is already lucrative and productive, but combined coastal shrimp and bivalve farming could tackle eutrophication.

South Asia (India, Myanmar)

Production in 2017: 13,000 t

Additional nutrients: discharge from agriculture on the Ganges delta, and intensive shrimp aquaculture.

Suitable species: *Crassostrea madrasensis*, *P. viridis*, *C. gigas*.

Food safety: nearshore plastic and sewage pollution means offshore farming is preferable over intertidal farming.

GDP per capita: US\$1000-2000.

Socioeconomic considerations: potential conflicts with the active fishing industry, but current fishing infrastructure could help service offshore farms.

7.4. Depuration

Bivalve food safety is a major hurdle. Even if human-derived pollutants are avoided through careful grow-out site selection, there is still potential for toxic cyanobacterial blooms or other bacteria to contaminate food¹⁸⁵. Depuration facilities, where bivalves are held for at minimum 48 hours after harvest in clean water, play a crucial role in ensuring food quality – but are lacking in regions trying to develop bivalve aquaculture such as Africa and India¹¹⁶.

Established and emerging methodologies can provide bivalve food safety solutions. Information transfer and investment from the European Union, US, and Australia could accelerate depuration facility development in the tropics – for example introduction of low-cost solar powered ultra-violet depuration systems¹¹⁶. Surveillance programmes such as

those used in the US can monitor toxic algal blooms and enforce increased depuration times when contamination occurs¹⁸⁵. The use of probiotics and antimicrobial peptides such as *Phaeobacter inhibens* and tachyplesin during depuration are new potential approaches for tackling bacterial contamination^{91,93}. In areas where funds are more limited, establishment of food safety monitoring programmes in relevant culture areas, as has been done through China's 2009 food safety legislations, may be the most efficient approach to cover multiple forms of contamination¹⁸⁶. A thorough economic assessment would still be required for any target region since each method may increase production costs and, if designed improperly, create a production bottleneck.

7.5. Infrastructure

The upscaling of bivalve aquaculture in the tropics and the establishment of necessary infrastructure raise important challenges. Increasing production will require significant investment in facilities, including harbours, transport, cooling and processing – and may be amplified in the tropics as high ambient temperatures and humidity mean food spoils more rapidly^{57,173,187}. Intensification will likely also raise the currently low environmental footprint of bivalve production (Table 7.1.).

Innovative financial approaches, research and industry development provide an opportunity to develop effective sustainable infrastructure. Microfinance institutions such as those utilised in India¹⁷⁹, overseas private investment from major producers in China and World Bank programmes such as PROFISH are suitable funding routes¹⁸⁸. An example of recent success is Peru, where a 50% reduction in corporation tax for aquaculture companies, incentives for investment from nationally prominent agri-businesses including Camposol Ltd and private investment in innovation centres allowed the bivalve industry to grow sevenfold between 2003-2015^{47,116,179}. Careful modelling and design of upscaled systems can ensure bivalve farming remains environmentally favourable relative to other food systems^{4,19}. Integrated facilities combining hatchery, grow-out, depuration and processing functions in urban areas could enable further productivity increases whilst providing additional infrastructure to support coastal production¹¹⁶. At the same time, waste streams from aquaculture and other industries could support bivalve aquaculture in recirculating aquaculture systems^{19,51}. Farming bivalves in a closed environment might even permit the

safe usage of fast-growing non-native bivalves to reduce production timescales, minimise accumulation of pollutants and increase production output ¹⁸⁹.

7.6. Consumer acceptance

Driving consumer uptake of bivalves as an appealing, nutritious, and sustainable food source is a key hurdle; unless properly tackled, it may render improvements across the rest of the value chain expendable. Societies in many tropical regions including Africa do not traditionally consume shellfish, contributing to a reinforcing loop between low production and low consumption¹¹⁶. Additionally, fears around food safety, and a lack of familiarity and knowledge around choosing, preparing, and cooking seafood are serious barriers to consumption ¹⁹⁰. Other types of meat and dairy can simply be more attractive ¹⁹¹.

A multi-pronged approach is required to stimulate consumer demand for bivalve shellfish. Pivotal to China's success were state-organised promotion of aquaculture as an affordable protein source and reform policies leading to the creation of a wide range of convenient, highly palatable, non-perishable processed bivalve products ^{177,179}. The increased consumer demand underpinned rapid aquaculture industry growth and will have contributed to the nation's economic expansion – a model other tropical nations could build on (Box 1). Looking into the future, consumer co-creation in food product development – possibly using new avenues such as social media – can drive innovations in bivalve food processing to meet the tastes of specific populations ¹⁹². Replacing conventional meat with bivalve meat within recipes or familiar processed foods may play a key role, for example shellfish paella in South America, clam stew in Africa, and battered bivalve meat in tropical urban areas ^{190–192}. Food processing developments could reduce the time and distance over which fresh bivalves need to be stored as well as the need for cooling infrastructure, and improve consumer perceptions of food safety (although an increased environmental footprint could result ¹⁹). Seafood quality certification offers promise to increase uptake and might lead consumers to pay more for this assurance ¹⁹³. There may also be opportunities for chefs and entrepreneurs in developing nations to improve their own livelihoods, establish bivalve-focussed food outlets and promote the consumption of bivalve meat in novel and creative ways ¹⁹.

7.7. A blue horizon

The global community is in great need of nutrient-rich food sources that can be produced without overburdening the environment. Bivalve shellfish are a promising alternative, particularly in the tropics. Vast areas of coastline are available for sustainable development, but several challenges must still be tackled for bivalve aquaculture to meet its potential. Leveraging new innovations and technologies can overcome these challenges, enabling bivalve aquaculture to provide a new source of income with the potential to feed nearly a billion people in the developing world.

7.8. Footnotes

Data accessibility: All datasets for this study are included in the chapter above and the supplementary files available at <https://doi.org/10.1038/s43016-020-0116-8>.

Author contributions: D.F.W. led the project and wrote the manuscript. D.C.A. contributed to study design, interpretation and reviewed the manuscript. Both authors gave final approval for publication.

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Chapter 8: Conclusion

The thesis aimed to apply breakthrough microencapsulated feed technology to research aiming to significantly improve the productivity of bivalve shellfish aquaculture, and thus help the bivalve industry to grow and play a leading role in meeting global food security goals. Novel microencapsulated feeds were developed, characterised, and proven digestible by commercially farmed bivalve species. Feeds enabled increased growth and improved survivorship in bivalve juveniles, enhanced the quality of broodstock and accelerated sexual development, and enabled fortification of adult bivalves with key nutrients for improved human health – results demonstrated in both laboratory experiments and industry trials. The thesis highlights the outstanding benefit of bivalves as a sustainable food source for a growing global population and the incredible open opportunity for industry expansion that could feed billions of people with nutrient rich food. Further research and stakeholder engagement to scale-up the use of microencapsulation technology, develop the bivalve value chain, and stimulate increased consumer demand can enable this opportunity to be realised.

8.1. Synopses of Research

The initial focus of the PhD was to assess the potential of microencapsulated diets as a problem-solving tool in the bivalve shellfish industry, and test viability via laboratory experiments. The critical review in Chapter 2 outlined the environmental and nutritional advantages of bivalve shellfish aquaculture as a strategic solution for food security, industry challenges that need to be overcome, and the potential of microencapsulation technology to tackle these challenges. The large beneficial impact a shift to bivalve aquaculture could have was highlighted – replacing just 25% of carnivorous fish aquaculture with bivalves would save 16.3 million tonnes CO₂, equivalent to half the annual emissions of New Zealand, an area of land larger than Wales (2.7 million ha), and 11.8 billion litres freshwater. Experimental work in Chapter 3 then demonstrated that the novel form of microencapsulated diet we developed known as BioBullets could be ingested by a commercially farmed bivalve; the blue mussel *Mytilus edulis*. The work emphasised the importance of carefully tailoring particle characteristics including size and buoyancy during

the next stages of research to avoid high levels of rejection in bivalve pseudofaeces or faeces.

The second phase of the PhD applied the new microencapsulation technology and aimed to improve the productivity of bivalve aquaculture in the hatchery setting – specifically regarding juvenile growth and broodstock conditioning. Research in Chapter 4, which was undertaken both in the laboratory and at a commercial hatchery, revealed that microencapsulated feeds could increase the growth and survivorship of European oyster (*Ostrea edulis*) juveniles relative to conventional algal diets. The studies highlighted a future opportunity for using microencapsulated diets to manage disease in bivalves; bacteria struggle to grow on the waxy encapsulant, and the capsules themselves could become a delivery vehicle for immunostimulants and probiotics. The importance of further optimising the nutritional profile of microencapsulated feeds was also emphasised; whilst omega-3 levels were high, protein levels were suboptimal, meaning microencapsulated diets performed worse on their own than when combined with conventional algae. Chapter 5 then demonstrated that a sustainable *Schizochytrium*-based microencapsulated diet could facilitate not only comparable but improved sexual development in *O. edulis* oyster broodstock relative to a conventional live algal diet. The broodstock research was investigating *Schizochytrium*-based microencapsulated diets for sustainability benefits and simply aimed to achieve comparable sexual development to algal diets. However, by demonstrating that sexual development could actually be improved, the research opened the potential opportunity for further commercial expansion in the bivalve shellfish industry through an increased capacity to produce bivalve seed.

The final phase of the PhD aimed to use both experimental and literature analyses to explore how microencapsulated diets and bivalve aquaculture could help tackle nutritional problems and meet food security goals. In Chapter 6 microcapsules containing vitamin A and D were designed, and an optimal dosing strategy to nutritionally fortify Pacific oysters *Crassostrea gigas* adults was quantified. Research revealed that just 2 such bivalves would provide enough vitamin A and D to meet human dietary RDAs. Tailoring the micronutrients encapsulated and the bivalve species reared could provide a low cost and highly sustainable mechanism to tackle regional nutritional deficiencies directly through the food supply and improve the lives of the 2 billion micronutrient deficient people worldwide. Chapter 7 built on this global perspective and reviewed how expansion of the bivalve aquaculture industry could help tackle food security goals specifically in the tropics. The review highlighted how

developing just 1% of the potential bivalve coastal production area in the tropics could feed nearly a billion people with all their protein needs by 2050. Key components of the value chain requiring further research, industry investment or policy changes to realise this potential were identified. Crucially, the chapter highlighted the importance of a multi-pronged approach to stimulate consumer demand for bivalve shellfish and alter perceptions regarding unfamiliarity, food quality, and taste.

8.2. Research and pathways for future impact

Research undertaken during this PhD has provided a platform to now unleash the potential of bivalve shellfish as a sustainable, nutritious food to help meet global food security goals. Future research will need to focus on optimisation and scale-up of the microencapsulation technology, strategies to realise the outstanding capacity for bivalve grow-out, and multidisciplinary research and stakeholder engagement to drive consumer demand.

8.2.1. Optimisation and scale-up of microencapsulation technology

Further optimisation of the microencapsulated feeding technology and broadening the contexts to which it can be applied is a crucial component of future research. The nutritional formulation of the microcapsules requires more specific tailoring to specific bivalve species and life stages in order to fully replace live algal feeds, and in general the protein content needs to be increased. Introducing new combinations of microalgae into microencapsulated feeds could lead to significant improvements in bivalve growth. Target species with high potential include *Isochrysis galbana*, *Chaetoceros calcitrans*, *Pavlova sp.* and *Chlorella sp.*¹⁹⁴. The potential of microencapsulated feeds to reduce disease incidence in hatcheries requires further exploration, and an appropriate first step in such research would involve including antimicrobial peptides such as tachyplesin in the feed formulation⁹³. In addition, industry research and proof-of-concept experiments will be important to better understand the potential options for applying microencapsulated feeds more broadly in aquaculture – for example in the production of fish fry.

Multidisciplinary applied research, stakeholder and industry engagement are required to scale-up the microencapsulated feed technology and enable potential to be

realised in improving the productivity of bivalve aquaculture. Risks of bivalve culture expansion need to be assessed, and opportunities for overcoming these risks should be exploited (Table 8.1.). The microencapsulation technology needs to be carefully targeted to intensify the time periods of bivalve production where it can yield the greatest beneficial impacts – specifically the juvenile rearing, depuration, and potential biofortification stages. All present the technology should not be applied to open water grow-out where the lack of a need for feed is a key advantage, although there would be potential to use the technology in intensive indoor grow-out systems. Formalised and detailed life cycle analyses on the sustainability and economics of using microencapsulated feed on a commercial level are required, including infrastructure development such as transport, distribution, and storage, and a consideration of other industries and externalities which would be altered by exploitation. Collaboration with manufacturing and engineering partners is required to develop methods to improve the efficiency of using microcapsules in hatcheries, for example automated feed delivery systems. There is a need for larger-scale trials of the feed in commercial bivalve aquaculture to identify any remaining catchpoints that need to be overcome. Routes to market and commercial exploitation will need to be navigated carefully. Many of the locations where the microencapsulated feed would be best applied are in overseas nations where there is an urgent need to establish productive hatcheries, and it will be important to ensure that the IP and technical knowledge developed during this BBSRC-funded PhD remains protected and an asset to UK research and innovation.

8.2.2. Realising the capacity in the bivalve production chain

Further work is required to realise the outstanding capacity for open-water bivalve grow-out. Microencapsulated feed was developed for application to key bottlenecks in bivalve aquaculture – hatchery production and depuration at harvest. The phase between these bottlenecks, grow-out, has huge potential capacity with over 1,500,000 km² of coastline worldwide suitable for production. However, to realise this capacity here is a requirement for improved knowledge transfer, regulation and policy regarding site selection and management. To maximise yield grow-out operators need to have information on the specific coastal areas with the highest levels of primary production, and the most appropriate grow-out structures to use. In order to ensure food safety, operators require improved access to data on hazardous pollutant distribution, so that site location and depuration protocols can be adjusted appropriately. In addition, as open-water grow-out

expands, conflicts with other industries such as fishing and oil drilling are likely to arise and improved policies such as Marine Functional Zoning will need to be applied.

Research innovation can also be used to further expand opportunities for bivalve grow-out whilst in parallel tackling additional environmental problems regarding food and sewage waste. Our cities currently produce over 23 billion tonnes of food waste and sewage sludge annually, on which heterotrophic algae could safely be grown and incorporated into sustainable microencapsulated feeds ^{195–197}. Research would assess whether such a feed could be used to grow bivalves under fully artificial urban conditions. Heated water effluents from industry could be used to maximise bivalve growth rates and reduce water pollution ¹⁹⁸. Hardy, fast growing, and even invasive species could be used without risk of escape ¹⁹. There is potential to develop a highly intensive, hygienic form of bivalve production that is close to consumers, minimising transport and distribution costs and mitigating concerns regarding open-sea water quality. Developing new grow-out options would also require investment in improved infrastructure and facilities, and this infrastructure could be used by conventional open-water operators to improve productivity.

8.2.3. Research and application to stimulate consumer demand

Improvements in bivalve production will yield little benefit to food security without parallel research efforts to identify how to increase consumer demand for the sustainable nutrient-rich food source. Today a lack of familiarity and knowledge around choosing, preparing, and cooking bivalve shellfish, alongside aforementioned food safety concerns, are serious barriers to consumption ¹⁹⁰ (Table 8.1.). Future research should include consumer co-creation trials, which could drive innovations in bivalve food processing to meet the taste of specific populations ¹⁹². Replacing conventional meats with bivalve meat within recipes or familiar processed food products is likely to play a key role, would reduce the time and distance over which fresh bivalves need to be stored, and could help improve consumer perceptions of food safety. Research also needs to identify key marketing levers that can be triggered to encourage consumption of sustainable nutrient rich bivalve foods in place of less sustainable meat and fish. Behavioural trials in food outlets could try approaches such as altering food positioning, presentation, range of choice, promoting health benefits, promoting sustainability benefits and adjusting prices to represent potential policy alterations. There is also potential for research to include the use of flavoured

microencapsulated feeds as a means to improve the palatability of bivalves to human consumers.

Alongside research, direct engagement and collaboration with the commercial food industry and stakeholders, government and policy representatives, and the media will be crucial to driving consumer demand. Collaboration with major seafood producers, retailers and international corporations will be vital to enable co-creation and marketing research to have real impact in the food industry. Engagement with stakeholders from food regulators and public health organisations worldwide would be needed to drive changes in healthy diet advice and food production policies that favour sustainable nutritious bivalve shellfish production. Publicity through social media and the press can also play a highly effective role in driving impact – evidenced by press articles based on the research of this thesis that included the concept of ‘bivalve fish fingers’ and ‘vitamin bullets’.

Table 8.1. Developing bivalve aquaculture – emerging risks and opportunities.

	Risk	Opportunity
Production	Over-harvesting of wild bivalve seed to support rapid grow-out expansion could damage the marine environment ^{34,35} .	Establishing new hatcheries and increasing efficiency can improve seed supply ³⁴ . For mussels particularly the default has been to use wild seed, but new technologies and the opportunity to produce productive triploid stock can change this default ¹⁹⁹ .
	Grow-out of fast growing but non-native bivalve species could harm local ecosystems ¹⁸⁴ .	Hatchery breeding can establish high-yielding varieties of bivalves local to specific geographical regions, for example <i>Crassostrea gasar</i> in West Africa and <i>Perna viridis</i> in India ³⁵ .
	Over-reliance on artificial feeds or microcapsules for bivalve production could increase the environmental footprint ³⁴ .	Feeding and microencapsulation can be applied to short, specific time stages of bivalve production, such as breeding, juvenile rearing, depuration and biofortification. This can

		maximise industry output, whilst allowing bivalves to rely on natural sources of feed during the longest stage of production; grow-out.
Consumer	Food safety problems could occur due to potential contamination of bivalves with bacteria, viruses, algal toxins, heavy metals, or microplastics.	Proper cooking eliminates the vast majority of pathogens, so education in food preparation would yield important benefits ²⁰⁰ . New depuration and processing strategies including the use of bacteriophages, metallothionein or chitosan chelating agents, and high-hydrostatic pressure can remove bacteria, viruses, heavy metals and microplastics from bivalves without cooking ²⁰¹ .
	Poor consumer demand for bivalves may persist due to low appeal, despite increased production capacity ¹⁹⁰ .	The consumption of bivalves in their natural form can be increased through work with high-profile chefs and the reinstatement of traditional seafood dishes. Innovations in food processing used in the development of lab-grown meats and vegetarian alternatives can also be applied to integrate bivalve tissue into familiar processed food products such as meat-style pieces and fish fingers ³⁶ .
	Feed or microcapsule producing companies may assert too much control and obtain monopolies within the industry ²⁰² .	Governmental policies can seek to democratise innovation, including a shortening of patent terms and use-it or lose it provisions, enabling competition to drive innovation and technological diffusion ²⁰³ .

8.3. Impact Summary

The PhD research has generated outputs that have pushed academic boundaries and that can have wide benefits through industrial application. Novel microencapsulation technology has been applied and demonstrated as a highly effective vehicle for delivering high quality nutrients to bivalve shellfish. The microencapsulated feeds are an order of magnitude cheaper to produce than conventional live algal feeds which currently account for 50% of bivalve production costs, contain high-quality nutrients sourced from sidestreams for sustainability, and take a dry powder form to remove risk of bacterial contamination. Research in the laboratory and commercial setting has proven microencapsulated diets as an effective solution for key bottlenecks in the bivalve shellfish industry regarding hatchery production of juveniles and conditioning of broodstock, and has opened an innovative new option for nutritionally fortifying seafood. Exploitation of research outputs from this PhD could enable rapid bivalve industry expansion and drive a step-change in sustainable global food production.

Research, development, and broader application of the outputs of this PhD can have a major beneficial impact on global food security and sustainability goals. Bivalve shellfish have a higher protein content than beef, are a rich source of essential fatty acids and micronutrients, are the most sustainable meat on the planet, and research has provided a platform to increase production of this food type. Every tonne of bivalve protein we produce instead of fish can spare 9 ha land, 67 tonnes CO₂ and 40,000 litres freshwater. Further innovations in bivalve grow-out could enable sustainable valorisation of nutrient-rich sidestreams from aquaculture and other industries. There are over 1,500,000 km² of coastlines worldwide suitable for highly productive bivalve aquaculture. If research outputs from this thesis could stimulate development of just 1% of this coastline for bivalve aquaculture, we could feed over 1 billion people worldwide with all their protein needs.

Appendix 1: From pest to profit – The potential of shipworms for sustainable aquaculture

Willer, D. F. & Aldridge, D. C. (2020). From pest to profit - The potential of shipworms for sustainable aquaculture. *Frontiers in Sustainable Food Systems*, 4:575416.

doi:10.3389/fsufs.2020.575416

We face a food crisis. Suboptimal diet is the biggest cause of death worldwide, food production the biggest greenhouse gas emitting sector, and by 2050 an extra 2.5 billion people need affordable nutrition. Current farming systems will fail to tackle this crisis, and there is an urgent need to diversify global food production and find effective solutions in currently underexploited food sectors. Shipworms, or shell-less *Teredo* clams, could prove a highly valuable component of such solutions. Historically viewed as a marine pest, they have unique physiological characteristics which make them an ideal food source, including exceptionally fast growth rates, the ability to feed on waste wood or sustainable microalgae, and a high protein and omega 3 content. Today only a select few traditional cultures in the Philippines consume shipworms, but there is considerable opportunity to develop mechanisms to farm shipworms and provide a sustainable, nutrient rich, affordable food source. This will require significant challenges to be overcome, ranging from fundamental research to industry development to food processing and marketing. Leveraging new innovations in breeding, aquaculture feeds, growth systems, food processing methodologies and consumer engagement can however offer powerful solutions, and could help turn what was once a maritime villain into a nutritional saviour.

A.1.1. Introduction

Our current food production system is failing us. Suboptimal diet is already the biggest cause of death worldwide and the global food system the single largest greenhouse-gas emitting sector. Yet by 2050 a further 2.5 billion people need access to affordable nutrition²⁰⁴. Farming of conventional crops including wheat, rice, and maize has received extensive investment over the last century, with productivity growing 2-3-fold between 1950

– 1990¹². However, yields are now beginning to stagnate, and the overreliance on this small variety of crops is a major explanation as to why 30% of humanity are now micronutrient deficient worldwide^{1,12}. Other more nutrient rich food sources have received considerably lower research attention (Figure A.1.1.). In order to meet planetary health goals, there is an urgent need to diversify global food production and to seek productivity improvements in currently underexploited food sectors that can provide sustainable, nutrient rich, affordable food solutions^{1,204}.

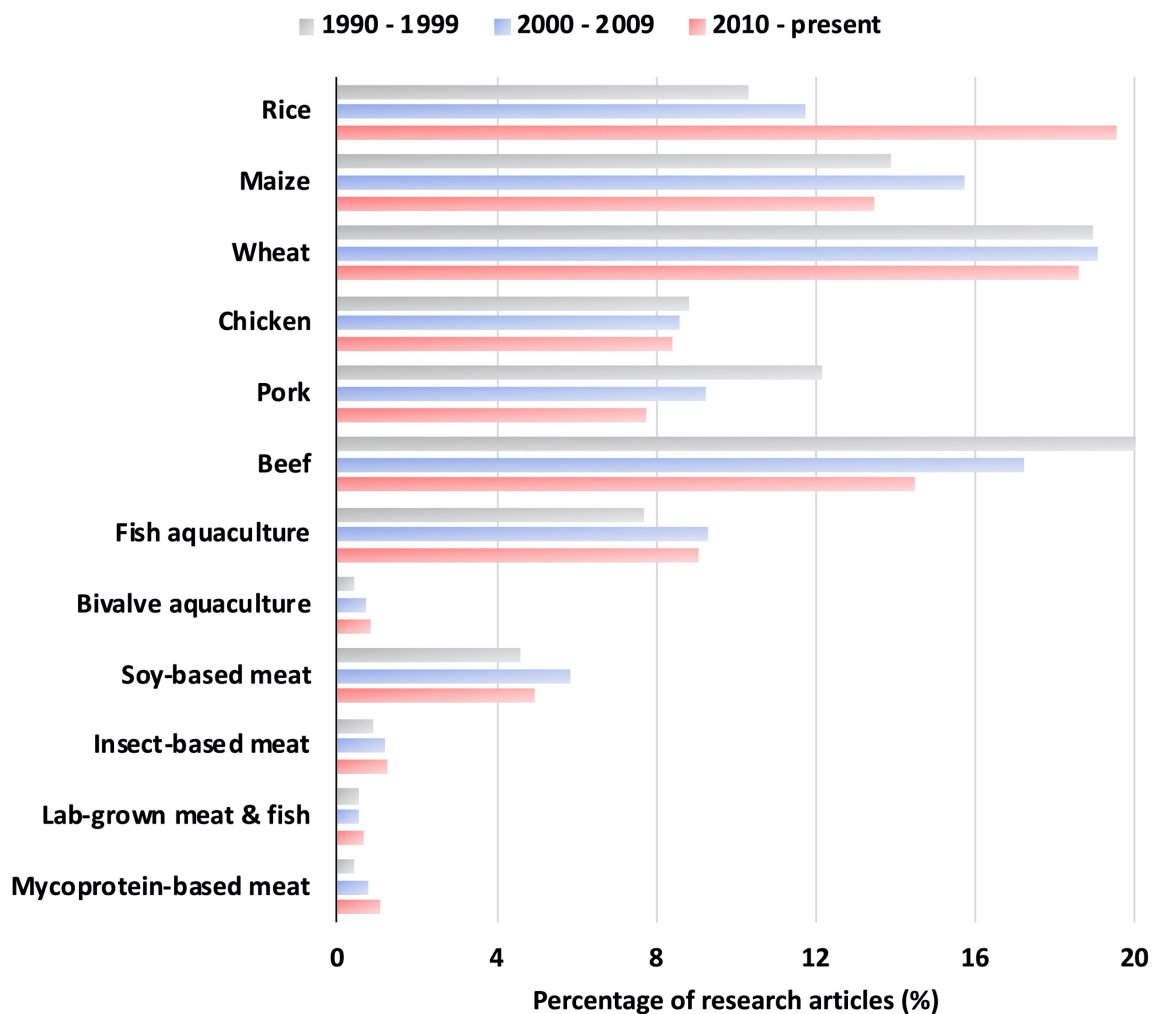


Figure A.1.1. Percentage of all research articles published corresponding to key food types across each decade. Many food sources with a high sustainability and nutritional potential (bivalve aquaculture and below) continue to receive relatively low levels of research investment. Scopus²⁰⁵ was used to count the total number of peer-reviewed publications corresponding to each food type between the dates 1990 – 1999, 2000 – 2009, and 2010 – 12/05/2020. Five most relevant search phrases were selected for each food type. To be

counted articles had to include any one of the search phrases in the abstract, title or keywords. Search phrases were as follows. Rice: 'rice farm', 'rice production', 'rice industry', 'rice food', 'rice field'. Maize: 'maize farm', 'maize production', 'maize industry', 'maize food', 'maize field'. Wheat: 'wheat farm', 'wheat production', 'wheat industry', 'wheat food', 'wheat field'. Chicken: 'poultry farm', 'poultry production', 'chicken farm', 'chicken production', 'broiler farm'. Pork: 'pork farm', 'pig farm', 'pork production', 'pig production', 'swine farm'. Beef: 'beef farm', 'cattle farm', 'cow farm', 'cattle production', 'beef production'. Fish aquaculture: 'fish farm', 'fish aquaculture', 'pisciculture', 'fish mariculture', 'fish hatchery'. Bivalve aquaculture: 'bivalve aquaculture', 'bivalve farm', 'clam farm', 'mussel farm', 'oyster farm'. Soy-based meat: 'tofu', 'tempeh', 'soy-based meat', 'soy protein', 'miso'. Insect-based meat: 'insect meat', 'insect flour', 'insect food product', 'insect snack', 'insect burger'. Lab-grown meat & fish: 'lab-grown meat', 'cultured artificial meat', 'lab-grown fish', 'in vitro meat', 'cell-based meat'. Mycoprotein-based meat: 'mycoprotein', 'quorn', 'fusarium venenatum', 'fungiculture protein', 'fungal protein food'.

There are major environmental and human health wins that could be made from research and industry investment in the farming of underexploited aquaculture species. Aquaculture is already the world's fastest growing food sector, growing 8.2% pa between 1970-2010^{12,47}. Growth has however been focussed on species such as salmon and shrimp, which are expensive to consumers and unsustainable to farm due to a reliance upon foods comprising predominantly of fish meal and oil from wild-caught fish³¹. The farming of bivalve shellfish (clams, mussels, oysters) is a highly attractive alternative. Bivalves have a higher protein content than beef, are a rich source of essential fatty acids and micronutrients, have a lower environmental footprint than all other meats and many plant crops, and are cheap to produce³¹. There is outstanding potential for growth of bivalve aquaculture; developing just 1% of the suitable coastline worldwide could fulfil the protein requirements of over one billion people^{13,18}. Yet the bivalve industry remains small scale and artisanal. There are bivalve species with high farming potential such as shipworms which could play a key role in sustainable aquaculture, where further research and industry attention is required in order to realise food production potential¹⁹.

Shipworms, or *Teredo* clams, are a saltwater bivalve species with unique life-history characteristics that could be leveraged to provide significant economic, sustainability, and nutritional benefits to human society. Shipworms grow exceptionally fast relative to other

aquaculture species, reaching 30 cm (or approximately 250 g wet weight) within 6 months, compared to shelled bivalves which typically take 2 - 3 years to reach a harvestable size of around 7 cm^{69,206}, presenting a highly productive and efficient possibility for producing animal protein. Shipworms lack a shell for protection, instead burrowing into and feeding on submerged wood. This wood digesting ability has historically made shipworms a pest in the maritime industry, where they have damaged traditional wooden hulls and marine piling; an issue now reduced with modern engineering materials²⁰⁶. Whilst *Teredo* clams can survive on wood alone, obtaining additional nitrogen via symbiotic nitrogen fixing bacteria in their gills, they can also filter feed on marine detritus like other bivalves²⁰⁷, thus presenting numerous options for low-environmental impact feeding in aquaculture production. Shipworms are rich in protein and essential omega-3 fatty acids, and like other bivalves can provide a highly valuable source of quality nutrition to humans^{13,207,208}.

Today shipworm harvest and consumption as a food is limited to a few select regions of southeast Asia, notably the Philippines and Thailand. Shipworms grow on decaying wood in the mangroves and are harvested by local populations. In some areas such as Bakhawan Eco Park (New Buswang, Panay, Philippines), the coastlines have been seeded with dead wood as part of mangrove restoration projects which in turn has increased the supply of shipworms for harvest, although there remain no formalised farming approaches²⁰⁹. Shipworms are sold raw in wet markets, and are particularly popular on Palawan island, Philippines where they are known as tamilok and in Trat Province, Thailand where they are known as priyang talay²¹⁰. Filipinos typically eat Tamilok raw and dipped in salt, chilli and vinegar in a dish known as kinilaw, whilst the Thais eat priyang talay in curries or braised with fish paste and bananas in a stew²⁰⁸⁻²¹⁰. Over the last decade increasing popularity of shipworms as a food for tourists and increasing ecological threats to mangrove swamps has put pressure on shipworm supply, and socioeconomic benefits could be yielded from increased mangrove protection or improved shipworm culture practices^{208,209}. Outside of southeast Asia, shipworms are still viewed as a pest. In Europe and the Americas climatic change is leading to increasing coastal salinity and temperature, making conditions more favourable to shipworms²¹¹. This could lead to the re-emergence of shipworms as a maritime nuisance, or alternatively with research and industry innovation and efforts to change consumer perception, could present a new opportunity to establish shipworm aquaculture.

A.1.1.1. Approach

Several approaches were leveraged to build a synthesis of the potential of shipworms as a food source and the key challenges and opportunities to establish aquaculture. Scopus was used ²⁰⁵ (Figure A.1.1., legend) to count the percentage of all research articles published corresponding to key food types across each decade since 1990, and highlight the relatively low research investment in bivalve aquaculture. We spoke to stakeholders across the aquaculture and food industry to gain an insight on the potential obstacles in creating a new food type and methods which could be effective in providing a solution. This provided direction for a thorough literature review. We searched Scopus ²⁰⁵ and Google Scholar ²¹² for studies on shipworms, aquaculture, and the development of unexploited food sectors. We used several key terms when covering the field: ‘shipworm’, ‘*Teredo*’, ‘bivalve’, ‘aquaculture’, ‘invasive species’, ‘sustainable nutrition’, ‘food safety’, ‘food processing’, ‘food marketing’; and included all relevant research up to and including 20th June 2020. Prior to our study, no studies had built a synthesis on the potential of shipworms for aquaculture.

A.1.2. Challenges and Opportunities

Production of shipworms could offer major advantages over other forms of aquaculture for food production. Farming would be significantly more sustainable than finfish aquaculture, with shipworms requiring waste wood or natural or sustainably grown algae rather than fish as a feed source, helping contribute towards circular economies in food production ^{19,207}. The disadvantages of conventional bivalve aquaculture – notably the slow growth rates and energy investment in a shell which cannot be used as food – would be avoided. Shipworms would provide a valuable source of protein and essential fatty acids to replace less sustainable and more expensive meat and fish products. In order for this potential to be realised there are key challenges that must be overcome, ranging from fundamental research to industry development to food processing and marketing. Novel technologies and new application of innovations from other industries can however provide exciting opportunities for success (Figure A.1.2.).

A.1.2.1. Fundamental Research

Fundamental research is required in several key areas to allow the exploration and development of shipworm aquaculture. There are approximately 65 species of shipworm worldwide and we do not yet know which would be most appropriate for efficient farming and human nutrition²¹³. The only small-scale attempts at culture to date have involved floating logs in coastal waters or seeding mangroves with dead wood to encourage *Teredo* proliferation²⁰⁸. When an appropriate species is identified, there will also be a need to develop scientific methodologies for trait selection and large-scale breeding of shipworm juveniles, which is challenging in bivalves due to high fecundity, self-fertilisation, genetic load and segregation distortion²¹⁴. The nutritional profile of shipworms also needs to be formally analysed – whilst they are recognised to have a similar profile to other bivalves no quantitative data has been published in peer-reviewed literature^{207,208}. The most appropriate abiotic conditions for farming shipworms also need to be identified, including temperature, salinity, and saltwater mineral composition. We must also develop a greater understanding as to how climatic change may affect these values, as this may affect site selection for farms without facilities to control temperature and salinity²¹⁵. Crucial to successful shipworm aquaculture will be the selection and development of appropriate feeds. Research indicates that filter feeding is in fact the preferred source of dietary nutrition for shipworms, with wood drilling performed primarily to provide shelter²⁰⁷, but we do not yet know what the most appropriate feed would be for shipworm farming. Infection control must also be considered. Disease is a major concern in aquaculture, costing the industry over US\$6 billion annually, yet there is an urgent need to reduce the widespread use of antibiotics which is driving bacterial resistance and increasing threat to human health²¹⁶.

Knowledge and methodologies gained from research in conventional agriculture and aquaculture can help tackle challenges in shipworm aquaculture. Current scientific literature suggests *Teredo navalis* may be a promising shipworm candidate for aquaculture, the same species as consumed by Philippine societies at present²⁰⁸. The development of whole genome sequences and genetic linkage maps, as has been done in other bivalve aquaculture²¹⁴ could be used to select for desirable traits such as faster growth, greater broodstock quality, and an improved profile regarding nutrition and palatability. Optimum temperatures and salinities for farming are likely to be between 10 - 25 °C and 10 - 30 ‰ respectively²⁰⁶; further research can refine these values, make predictions as to which coastal locations

might become more ideal under climate change, and make use of new innovations in artificial salt formulations (e.g. Homarsel, produced by Zoutman, Belgium). Regarding feed for shipworm aquaculture, waste wood, live algae, and artificial or microencapsulated feeds are all viable options. The use of wood may allow the recycling of waste, and the use of microencapsulated feeds^{32,33} would avoid major challenges faced by conventional algal feed including quality inconsistencies, contamination, and poor shelf life³¹. Research would allow identification as to whether a combination of these feeds or a single feed is most optimal for growth. There are also emerging new opportunities for more sustainable disease control, including the use of probiotics and antimicrobial peptides^{93,217}, and it would be advisable to quantify the efficacy of their use in shipworms.

A.1.2.2. Industry Development

Industry will play the central role in the establishment of shipworm aquaculture, and will need to overcome challenges regarding facility design, safety and economics in order to develop and expand successfully. The practical aspects of setting up shipworm farms pose an initial hurdle. The invasive nature of shipworms means that they can represent a hazard if they escape into the natural marine environment²⁰⁶ and farming systems and infrastructure need to be designed to nullify this risk. Overzealous farming of other bivalves such as *Magallana gigas* has already been shown to displace important native species and modify marine ecosystems¹⁸⁴. Food safety is also a major concern when farming filter feeding bivalves, and in polluted waters there is high potential for hazardous substances such as heavy metals, microplastics and toxic cyanobacteria to accumulate in bivalves – with oysters farmed in the South China Sea for example now containing approximately 11 microplastic particles per individual^{35,182,183,185}. Ocean water quality is likely to further decline as the human population expands and shipworm farms should be designed to cope with these changes²¹⁸. Coastal areas will undergo further urbanisation and development, which could both restrict and provide new opportunities as to where shipworm farms could be located. The industry will also need to develop automated mechanisms to efficiently monitor shipworms and at harvest remove them from the woody substrates they burrow in. In conventional bivalve production the shucking (de-shelling) process in manufacturing packaged bivalve foods such as tinned clams is highly labour intensive and a major contributor to food costs²¹⁹, and while shipworms lack a shell it will be important to find cost-effective processing systems. Finally and crucially, a thorough economic assessment of

the entire proposed production value chain will be required in order to ensure shipworm aquaculture is scalable and financially viable. Inadequate assessment could lead to expensive bottlenecks in production ³¹, or at worst could result in complete failure of shipworm aquaculture ²²⁰.

Lessons learned from the commercial success of other forms of aquaculture could help ensure effective development of the shipworm industry. To mitigate risk of shipworms escaping into the open sea, farming of shipworms could involve the use of enclosed saltwater tanks, either semi-submerged on the coastline or onshore. The installation of flood protection structures and nearshore or offshore wind farms as a means to mitigate climatic change and its impacts could provide a new opportunity for mounting shipworm growth tanks. Larger enclosed areas could also be constructed within mangrove restoration projects, in parallel improving water quality and coastal protection ²⁰⁹. There may also be an option to setup enclosed tanks in urban areas away from the sea as is seen in finfish aquaculture ²²¹, which would further reduce the risk of escape due to the lack of surrounding saltwater. Enclosed tanks would enable optimisation of temperature and salinity to improve growth rates, and also the use of artificial seawater or clean deputed seawater which would dramatically increase food safety ^{28,221}. Given that consumer fears regarding safety are one of the biggest barriers to increased bivalve consumption, the use of enclosed tanks could be the underpinning factor in the commercial success of shipworms as a food source ¹⁹⁰. Automation during growth and at the point of harvest through the use of optical sensors, machine vision systems and low-cost robotics could help avoid excessive labour costs in shipworm production, with this approach already yielding great benefits in finfish aquaculture ²²². Identifying the minimal requirements and optimal arrangement of woody substrates for shipworm farming would further increase operating efficiency during growth and harvest. There is also the opportunity to use circular economies to divert side-streams of shipworm production back into the system for example for feed or wood production. To ensure commercial viability of shipworm farming, bioeconomic modelling methods that have recently been tried and tested across salmon, tilapia and shrimp aquaculture can be applied. Compared to using simple cost-benefit analyses alone, the use of scenario simulations and algorithm-based approaches can ensure that farming solutions identified are more resilient to changes in external economic and environmental factors ²²³.

A.1.2.3. Food Processing & Marketing

Research and industry development will provide the foundation for shipworm aquaculture, but unless challenges in food processing and marketing are tackled these efforts will be in vain. The first major challenge will be in turning shipworm meat into a product that is palatable for human consumption. Invertebrate-based foods such as bivalves and insects are viewed with fear and disgust by many western societies, and innovations in food manufacturing are being developed to overcome this hurdle ²²⁴. Molluscs also have a different muscle structure (obliquely striated, cross-striated, and smooth) to the striated muscles of fish and mammals, which may pose additional food processing challenges ²²⁵. To ensure widespread retail demand, it will also be essential to ensure that products developed fit a wide range of consumer tastes and cultural preferences ²²⁶, and this will be of equal importance in the restaurant and hospitality market alongside supermarkets. Products and dishes will also need to remain affordable to consumers. This requires measures to ensure production is efficient as well as approaches to avoid market monopolisation which could drive up prices and reducing industry innovation, as has unfortunately been seen in the agricultural and mycoprotein industries ²²⁷. Paramount to the overall strategy will be for researchers and industry to work together to identify the most effective methods to market shipworm food products and promote consumer uptake.

There are a wide array of effective solutions that can be applied to the discussed challenges in the processing and marketing of shipworm-based foods. Innovations in food manufacturing made during the development of mycoprotein and insect-based foods can be applied to shipworm meat. These range from simple dehydration, powdering, and reconstitution, to thermoplastic extrusion and fibre spinning of meat proteins into a completely new form, which could help with the potential challenge of processing the different molluscan muscle types ^{226,228}. This could enable the development of food products with a wide range of forms to fit a range of cultures, from burgers and fish fingers to meat-style pieces to use in traditional stews, and is an approach that has led to great commercial success for products including Quorn (USA), Beyond Meat (USA), and Eat Grub (UK) ²²⁹. Working with high profile chefs and finding methods to explicitly present shipworms undisguised in a highly palatable form on a plate will also play a key role. Historically in Asia and increasingly in the West this strategy of including insects in premium dishes has been central to establishing a high level of cultural acceptance for insect-based foods ^{224,229}. Marinated shipworm kinilaw is already popular with both locals and tourists in the

Philippines, and gaining further insight from Filipino culinary practices could help such dishes become a global delicacy ²⁰⁸. To ensure shipworm-based foods are easily affordable to consumers several mechanisms could be deployed. These include governmental and private investment to allow the industry to scale rapidly and achieve lower operating costs, subsidies to encourage purchase of nutritious shipworm-based food in place of less sustainable meat products ²³⁰, and enforcement of industry regulations to promote competition in shipworm production and food processing ²³¹. Marketing will also play a pivotal role in encouraging consumer selection and purchase of bivalve based foods. Mass surveys, consumer co-creation activities, and domestic trials can help identify the most effective marketing levers to pull. A promotion strategy in which shipworms are double framed as an environmental solution to excessive meat consumption and as a protein packed superfood, an approach already being used in the seafood and insect industry ²³², may prove one of the most effective mechanisms.


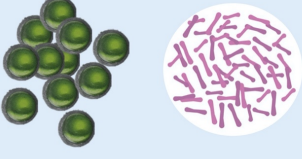



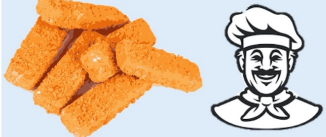
Challenges		Solutions and Opportunities
Species selection	 <p>Fundamental Research</p> 	Numerous options, <i>Teredo navalis</i> is the most likely candidate
Breeding & trait selection		Use methodologies established in clams & oysters to select for fast growth, desirable nutritional profile & high quality broodstock.
Temperature & salinity optimisation		Current research supports ranges between 10-25 °C and 10-30 ‰, further research is needed to find an optimum value
Saltwater mineral composition		Research can establish a formulation for optimal growth
Feed type		Options include wood, live microalgae & microencapsulated feeds
Disease control		Research into use of probiotics and antimicrobial peptides
Risk of shipworms escaping into the natural environment	 <p>Industry Development</p> 	Use of enclosed saltwater tanks
Ensuring high water quality for food safety		Use of artificial seawater formulations in tanks in place of natural seawater
Ensuring easy harvesting of shipworms		Identifying minimal wooden substrate requirements to maintain growth, creating effective shipworm removal mechanisms
Ensuring production is scalable and economically viable		Formal analyses in collaboration with experts from similar industries
Turning shipworm meat into a palatable meat for human consumption	 <p>Food Processing & Marketing</p> 	Collaboration with companies with expertise in the production of meat alternatives such as mycoprotein, insects & soya
Ensuring widespread retail demand for shipworm-based meat		Create convenient products and adapt recipes to market & cultural preferences
Creating demand for shipworms in the restaurant industry		Work with high-profile celebrity chefs
Ensuring meat remains affordable		Legislation to promote competition & avoid monopolisation
Identifying additional marketing levers		Could include promotion of sustainability, nutrition & cost advantages

Figure A.1.2. Key challenges, solutions and opportunities in the development of shipworm aquaculture.

A.1.3. Conclusion

As the world's population grows by 200,000 people every day, we have a global responsibility to find new ways to feed everyone without further depleting the planet's already stretched natural resources. Finding viable, sustainable, affordable solutions will mean thinking outside-the-box and considering the previously inconceivable. Shipworm aquaculture has outstanding potential to become a component of a broader global solution. Challenges need to be overcome, ranging from fundamental research and viability assessments to industry development to food processing and marketing. Yet there is an opportunity to provide our global community with a fast growing, sustainable, nutritious food source that could help remediate the catastrophic damage current food production systems are causing to environmental and human health. Policy changes will play a key role in stimulating research and industry efforts. Financial incentives that support sustainable shipworm research and aquaculture in place of less sustainable meat production, legislations that support mangrove restoration, coastal and urban aquaculture, and tax breaks on the production and retail of sustainable nutrient-dense foods are all options. Changing industry practice and consumer behaviour will not be easy, but rewards are seldom reaped without new ways of thinking. Shifting our perception of shipworms as a pest and giving them a place on our plates is one change in our thinking that could yield great rewards.

A.1.5. Footnotes

Data availability: All datasets for this study are included in the article above.

Author contributions: D.F.W. led the investigation and wrote the manuscript. D.C.A. contributed to design, interpretation and reviewed the manuscript.

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Appendix 2: Matches and Mismatches Between Global Conservation Efforts and Global Conservation Priorities

Willer, D. F., Smith, K. & Aldridge, D. C. (2019). Matches and Mismatches Between Global Conservation Efforts and Global Conservation Priorities. *Frontiers in Ecology and Evolution* vol. 7 297. doi:10.3389/fevo.2019.00297

Species extinctions are occurring at an unprecedented rate and there is a global need to understand whether conservation effort is appropriately allocated to protect those species at risk. In this study three major measures of global conservation effort across IUCN Red List Threats and Habitats were assessed; staff time spent by the largest cluster of conservation organisations in the world - Cambridge Conservation Initiative, efforts by international NGOs through social media, and global conservation research publications since the year 2000. We find global conservation effort is generally aligned with global conservation priorities, but there are important outliers. Shrublands and rocky areas receive disproportionately little investment across all effort measures relative to the number of high extinction risk species, threats from residential and commercial development receive relatively low research and time investment despite social media attention, while marine areas and climate change receive more attention than expected. Governments and society must make critical conservation decisions in the context of rapid global change, and there is potential for key Threats or Habitats to receive less attention than required. The global conservation community would be wise to carefully consider and improve its understanding of effort-priority mismatches if the greatest number of high extinction risk species are to be protected.

A.2.1. Introduction

Conserving biodiversity is recognised as the cornerstone of protecting global ecosystem services, which are estimated to be worth over USD \$127 trillion yr⁻¹ 7,233. Scientists believe we are now driving Earth's sixth mass extinction event²³⁴, and with continued ineffective management the annual value of our ecosystems could halve by 2050

7. Conservation organisations are working hard to protect our natural capital, focusing on particular threats, habitat, geography or taxa, with their contributions ranging from policy engagement to running projects on the ground^{235–237}. Given effort investment by conservation NGOs is increasing but resources and time remain scarce, it is becoming increasingly important to understand if effort is being spent where it is most needed²³⁸.

The International Union for Conservation of Nature (IUCN) Red List represents an invaluable tool for conservation organisations to appropriately allocate their effort. The Red List is recognised as the world’s most comprehensive inventory of species’ conservation status, with over 91,000 species assigned metrics including extinction risk, Habitats occupied and Threats exposed to²³⁹. Agriculture, aquaculture and biological resource use currently threaten the largest number of high extinction risk species, while forests and wetlands contain the most (Figure A.2.1.).

We asked whether global effort in terms of research, social media, and time investment is aligned to biodiversity priorities set out by the Red List. Specifically: (1) How has conservation research been distributed across the Red List Threat and Habitat categories since the year 2000; (2) What is the distribution of Twitter posts from international conservation NGOs across Red List Threat and Habitat categories; (3) What proportion of time do academics and NGO staff working within the largest cluster of conservation organisations in the world, the Cambridge Conservation Initiative (CCI), spend on each Red List Threat and Habitat; (4) How does the amount of research, Twitter posts, and time allocation on each Red List Threat and Habitat correlate with the number of threatened species, and where are the key mismatches?

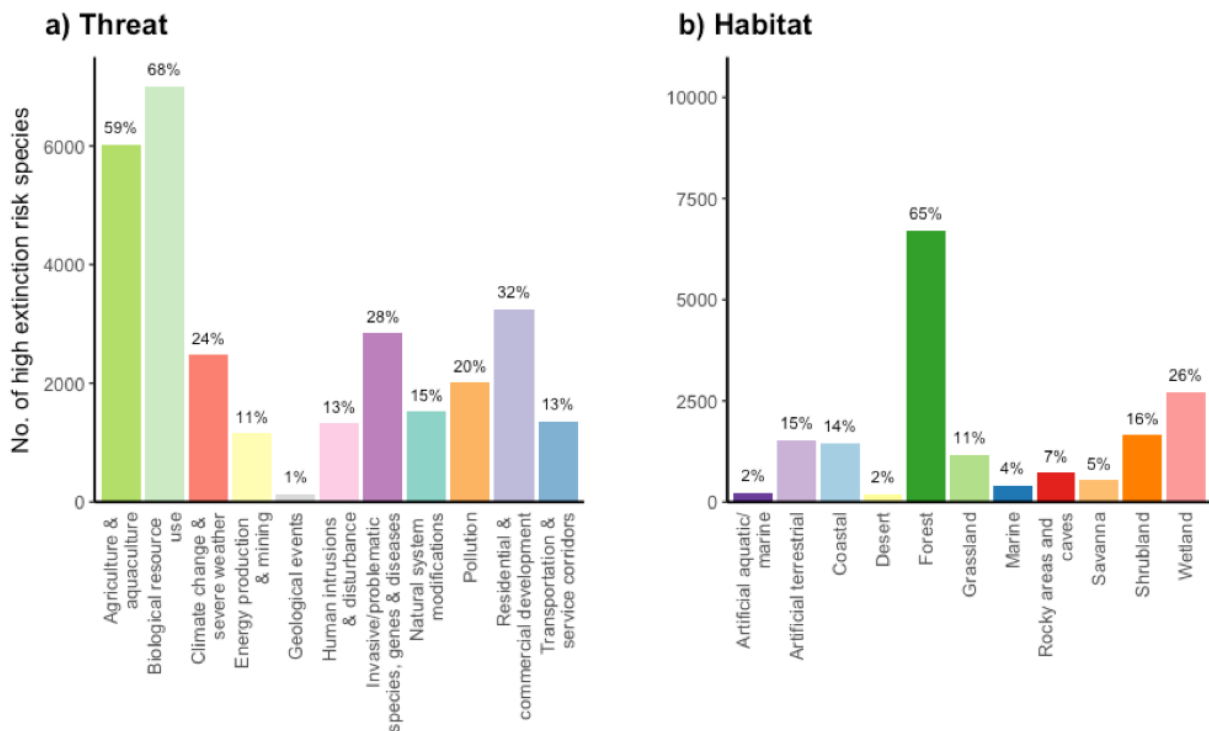


Figure A.2.1. The number of high extinction risk species in each Red List Threat and Habitat class. Percentages on bars refer to the percentage of high extinction risk comprehensively assessed taxa that occupy the habitat or are exposed to the threat. Data are from Red List version 2017.3 (see Table S1). High extinction risk refers to species in six of the eight Red List categories; Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR), Extinct in the Wild (EW), Extinct (EX). Least Concern (LC) and Data Deficient (DD) are the remaining two categories. To date 35,306 of the >91,000 Red List species have been comprehensively assessed, and of these 10,249 are classed as high extinction risk.

A.2.2. Materials and methods

A.2.2.1. Red List analysis

Red List data (version 2017.3) were obtained using the International Union for Conservation of Nature (IUCN) Red List API and R Statistics^{239 100} (see R Code S1 in Supplementary Material). The broadest or ‘level 1’ Threat and Habitat data for all species in the 31 taxonomic groups comprehensively assessed by the IUCN (Table S1) were retrieved; the number of comprehensively assessed groups continues to grow as the IUCN work down a priority list. The data were then filtered to leave only species in classifications we tagged as ‘high extinction risk’; Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR), Extinct in the Wild (EW), Extinct (EX). The remaining two classifications we

did not class as 'high extinction risk' were Least Concern (LC), and Data Deficient (DD) for those species where not enough information is available to assign a category of extinction risk. A few Habitats were combined; 'rocky areas' and 'caves and subterranean habitats' were merged into 'rocky areas and caves'; 'marine neritic', 'marine intertidal' and 'marine coastal/supratidal' were merged into 'coastal'; 'marine oceanic' and 'marine deep benthic' were merged into 'marine'; and 'introduced vegetation' was added to the 'artificial terrestrial' Habitat. This created a total of 11 Threats and 11 Habitats. The number of species in 'high extinction risk' classifications from comprehensively assessed taxonomic groups in each Threat and Habitat category were then counted (Table S1).

The number of threatened species in comprehensively assessed taxonomic groups is regarded by the IUCN as the best available representation of global conservation priorities. The comprehensive assessment methodology prioritises generating and updating datasets for species groups which are known to contain many threatened or near-threatened species, and there is not a bias towards specific Threats or Habitats. The number of threatened species in each Threat or Habitat class is thus a fair metric to represent the proportion of all species in those classes that are threatened^{240,241}. Alternative metrics such as the proportion of threatened species in comprehensively assessed groups are less useful, because they misleadingly inflate the proportion of all species that are threatened, due to the preferential selection of threatened species in the comprehensive assessment process^{240,242}. Following IUCN guidance our study used the number of threatened (high extinction risk species) as the conservation-priority metric to compare against effort metrics, and this also enabled comparison of our data with other studies which have used the same metric^{242–244}.

A.2.2.2. Search keywords

The keywords used for the research and Twitter analyses were obtained from 'level two' of the IUCN Threat and Habitat classification criteria²³⁹. 'Level two' contains a wide selection of keywords for each 'level 1' Threat and Habitat category, and was used as a standardised criteria to ensure our search keywords would capture as many relevant Twitter and research posts as possible. Table S2 shows the keywords used for each Threat and Habitat category.

A.2.2.3. Research analysis

The Scopus literature database API was used to search for all scientific articles published between 01/01/2000 and 31/12/2017 containing ‘conservation’ and ‘biodiversity’ and at least one Habitat or Threat keyword, in the title, abstract, or keywords²⁰⁵. The number of articles corresponding to each Threat and Habitat for each year were recorded (Table S3).

A.2.2.4. Twitter analysis

Twitter data were obtained using the Twitter API and R Statistics^{100 245}. A list of 85 international NGOs was selected from the IUCN members list of 107 international NGOs; the 22 NGOs with no Twitter profiles or profiles with no direct conservation relevance were excluded. Profile data and the most recent 3200 tweets, excluding retweets, from all 85 NGOs were downloaded on 29/05/2018. To ensure fair comparison between NGOs, 150 days of data between 31/12/2017 and 29/05/2018 were selected. This was the longest period that could be selected across all organisations, as several NGOs had their 3200th most recent tweet on 31/12/2017, and no more than 3200 tweets can be retrieved due to limits imposed by the Twitter API. The selected tweets were then searched using the keywords for each Threat and Habitat, and the number of tweets corresponding to each Threat and Habitat was counted (Tables S4 and S5, R Code S4).

A.2.2.5. Time analysis

Time data were obtained using a SurveyMonkey survey sent out to nine conservation organisations within the Cambridge Conservation Initiative (CCI) (Table S6)²⁴⁶. The nine organisations were: Royal Society for the Protection of Birds, BirdLife International, International Union for Conservation of Nature, United Nations Environment Programme World Conservation Monitoring Centre, Cambridge Conservation Forum, Fauna and Flora International, TRAFFIC, British Trust for Ornithology, and the University of Cambridge Conservation Research Institute. Insurance was provided by the University of Cambridge, and ethical approval by the Psychology Research Ethics Committee, University of Cambridge. Individuals were asked the following six questions: ‘Which organisation do you work for?’; ‘Estimate the percentage of your time that was spent working on the following biodiversity Threats over the past year’; ‘Estimate the percentage of your time that was spent working on the following Habitats over the past year’; ‘Imagine funding and job requirements were

not an object, and you were acting in the best interest for conservation. What percentage of your time would you spend working on the following biodiversity Threats over the next year?'; 'Imagine funding and job requirements were not an object, and you were acting in the best interest for conservation. What percentage of your time would you spend working on the following Habitats over the next year?'; and 'Any other comments?'. Some individuals did not fully complete the survey, so there were fewer responses to the later questions. Of the 68 individuals who completed every question, 11 were from academic and 57 from non-academic organisations. Data in the 'other threats' category, a category added to the survey to make completion by participants easier, were not used in the analysis.

A.2.2.6. Data presentation and statistics

All data were analysed and plotted using R Statistics (Tables S1 – 8 and R Code S1 – 6)¹⁰⁰. For the time data (Figure A.2.4.), paired sample Wilcoxon tests identified differences in how people spent time last year compared to how they would spend it next year on Threats and Habitats, see Table S7 for V and p values. Median and mean response values for each Threat and Habitat for last year and next year were then calculated. Means were standardised so that each Threat and Habitat total was out of 100 for use in Figure A.2.4. and the manuscript (medians were not used for this purpose due to the low resolution of responses given by any individual; employees tended to give scores to the nearest 10%). For the scatterplots (Figure A.2.5.), major axis models were used to test for positive correlation between each conservation effort variable and the number of high extinction risk species, and also between research and time effort. Equations and statistics are presented in the results.

A.2.3. Results

A.2.3.1. Research

Our findings reveal conservation research is growing rapidly but is not proportionately distributed across the number of high extinction risk species in each Red List Threat and Habitat. In 2017 over 3700 peer reviewed scientific articles were published with both 'biodiversity' and 'conservation' in the title, abstract, or keywords; a seven-fold increase since the year 2000²⁰⁵. Over the past five years climate change and agriculture

received nearly 40% of research attention, whilst invasive species, energy production and transportation combined received just 13% (Figure A.2.2a.). Forest Habitats accounted for 37% of research across all 11 Habitats, whilst shrublands and rocky areas both made up less than 1% (Figure A.2.2b.).

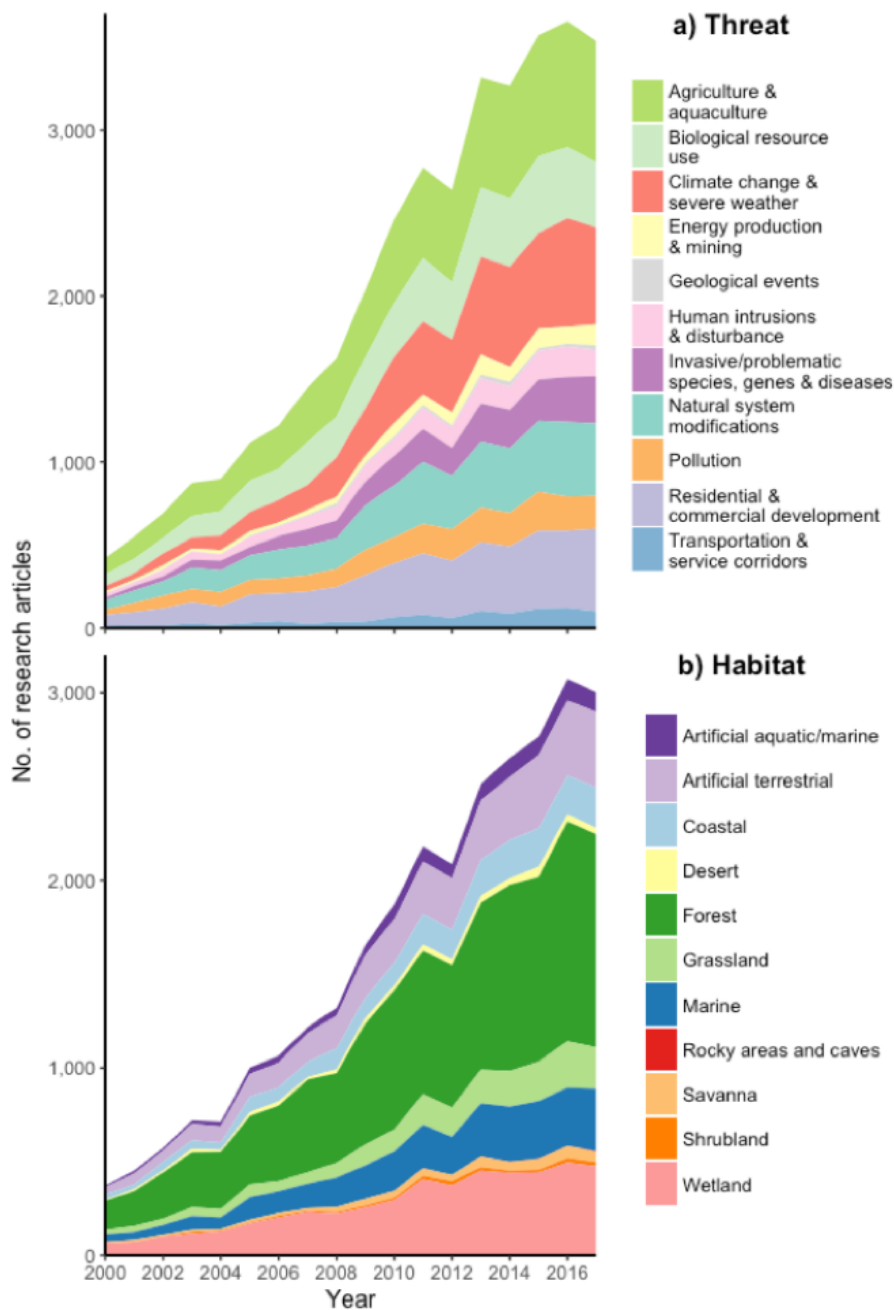


Figure A.2.2. The number of research articles relating to Red List Threats and Habitats.

Research articles allocated to a specific Threat or Habitat contained ‘biodiversity’ and ‘conservation’ and at least one keyword corresponding to the Threat or Habitat, in the title, keywords, or abstract of the article. Data are for 01/01/2000 to 31/12/2017 and obtained from Scopus literature database ²⁰⁵.

A.2.3.2. Social media

Twitter posts from large international conservation NGOs were also focussed towards certain Habitats and Threats. Climate change, agriculture, and biological resource use accounted for 52% of Threat-related Twitter posts, from all 85 assessed NGOs over 150 consecutive days in 2018. Invasive species and transportation each received just 2%. Nearly 70% of Habitat posts concerned forests or marine environments, whilst rocky areas and shrubland received zero attention. Unsurprisingly, some Habitat-specific NGOs displayed especially narrow focus, for example the African Wildlife Foundation on savannas, and the Rainforest Alliance on forests (Figure A.2.3.).

A.2.3.3. Time investment

Conservation practitioners within CCI invest the majority of their time on a few key Habitats and Threats and would not choose to dramatically alter this. Over the past year forest, wetland and marine Habitats received 55% of time, whilst rocky areas and deserts received just over 1% each. For Threats, 39% of time was spent on agriculture and biological resource use, while invasive species, natural system modifications, transportation, pollution and development combined received only 28% (Figure A.2.4., left). When conservation practitioners were asked how they would spend their time to best help conservation if funding and job requirements were not an object, they gave a response which suggested they would only slightly alter their time allocation, but with the same few Habitats and Threats still receiving the majority of attention. Forests, wetlands, and marine Habitats would still receive 58% of time, whilst deserts would receive significantly more time (twice current 1%) (paired sample Wilcoxon, $V = 40$, $p < 0.05$). Agriculture and biological resource use would still receive 36% of time, although climate change would receive significantly more attention (5% on top of current 8%) (paired sample Wilcoxon, $V = 249$, $p < 0.01$) (Figure A.2.4., right).

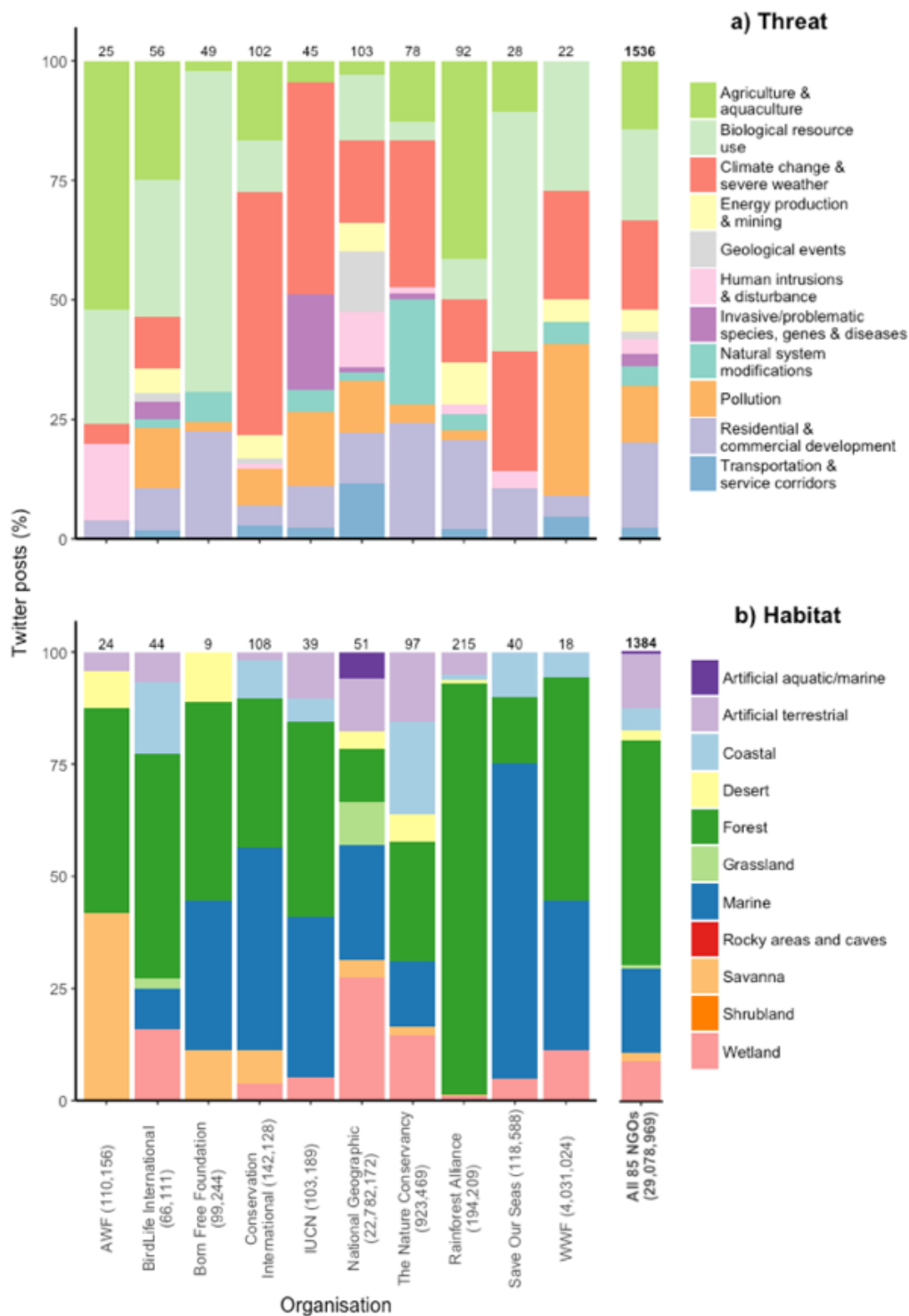


Figure A.2.3. Twitter posts on Red List Threats and Habitats. Twitter posts allocated to a specific Threat or Habitat contained at least one keyword corresponding to the Threat or Habitat. The right-hand bars are a total from the 85 international NGOs with Twitter profiles who are members of the IUCN. The left-hand bars are the top 10 most followed NGOs of the 85. Data are from 150 days between 31/12/2017 and 29/05/2018, and the number of posts is shown on top of the bars for each NGO. The number of followers on 29/05/2018 is shown after each NGO name.

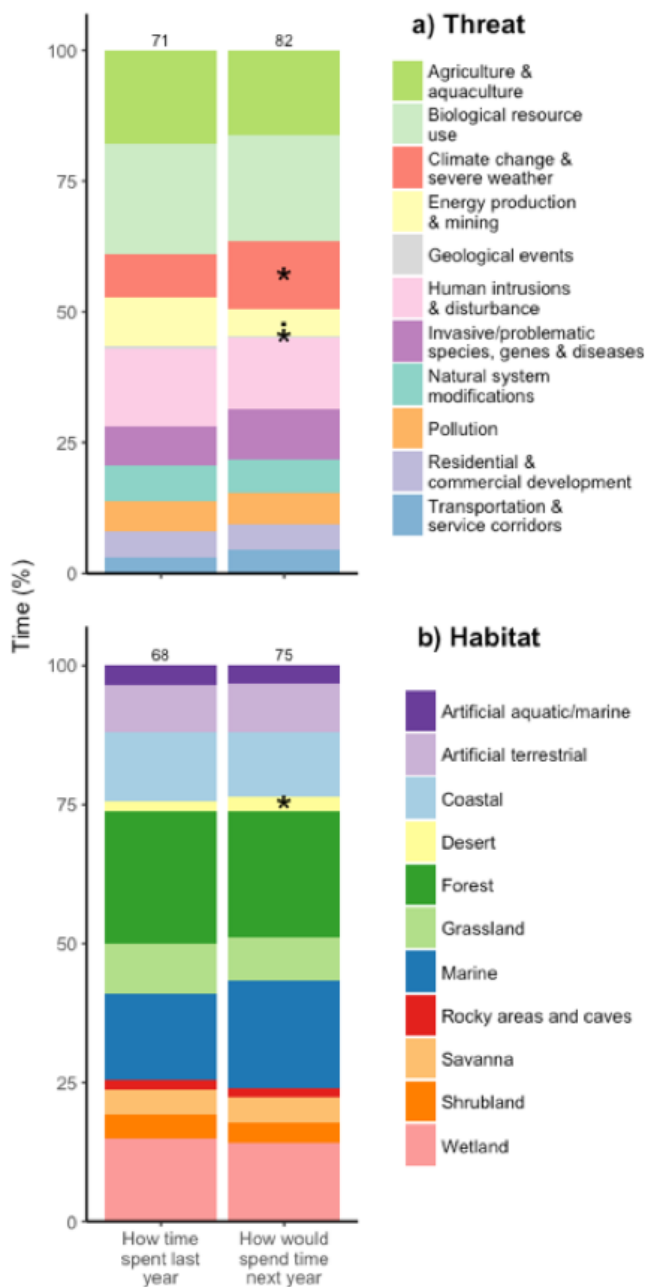


Figure A.2.4. How conservation practitioners spent their time last year and how they would spend it next year. Survey responses are from nine leading internationally-focused biodiversity conservation organisations (see Methods 2.5. Time analysis). ‘Last year’ is 01/08/2017 – 31/07/2018. ‘How would spend time next year’ assumed that conservation practitioners were acting in conservation’s best interest and that funding and job requirements were not an object. The * indicates a significant (Paired Wilcoxon test, $p < 0.05$) and near significant ($p = 0.07$) difference between ‘last year’ and ‘next year’ for threats or habitats. The number of individual responses to each question is shown on top of each bar.

A.2.3.4. Conservation effort and priorities

The amount of research, social media, and time allocated to Red List Threats and Habitats does positively correlate with number of high extinction risk species present in each category, but there are a few key outliers (Figure A.2.5.). Shrublands have the third greatest number of high extinction risk species of all Habitats (1650 species), yet along with rocky areas receive consistently less than expected attention across all effort measures (Figure A.2.5, right). In comparison, marine Habitats contain relatively fewer (422) high extinction risk species yet receive greater than expected attention across all measures. Patterns of outliers are not completely consistent across research, social media, and time components. There is a tendency for Twitter to give a lot of attention to development, an area which receives little time or research relative to the number of high risk species in this category (Figure A.2.5, left). Invasive species receive very little attention on Twitter, despite posing a high extinction risk to over 10,000 species. Climate change receives a relatively large amount of research and social media attention relative to the number of high extinction risk species, although this is not reflected in staff time. More broadly though, the focus of the academic community and conservation NGOs is closely aligned; there is a highly significant correlation between research and time effort for Habitats ($p < 0.001$), and a near-significant correlation for Threats ($p = 0.06$) (see Figure S1 in Supplementary Material). Full equations for the positive correlations between effort variables and species are as follows: research vs species for Threats $y = 319.80 + 0.41x$, $r^2 = 0.40$, $p < 0.05$; Twitter vs species for Threats $y = 39.12 + 0.038x$, $r^2 = 0.52$, $p < 0.05$; time vs species for Threats $y = 2.81 + 0.0024x$, $r^2 = 0.62$, $p < 0.01$; research vs species for Habitats $y = 29.26 + 0.79x$, $r^2 = 0.84$, $p < 0.001$; Twitter vs species for Habitats $y = 0.095x - 23.23$, $r^2 = 0.74$, $p < 0.001$; time vs species for Habitats $y = 4.37 + 0.0030x$, $r^2 = 0.63$, $p < 0.01$. The positive correlations between research and time effort: for Threats $y = 306.82x - 1394.32$, $r^2 = 0.35$, $p = 0.06$; for Habitats $y = 233.34x - 847.87$, $r^2 = 0.84$, $p < 0.001$.

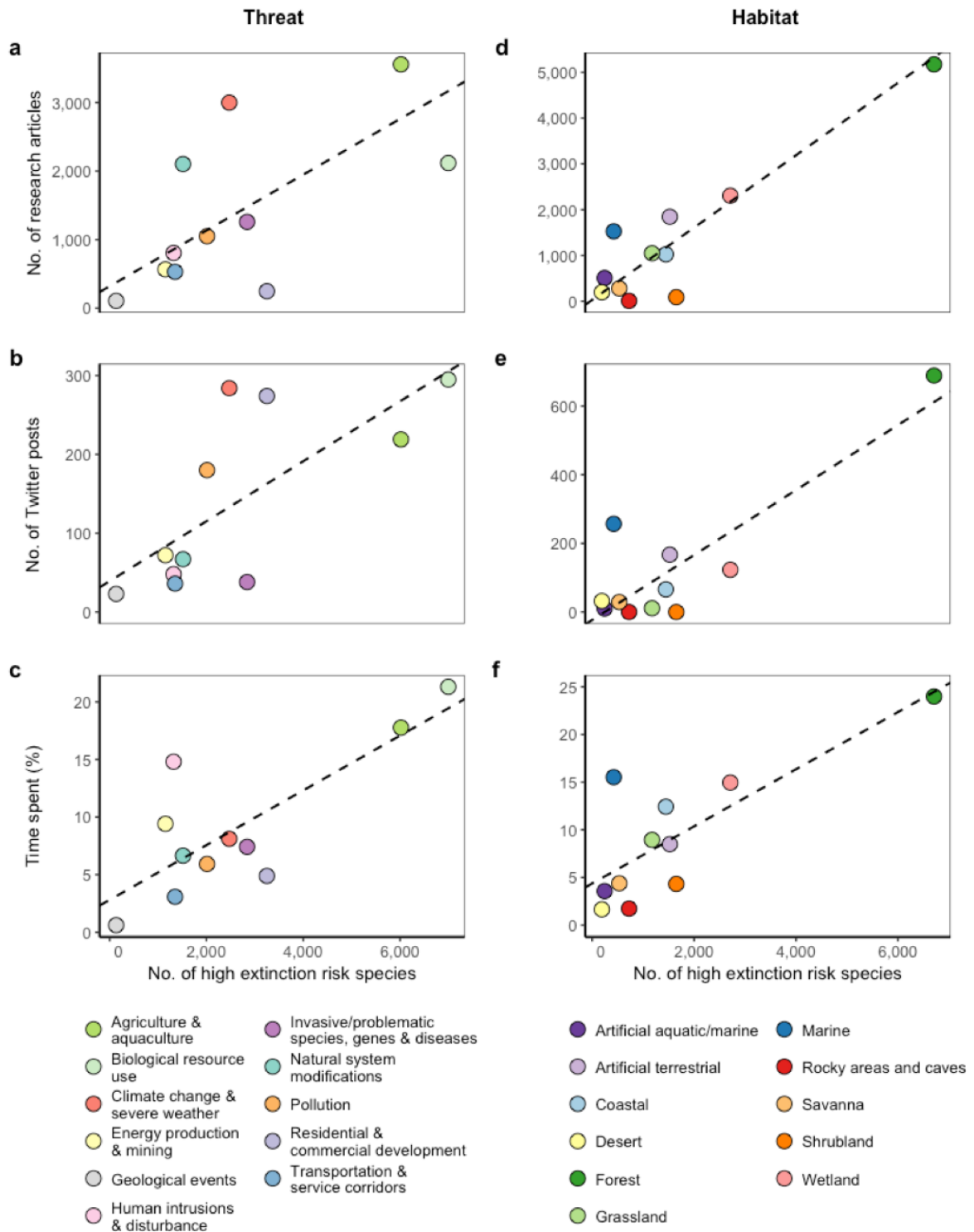


Figure A.2.5. Conservation effort relative to the number of high extinction risk species across Red List Threats and Habitats. Research data covers five years 01/01/2013 – 31/12/2017, Twitter data 150 consecutive days 31/12/2017 – 29/05/2018, and time data one year 01/08/2017 – 31/07/2018. Black dotted lines are major axis models (for A – F: $r^2 = 0.40, 0.52, 0.62, 0.84, 0.74, 0.63$ respectively; all $p < 0.05$). Points above the line receive above average effort per species, points below the line receive below average effort per species.

A.2.4. Discussion

The focus of global effort and conservation Red List priorities do in general line up, but there are key mismatches that cannot be ignored. Shrublands and rocky areas receive disproportionately little investment across research, social media and staff time effort measures relative to the number of high extinction risk species. Threats from residential and commercial development receive relatively low research and time investment despite high social media attention, and invasive species receive little media attention. Marine areas and climate change receive more attention than expected across all effort measures.

The presence of these mismatches corroborates with recent studies that are just beginning to understand whether the global community is distributing its conservation efforts appropriately. In 2018 it was shown that drivers of biodiversity loss do not always align with research efforts; 'habitat change' and 'pollution' drivers were falling behind conservation targets, a parallel seen in the lack of effort on residential and commercial development threats in our study²⁴⁷. Mazon et al. also showed there was insufficient conservation progress on 'invasive species', reflected in the low media attention in our study, and concerning given invasive species are the biggest factor in species extinction²⁴⁴. We also now know that biodiversity research is biased towards temperate regions^{242,243}. Our study did not assess geographic bias directly, but the high prevalence of European and North American conservation organisations in the Twitter and time investigations, and the fact that CCI as the largest cluster of conservation organisations in the world is based in Cambridge, UK, would support the presence of a temperate bias.

To avoid further expansion of mismatches, the global community should be made more aware of and improve its understanding of mismatches now. More species data are needed; at the time of this study, only 33,536 of the 91,000 species on the IUCN Red List had been comprehensively assessed. More information on the activities of governmental organisations is required, with studies to date focussing primarily on researchers and NGOs. We should also consider carefully which effort measures exhibit the most influence, whether it be research, social media, time, or others, to avoid mistakes and ineffective conservation action. Scientists caution that if tweets do not accurately convey conservation priorities misinformation can cascade through social media, and the same will apply across other

communication and organisational networks²⁴⁸. Today perfect allocation of conservation funds and resources to Threats and Habitats is perhaps an unrealistic goal, but improving our understanding of mismatches can help us come closer to achieving this goal.

Effort in conservation continues to grow as the threats posed to global biota become increasingly pressing. While maintaining species diversity may not be the primary goal of all conservation efforts, it is certainly a very important one²⁴⁹. Conservation researchers and practitioners would therefore be wise to consider which Habitats are at greatest risk of biodiversity loss and which Threats are the most important drivers when designing and implementing conservation strategies. In doing so, the greatest gains for global biodiversity may be achieved. Resources to protect biodiversity and our ecosystems are limited and the environmental, human, and economic costs of failure are of enormous magnitude. Effective allocation of our resources should be seen as a top global priority.

A.2.5. Footnotes

Data availability: All datasets for this study are included in the article above and the supplementary files available at <https://doi.org/10.3389/fevo.2019.00297>.

Author contributions: D.W., D.C.A. and K.S. participated in the design of the study and wrote the manuscript. All authors gave final approval for publication.

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Appendix 3: Feasting on terrestrial organic matter: Dining in a dark lake changes microbial decomposition

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Boreal lakes are major components of the global carbon cycle, partly because of sediment-bound heterotrophic microorganisms that decompose within-lake and terrestrially derived organic matter (t-OM). The ability for sediment bacteria to break down and alter t-OM may depend on environmental characteristics and community composition. However, the connection between these two potential drivers of decomposition is poorly understood. We tested how bacterial activity changed along experimental gradients in the quality and quantity of t-OM inputs into littoral sediments of two small boreal lakes, a dark and a clear lake, and measured the abundance of operational taxonomic units and functional genes to identify mechanisms underlying bacterial responses. We found that bacterial production (BP) decreased across lakes with aromatic dissolved organic matter (DOM) in sediment pore water, but the process underlying this pattern differed between lakes. Bacteria in the dark lake invested in the energetically costly production of extracellular enzymes as aromatic DOM increased in availability in the sediments. By contrast, bacteria in the clear lake may have lacked the nutrients and/or genetic potential to degrade aromatic DOM, and instead mineralized photo-degraded OM into CO₂. The two lakes differed in community composition and the higher concentration of dissolved organic carbon in the clear lake was associated with the community assemblages in the clear lake. Furthermore, functional genes relating to t-OM degradation were relatively higher in the dark lake. Our results suggest that future changes in t-OM inputs to lake sediments will have different effects on carbon cycling depending on the potential for photo-degradation of OM and composition of resident bacterial communities.

A.3.1. Introduction

Inputs of terrestrially derived organic matter (t-OM) are a major driver in lake ecosystems, but the ecological consequences of changes in their quantity and quality remain poorly understood²⁵⁰. Nearly a third of all the terrestrial carbon (C) input into inland waters is buried into lake sediments²⁵¹. Much of this burial, especially of larger particulate material, occurs in nearshore environments of small lakes through mechanisms including sedimentation and flocculation, where heavier matter and coagulated dissolved organic matter (DOM) fall out of suspension and accumulate^{252,253}. However, littoral sediments remain understudied relative to pelagic environments, especially in the context of future changes to t-OM exports. Terrestrial organic matter is increasingly exported into receiving waters, due to mechanisms including declines in anthropogenic sulfur deposition^{254,255} and increases in terrestrial primary production²⁵⁶ and soil decomposition²⁵⁷, and should thereby enhance sedimentation^{258,259}. Additionally, northward shifts in deciduous forests²⁶⁰, and the spread of fires and insect outbreaks²⁶¹, are expected to change the composition of t-OM that will be buried into receiving waters²⁶².

Although heterotrophic decomposition, particularly by bacteria, is the primary mechanism by which t-OM is assimilated into aquatic ecosystems^{250,263}, relatively little is known about how and why bacterial activity changes along gradients in the quantity and quality of buried t-OM. For example, bacterial production (BP) has been found to vary by 70% across freshwater sediments with different DOM concentrations²⁶⁴. One explanation for this is that bacteria assimilate low molecular weight (LMW) labile DOM quickly and easily so their production can be elevated in the presence of LMW compounds^{263,265,266}. By contrast, high molecular weight (HMW) compounds, including lignin, humic acid and aromatic molecules, require the production of enzymes that hydrolyze or oxidize complex structures into LMW compounds before they can be assimilated^{263,267–269}. Therefore, while HMW compounds are thought to be less amenable to microbial processing, the production of extracellular enzymes can allow bacteria to utilize these substrates and access both C and nutrients associated with terrestrially derived OM^{258,270–272}. Extracellular enzyme activity (EEA) should increase with concentrations of t-OM in lakes with limited LMW exudates^{273,274}, providing a source of C and nutrients, but may result in less bacterial growth as it comes with a high metabolic cost^{275,276}. Because extracellular enzymes are energetically

costly to produce, bacterial growth may even be stagnant or negative with increasing concentrations of HMW OM ²⁷⁷.

Environmental conditions, such as sediment light exposure and nutrient availability, will also interact with t-OM supply to dictate how bacterial communities will respond to future increases in the sedimentation of t-OM. For example, high light levels at the sediment-water interface can increase the availability of LMW phytoplankton exudates and photo-oxidize up to 70-95% of the HMW t-OM found in lakes into LMW compounds ^{263,278,279}. This process can therefore negate the reliance of bacterial communities on EEAs. By contrast, lakes with lower sediment light exposure will have reduced oxidation of dissolved compounds at the sediment-water interface, increasing their availability to bacteria and their ability to settle into sediments by forming organic particles ²⁵⁹. Bacteria may consequently rely on EEAs to acquire LMW C from HMW t-OM ²⁶³. Because EEA is the rate-limiting step for making HMW C available to bacteria, the total pool of available LMW C could also be smaller in these cases ²⁸⁰⁻²⁸². Enhanced BP from LMW compounds will also depend on an adequate supply of nutrients for building proteins and cells ^{266,283-286}. Changes in bacterial functions involved in heterotrophic decomposition, including EEA, may also arise because the ability to degrade HMW compounds is taxonomically restricted ^{274,287-289}. Consequently, shifts in bacterial community composition with increasing t-OM inputs will alter bacterial function ²⁹⁰. The functioning of whole communities can also change independently of composition if there is a high degree of redundancy amongst taxonomically distinct lineages ²⁸⁸. However, irrespective of the underlying cause of change, no study has yet connected actual and potential functioning of sediment bacterial communities to the quantity and quality of t-OM input into lake sediments. Understanding this connection can help improve predictions of future changes in diverse ecosystem processes such as C cycling, food web production, and water quality ²⁵⁰.

Here our aim was to characterize how bacterial activity changed along fine-scale gradients in the quality and quantity of t-OM in the littoral sediments of small boreal lakes. We addressed this aim by analyzing OM from pore water collected at the interface of the sediment and overlying lake water, where t-OM is primarily deposited ²⁹¹. We focused on the effect of terrestrially-sourced particulate OM as this is the primary way in which t-OM enters the littoral zone and benthic food web ^{292,293}. Boreal lakes also have varying water clarity and dissolved organic carbon (DOC) concentrations, ranging five-fold from approximately 4 to 20 mg C L⁻¹ ^{252,294}, so can have very different responses to changes in t-

OM additions because of these differences in overlying water quality²⁹⁵. We predicted that sediment bacteria would allocate carbon differently, either to enzyme production or CO₂ production, in a clear and dark lake with increasing additions of terrestrially derived OM, and we used metagenomics to reveal the underlying mechanisms for these changes. We tested our prediction in sediment mesocosms that mirror natural ecosystems in their biogeochemical dynamics and provide a controlled way to replicate t-OM inputs across lakes with contrasting water quality and clarity²⁹⁶. Microbial community composition and the environment are closely linked, and a major challenge is to decouple these two effects on microbial function²⁹⁰.

A.3.2. Materials and Methods

A.3.2.1. Study site

Our experiment was deployed in the nearshore region of two lakes near Sudbury, Ontario Canada: Lake Laurentian (46°27'9.74"N, 80°56'35.42"W) and Swan Lake (46°21'58.96"N, 81° 3'48.58"W). The sites have minimal human disturbance and are surrounded by similar early-successional forest, but differ in their overlying water quality. Swan Lake is more oligotrophic than Lake Laurentian, with mean (\pm SE) total phosphorus concentrations (mid-lake surface grabs taken during our sampling) of $9.3 \pm 0.4 \mu\text{g L}^{-1}$ versus $35.2 \pm 2.5 \mu\text{g L}^{-1}$ respectively. Swan also had more than four-times higher light levels at the sediment surface over the course of our experiment. Mean (\pm SE) light levels in Swan were $6378 \pm 149 \text{ lx}$ versus $1482 \pm 28 \text{ lx}$ in Laurentian, as measured every 60 minutes over the duration of our experiment using Hobo UA-002-64 light loggers. The loggers were installed on the sediment surface to measure light intensity reaching the sediment-water interface of the 24 mesocosms per lake. These differences were consistent with the overlying lake water, as DOC concentrations were 2 mg L^{-1} in Swan versus 7 mg L^{-1} in Laurentian. We hereafter refer to the two lakes as the “clear” and “dark” lake, respectively.

A.3.2.2. Experimental design

We submerged sediment amended with different types of t-OM on bottom of the study lakes in the nearshore environment (0.30 - 0.75 m depth) during July 2015 after Tanentzap et al. (2017)²⁹⁶. Submergence exposed the experimental sediments to natural

overlying water conditions. Briefly, sediments composed of 0, 5, 25, 35, and 50% t-OM (dry-weight basis) and locally sourced inorganic material were mixed with particle sizes and vertical structuring of all material mimicking natural lake sediments ²⁹⁶. We then filled 17.5 L, 50.8 × 38.1 × 12.7 (height) cm HDPE containers with 8 cm of sediments (total sediment volume ~ 15 L). For each t-OM quantity, material was added in a 1:2, 1:1, or 2:1 dry-mass ratio of deciduous to coniferous litterfall collected from nearby forests. Each of the 5 quantities × 3 qualities combinations were replicated 3 times in each lake. Mesocosms were arranged in a block design between two sampling bays, submerged in rows at increasing distance from the shoreline, and covered with a 1mm × 1mm nylon mesh screen to standardize shading and resuspension within lakes. Importantly, sediment pore water samples taken from our mesocosms have been found to reflect the biogeochemistry of natural lake sediments ²⁹⁶, allowing us to extrapolate our findings to field conditions.

The sediment manipulations interacted with lake conditions to produce experimental gradients in pore water OM quality and quantity within each lake, which we directly measured from optical properties and DOC concentrations, respectively (Figure S1). These pore water dissolved pools were distinct from the overlying lake water and represented the outcomes from mineralization of the added (i.e. sedimented) terrestrial OM. We then followed recommendations to analyze our responses in relation to these continuous gradients rather than the original factorial levels ²⁹⁷. Avoiding the use of categorical variables to represent predictors that are clearly continuous, e.g. levels of DOM released from sediment additions, has the added benefit of allowing us to develop predictive models that can demonstrate scientific understanding (see Houlihan et al. 2017 ²⁹⁸ for further discussion).

A.3.2.3. Water chemistry and gas sampling

We collected pore water samples from our mesocosms during three sampling periods in June, July and August of 2016. A 3 mL polypropylene syringe was secured horizontally immediately beneath the sediment surface along one side of the HDPE container prior to submergence in the lake. The wall of the syringe that faced the sediment was removed and covered in ca. 250 μm nylon mesh. Each sampling syringe was then connected to 122 cm of nylon tubing that was purged of water before any sample collection.

On each sampling period, we extracted 45 mL of pore water into an air-tight 60 mL syringe. pH was immediately measured with a handheld meter (HI 9126, Hanna instruments,

Woonsocket, RI, USA), before filtering 25 mL of each sample through a 0.5 μm glass fibre filter (Macherey-Nagel MN 85/90) and into a 20-mL glass scintillation vial. Glass vials were pre-acidified for a sample pH of approximately 2-3 to counter the well-documented effects of metal quenching of DOM fluorescence that becomes negligible below a pH of 3.0^{299,300}. In the lab, we measured two widely used DOM metrics using a Cary 60 UV Vis spectrophotometer and a Cary Eclipse fluorescent spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), after analyzing the samples for DOC concentration on a Shimadzu TOC-5000A (Shimadzu Co, Columbia, MD, USA). The first DOM metric was the specific UV254 absorbance (SUVA), an indication of the average aromatic fraction of DOM per unit DOC. Higher values of SUVA indicate a more reduced state due to intact ring structures which have yet to be oxidized, and tends to be HMW DOM³⁰¹. All UV absorbance values were corrected for total iron concentrations, because iron absorbs UV at a similar wavelength to SUVA, and can artificially increase measured SUVA values^{302,303}. Iron was measured using the FerroVer method (Hach Company 2014) with a Hach DR3900 spectrophotometer (HACH, Loveland, CO, USA). The second metric we measured was the humification index (HIX), for which higher values correspond to longer wavelengths of fluorescing molecules as humification of DOM proceeds³⁰⁴. In addition to DOC and DOM, we also measured total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) using persulfate digestion and ascorbic acid methods, respectively (Hach Company 2014³⁰⁵), with a Hach DR3900 spectrophotometer. TDP and TDN values that were below the detection limit were set to the minimum detection value (0.05 mg L⁻¹ and 1.0 mg L⁻¹, respectively).

We also inferred dissolved CO₂ concentration from the total inorganic carbon concentration, pH and temperature on the day of sample collection from a 45 mL water sample. 2 mL of 0.5M HCL were injected into the sample before drawing in 15 mL of ambient air, closing the stopcock, and shaking for 2 minutes to equilibrate the air and acidified sample. 10 mL of the headspace air was then collected in a gas syringe for measurement of CO₂ concentration on a SRI 8610C gas chromatograph within 48 hours of collection (SRI Instruments, Torrance, CA, USA). Concentration of CO₂ in the ambient air was measured from a volume of 10 mL and used to correct the headspace measurements. We calculated final pore water concentrations of CO₂ following methods from Aberg and Wallin (2014)³⁰⁶ by applying the Bunsen solubility coefficient and ideal gas law, accounting for pH and the ambient air concentration of CO₂.

A.3.2.4. Bacterial production

Bacterial production (BP) was measured using ^3H leucine incorporation after Pace et al. (2004)³⁰⁷. Duplicate 1.5 mL pore water samples were collected on ice in each of June, July and August 2016 approximately two weeks after sampling water chemistry to analyze how bacteria responded to antecedent DOM conditions. In the laboratory, we added 100 μL of 17.5 nM L-[4,5- ^3H] leucine (1 mCi mL⁻¹, PerkinElmer, Waltham, MA, USA) to each sample. Duplicate “kill” samples were prepared for each combination of OM quality and quantity in each lake. These samples immediately received 0.3 mL of 50% trichloroacetic acid (TCA), killing any live cells, and providing a measure of background ^3H leucine incorporation that was later subtracted from the incubated values. After incubating for 1 hour, each sample received 0.3 mL of 50% TCA to stop ^3H leucine uptake. Samples were subsequently centrifuged at 14,000 rpm for 10 minutes and the supernatant was discarded. Each sample was then washed twice with 5% TCA, dried using a vacuum centrifuge, and stored at -20 °C. ^3H incorporation was later measured using a Beckman Coulter LS6500 liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Prior to counting, each sample received 1 mL of Optiphase Hisafe 2 Scintillation fluid and was vortexed for 30 seconds. This step was repeated twice more for a total sample volume of 3 mL. Decays per minute measured on the scintillation counter were then converted to BP using standard conversion factors³⁰⁸.

A.3.2.5. Extracellular enzyme activity

We assayed five hydrolytic enzymes (β -1,4-glucosidase, β -^D-1,4-cellobiosidase, β -1,4-xylosidase, leucine aminopeptidase, and phosphatase) and two oxidative enzymes (peroxidase and phenol oxidase) in pore water collected in July and August of 2016. Samples were taken one week after water chemistry sampling to test how bacterial enzyme exudation responded to DOM, and one week before BP sampling to test how enzyme activities affected subsequent bacterial C utilization. Pore water samples were assayed directly without dilution²⁶⁷, and without buffer to capture the in-situ pH³⁰⁹. Optimal incubation times for hydrolytic enzymes were determined by standard time trials as recommended and described in German et al. (2011)³⁰⁹. We found these times to be 2 hours for β -1,4-glucosidase and leucine aminopeptidase and 1 hour for β -^D-1,4-cellobiosidase, β -1,4-xylosidase, and phosphatase. Timings of oxidative incubations were performed as suggested in Sinsabaugh et al. (2003)³¹⁰. Fluorescence and absorbance were read on a Synergy H1 microplate reader (BioTek, Winooski, VT, USA). β -^D-1,4-cellobiosidase,

β -1,4-xylosidase, and β -1,4-glucosidase were summed as total hydrolase activity, as these enzymes collectively target structural components of plant-derived OM³¹¹. Similarly, peroxidase and phenol oxidase activity were expressed as total oxidase activity because they have a similar functional role^{273,310}.

A.3.2.6. Metagenomics shotgun sequencing

DNA was extracted in duplicate from each mesocosm in August 2016 using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc, Carlsbad, CA, USA), and following the manufacturer's protocol. Sequencing libraries were prepared with 1 ng of genomic DNA per sample using the Nextera XT DNA Sample Preparation and dual-barcoding with Nextera XT Indexes (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Libraries were quantified on a Qubit 3.0 Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and on a Bioanalyzer HS DNA chip (Agilent, Santa Clara, CA) and pooled in equimolar concentrations into a single sample. Samples were sequenced on an Illumina platform using a NextSeq 500/550 Mid Output Kit v2 (300 cycles, paired-end).

Raw sequences were processed at a read depth of approximately 3.3 million reads per sample following the European Molecular Biology Laboratory-European Bioinformatics Institute (EBI) pipeline version 3.0³¹². In brief, the SeqPrep tool (<https://github.com/jstjohn/SeqPrep>, version 1.1) was used to merge paired-end overlapping reads, Trimmomatic (version 0.35)³¹³ was used to trim low quality ends and sequences with >10% undetermined nucleotides, and sequences <100 nucleotides were removed using Biopython (version 1.65)³¹⁴. Sequences were functionally annotated by predicting coding sequences (pCDS) above 60 nucleotides with FragGeneScan (version 1.20)³¹⁵. Read matches were then generated against pCDS using a subset of databases from InterProScan (version 5.19-58.0)³¹⁶ and summarized using the Gene Ontology terms. Sequences were taxonomically annotated with QIIME (version 1.9.1)³¹⁷. Representative 16S sequences were classified using the SILVA reference database (release 128)³¹⁸ at 97% sequence identity following the open-reference Operational Taxonomic Unit (OTU) picking method with reverse strand matching enabled³¹⁷. The sequences were deposited in EBI Metagenomics under the project accession number ERP019980. We normalized both the functional and OTU datasets by transforming the abundance of annotated sequences to abundances relative to the per sample total to account for sequencing bias^{319,320}. We focused our analysis on the subset of functional genes that matched the groupings of

hydrolytic ("cellulase activity", "glucosidase activity", and "xylan 1,4-beta-xylosidase activity") and oxidative enzymes ("peroxidase activity" and "catechol 1-2-dioxygenase activity") measured for extracellular activity, as well as genes required to catabolize aromatic DOM ("aromatic compound catabolic process") and genes for oxidoreductase activity involving oxygen ("oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen"), an important step in the decomposition of aromatics²⁷³.

A.3.2.7. Statistical analysis

We tested the effects of DOM quantity and quality on bacterial responses using linear mixed-effects models with the *lmer* function in the *lme4* package in R v3.2³²¹. Bacterial responses included BP, CO₂ concentrations, hydrolase activity, oxidase activity, phosphatase activity, and leucine amino peptidase activity. We transformed responses with a quarter-power rather than logarithmically because small values in some of the responses resulted in heavy left-tail-distributions with the latter. Each response was then predicted as a function of DOC, SUVA and HIX, in addition to sampling month and environmental variables pH, TDN and TDP. The model for CO₂ also included BP as a predictor because CO₂ is a combined product of bacterial respiration and photo-mineralization, which can account for up to 50% and 90% of total production respectively, depending on environmental conditions^{263,279}. We accounted for the blocking design of our experiment, whereby the mesocosms were distributed around two sampling bays, by including bay as a fixed factor. We also accounted for repeated measurements of the same mesocosm and blocking row to account for proximity to shoreline and decreasing depth by including these factors as random effects. Finally, we allowed mean responses to vary between the dark and light lake (i.e. lake fixed effect) and tested whether the effects of DOC, DOM quality, and BP (for CO₂ response) differed between lakes with contrasting light penetration (i.e. a statistical interaction). Temperature was excluded from the models because it did not vary among mesocosms within lakes (mean \pm SE of 15.85 ± 0.03 °C and 14.0 ± 0.02 °C in dark and clear lake, respectively).

We determined the best-fitting model that explained our responses using backwards stepwise selection. Models were compared using the Akaike Information Criterion (AIC) and we sequentially removed predictors that did not increase AIC by more than 2, ensuring the more parsimonious models also never had AICs that were more than 2 values higher than the lowest observed value across the entire model set. Main effects were only dropped after

their interactions. We assessed the significance of factors in the best supported model with p -values calculated from a t -distribution using the R package *lmerTest*³²². Degrees of freedom were adjusted with the Kenward-Roger method, which reduces bias in small sample settings³²³.

We also analyzed the relative abundances of OTUs and functional genes relating to t-OM degradation to identify potential reasons for lake-dependent bacterial responses. First, we tested for a compositional difference between lake bacterial communities using a permutational multivariate analysis of variance (perMANOVA) with Bray-Curtis distances between samples. We also tested whether compositional differences were associated with sediment pore water DOC, SUVA, HIX, and pH within mesocosms of each lake by including these variables in the perMANOVA. Significance of marginal effects was assessed with 1000 permutations of the raw data, which were restricted to each lake for the covariates, using the *adonis2* function in the R package *vegan* version 2.5-2³²⁴. We did not include TDN and TDP in these analyses to minimize collinearity; this issue was not a concern for linear mixed-effects models as none of the parameter estimates were strongly inter-correlated ($r > 0.75$). Differences in OTU relative abundance between lakes were visualized with a non-metric multidimensional scaling (NMDS) ordination. We also used a perMANOVA with Bray-Curtis distances to test whether the relative abundances of the subset of functional genes could be explained by environmental variables and lake identity. Again, we visualized statistically significant factors ($p < 0.05$) using a NMDS ordination. In a separate perMANOVA, we tested whether the original manipulations of t-OM quantity and quality were also correlated with the composition of pore water bacterial communities and functional genes. Finally, we tested if the relative abundance of the subset of functional genes differed on average between lakes using paired t -tests. We paired mesocosms that were in the same position in our experimental block design but located in different lakes.

Finally, we quantified the difference in bacterial community composition between the natural sediments of the two study lakes at the start of the experiment. We calculated the Bray-Curtis dissimilarity index between six pooled samples from each lake, where a value of 0 indicated communities were identical and a value closer to 1 indicated communities were entirely different, i.e. no overlapping OTUs³²⁵.

A.3.3. Results

A.3.3.1. Bacterial responses to t-OM addition depended on lake clarity

We found that BP decreased with the aromatic fraction of DOM (i.e. SUVA) across both lakes ($t = -2.04$, $df = 156$, $p = 0.042$), such as if the bacteria expended more energy to break down aromatic DOM than to grow. For example, a 25% increase above the mean aromaticity of $3.37 \text{ L mg-C}^{-1} \text{ cm}^{-1}$ decreased BP below its mean of $0.54 \text{ g C L}^{-1} \text{ d}^{-1}$ by 14.5% (Figure A.3.1a.). The decrease in BP with an increasing fraction of aromatic pore water DOM in the dark lake was associated with a small increase in CO_2 production ($t = -2.68$, $df = 158$, $p = 0.008$, Figure A.3.1b.), suggesting that bacteria in the dark lake were incurring a higher metabolic and respiratory cost associated breaking down aromatic OM. Specifically, CO_2 decreased by 2% below the mean value of $8.59 \text{ mg-CO}_2 \text{ L}^{-1}$ with a 25% increase in BP. In contrast to the dark lake, greater UV exposure at the sediment-water interface in the clear lake may have led to photo-oxidation of DOC that increased CO_2 production (lake effect: $t = 13.38$, $df = 155$, $p < 0.001$). Greater photo-degradation in the clear lake may have also resulted in a lower concentration of aromatic DOM relative to the dark lake (two-sample t -test: $t = -2.64$, $df = 143$, $p = 0.009$, Figure A.3.2a.). Despite the higher pore water DOC concentration in the clear lake, of which less was aromatic and presumably more was LMW C (Figure A.3.2a-b.), BP was lower on average ($t = -6.54$, $df = 120$, $p < 0.001$). The lower BP likely arose because of nutrient limitation that was inseparable from the lake-level effect (Figure A.3.2d-e.). On average, the clear lake always had 13.6 and 15.0% less available N and P, respectively, relative to C (i.e. higher pore water C:N and C:P ratios) when comparing the same mesocosms between lakes ($t = 7.59$, $df = 118$, $p < 0.001$; $t = 16.74$, $df = 100$, $p < 0.001$). Full results of model selection are given in Table S1.

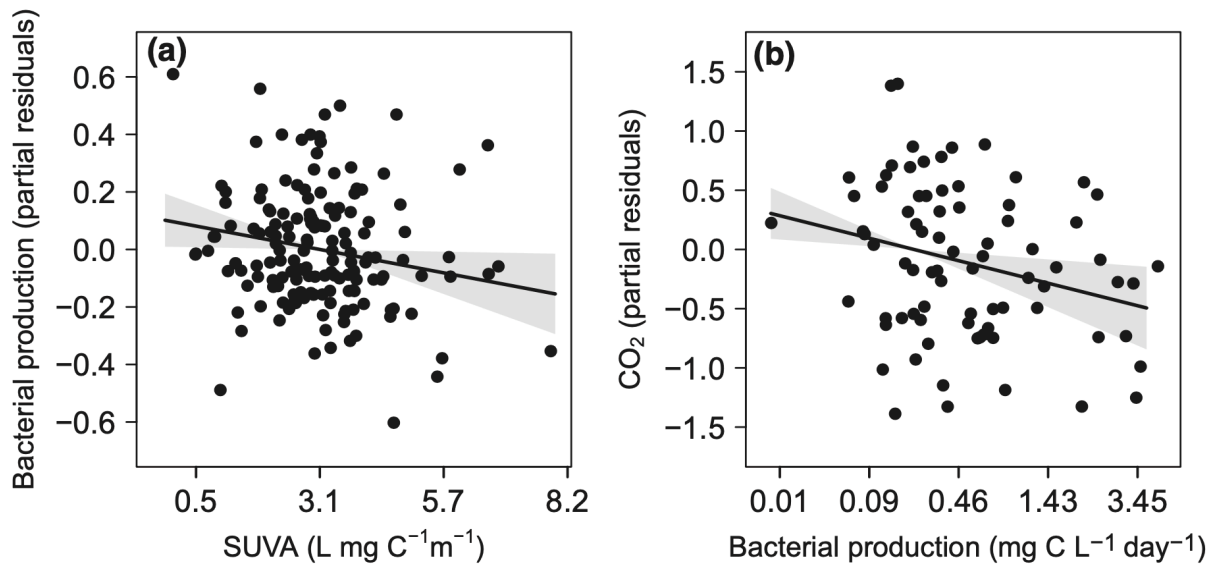


Figure A.3.1. Energetic tradeoffs in the dark but not the clear lake. (a) BP decreased with specific ultraviolet absorbance (SUVA) across both lakes, resulting in (b) more CO₂ production where BP was lower, but only in the dark lake. Solid lines are mean effects \pm 95% confidence intervals

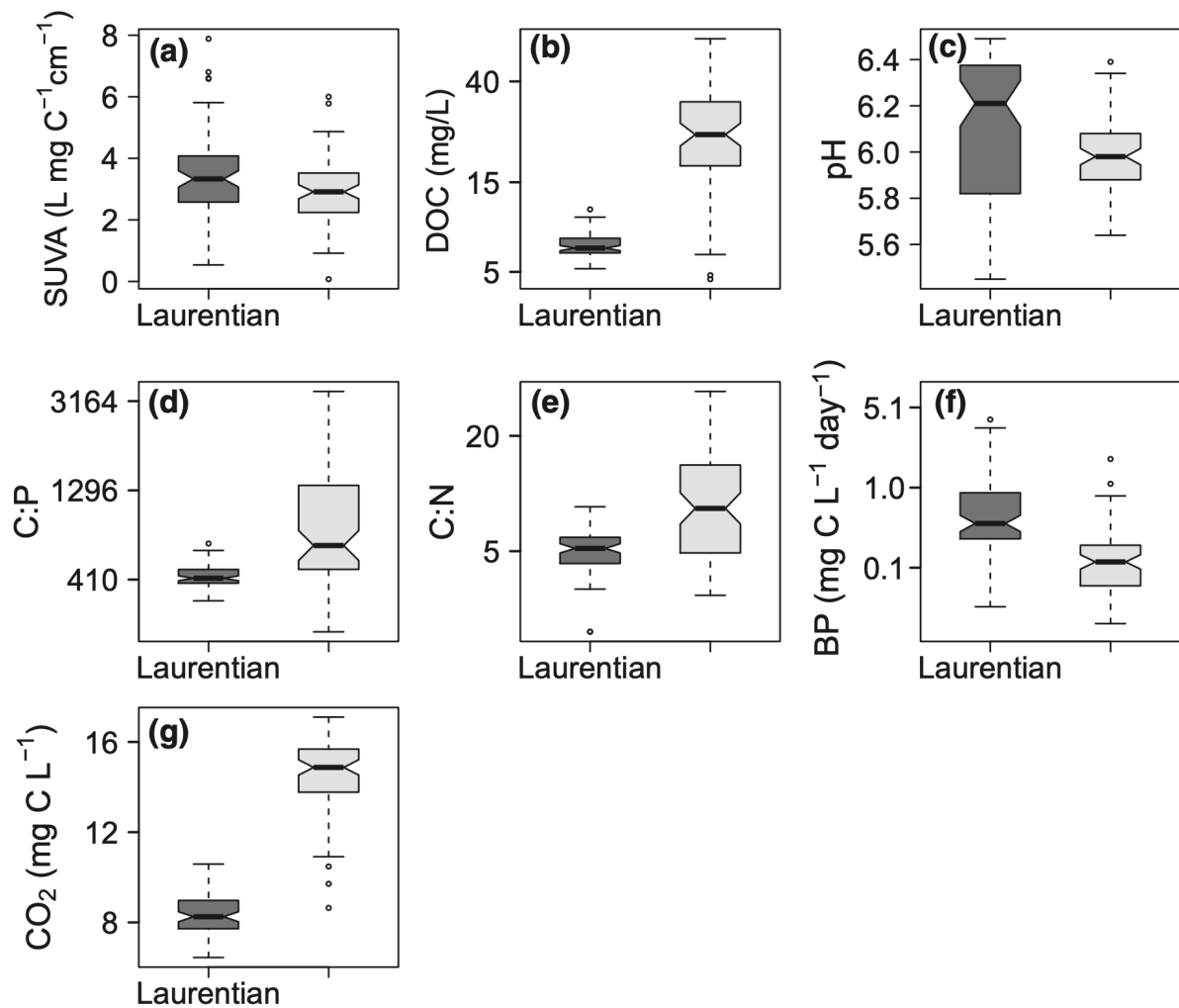


Figure A.3.2. Pore water differed between mesocosms in the dark (LAU) and clear (SWA) lake. The dark lake had (a) more SUVA, (b) lower DOC concentrations, (c) slightly higher pH, (d) lower C:P and (e) C:N ratios, (f) more BP, and (g) less CO₂ production. Nonoverlapping notches indicate differences in the two medians based on 95% confidence intervals³²⁶. The upper and lower whiskers extended 1.5 times the interquartile range, with points outside of this range plotted

A.3.3.2. Extracellular enzyme production enabled aromatic OM decomposition in the dark lake

The decrease in bacterial productivity in the dark lake corresponded with a positive association between hydrolytic and oxidative enzyme activity and the fraction of aromatic pore water DOM. Bacteria were presumably limited for LMW substrates in the dark lake and subsequently produced more hydrolytic and oxidative enzymes with higher fractions of SUVA ($t = 2.09$, $df = 99$, $p = 0.039$ and $t = 4.27$, $df = 111$, $p < 0.001$, respectively; Figure A.3.3.). For example, a 25% increase in the mean aromatic fraction of $3.37 \text{ L mg C}^{-1} \text{ cm}^{-1}$

increased oxidative and hydrolytic enzyme activity by 14.6% and 11.4% above the mean values of $0.05 \mu\text{mol mL}^{-1} \text{h}^{-1}$ and $230.0 \text{ nmol mL}^{-1} \text{h}^{-1}$, respectively. There was no response to aromaticity in the clear lake for oxidative ($t = -1.33$, $df = 112$, $p = 0.100$) or hydrolytic enzymes ($t = 0.66$, $df = 106$, $p = 0.419$), suggesting that bacteria were not utilizing terrestrially derived OM for growth. Both phosphatase and leucine aminopeptidase had no response to DOM quality or DOC concentration (Table S2).

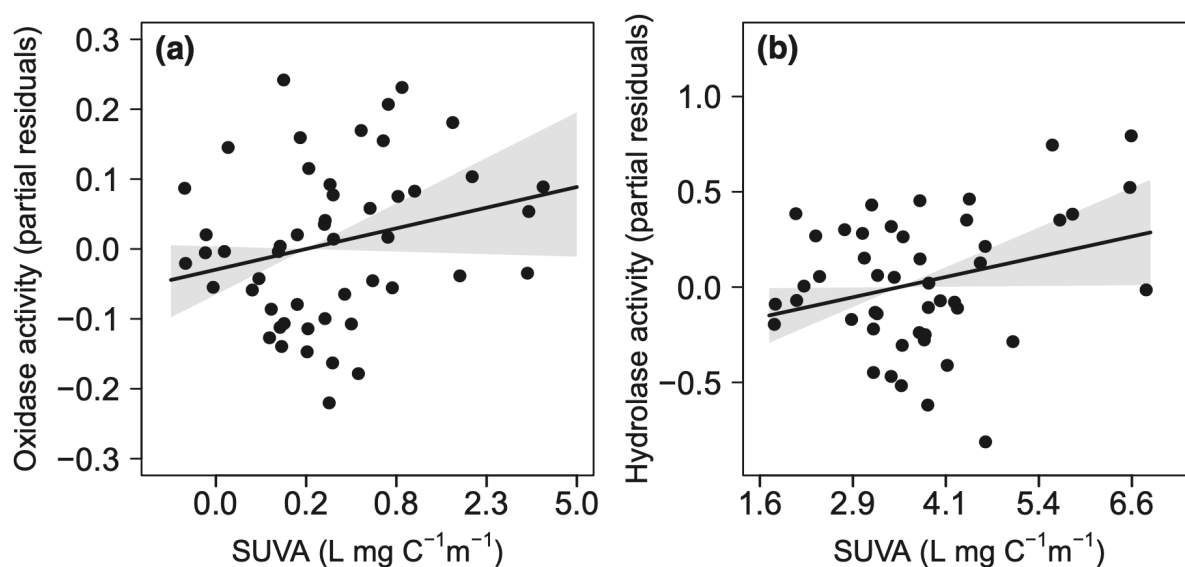


Figure A.3.3. Enzyme activities increased with aromaticity of DOM in the dark lake. (a) Oxidase and (b) hydrolase activity increased with specific UV254 absorbance (SUVA). Solid lines are mean effects $\pm 95\%$ confidence intervals

A.3.3.3. Different bacterial communities underlie varying responses to t-OM addition

We found evidence that, in addition to differences in available nutrients, bacterial activity might have also differed between the two lakes because of underlying differences in community composition and subsequent functional potential to decompose t-OM. The relative abundance of pore water bacterial OTUs differed significantly between the two lakes across all mesocosms ($F_{1,49} = 2.01$, $p = 0.001$, Figure A.3.4a.). Correspondingly, we found that genes relating to oxidative enzymes ($t = 4.42$, $df = 24$, $p < 0.001$), aromatic catabolism ($t = 9.22$, $df = 24$, $p < 0.001$) and oxidoreductase activity ($t = 18.71$, $df = 24$, $p < 0.001$) were on average 1.1 – 3.0 more times abundant in the darker lake where microbial activity responded more strongly to increasing fractions of aromatic OM (Figure A.3.5a-c.). There were slightly higher abundances of hydrolytic genes in the clear lake ($t = -2.38$, $df = 24$, $p =$

0.025, Figure A.3.5d.), but because enzyme expression and exudation may be more strongly induced by low concentrations of LMW substrates than the presence of HMW DOM, the clear lake bacterial communities did not produce more enzymes in response to increasing DOC quantity (Table S2).

The observed differences in bacterial community composition and function may have arisen because in-lake processes, including photo-oxidation at the sediment-water interface, caused the added t-OM to diverge between lakes. Communities varied with pore water DOC ($F_{1,49} = 1.42$, $p = 0.037$) and pH gradients in each lake ($F_{1,49} = 1.48$, $p = 0.009$; Figure A.3.4a.), which themselves differed between lakes (Figure A.3.2.). Functional genes capable of degrading inputs of t-OM also differed between the two lakes ($F_{1,49} = 4.13$, $p = 0.035$) and varied along gradients of humic DOM ($F_{1,49} = 4.63$, $p = 0.030$) and pH in sediment pore water of each lake ($F_{1,49} = 5.24$, $p = 0.031$; Figure A.3.4b.). Consistent with these results, we found that the composition of pore water bacterial communities changed with the original quantity and quality of t-OM added in each lake ($F_{1,50} = 2.75$, $p = 0.016$; $F_{3,50} = 3.48$, $p = 0.001$, respectively). The presence of functional genes mirrored these results ($F_{1,50} = 2.22$, $p = 0.001$; $F_{3,50} = 1.80$, $p = 0.001$, respectively). Both lakes also had very different resident bacterial communities at the start of our experiment, as expected if the existing environment was responsible for altering the quality of t-OM received by the sediment bacteria (Bray-Curtis dissimilarity between lakes = 0.90).

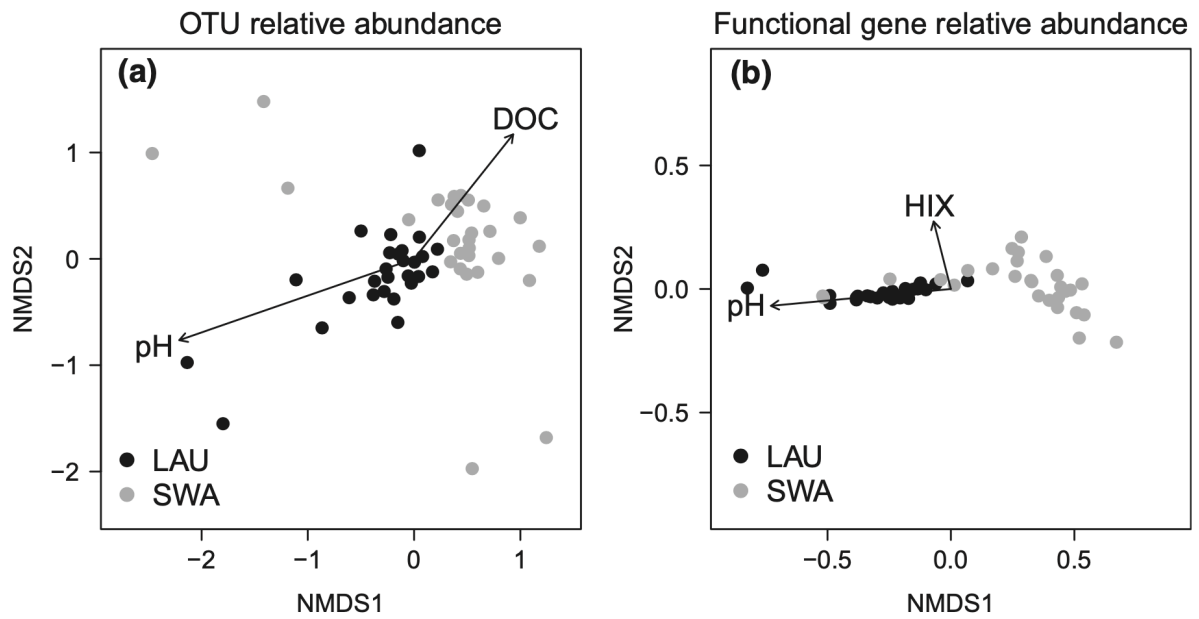


Figure A.3.4. Bacterial community and functional composition differ between the dark (LAU) and clear (SWA) lake. (a) Nonmetric multidimensional scaling (NMDS) of OTU abundance in individual mesocosms (stress = 0.13), where lake, pH, and dissolved organic carbon (DOC) were correlated with OTU relative abundances within mesocosms (for all, $p < 0.05$ with perMANOVA). (b) NMDS of relative abundance of functional genes related to t-OM degradation (stress = 0.02), where lake, pH, and HIX and were correlated with functional gene composition within mesocosms (for all, $p < 0.05$ with perMANOVA)

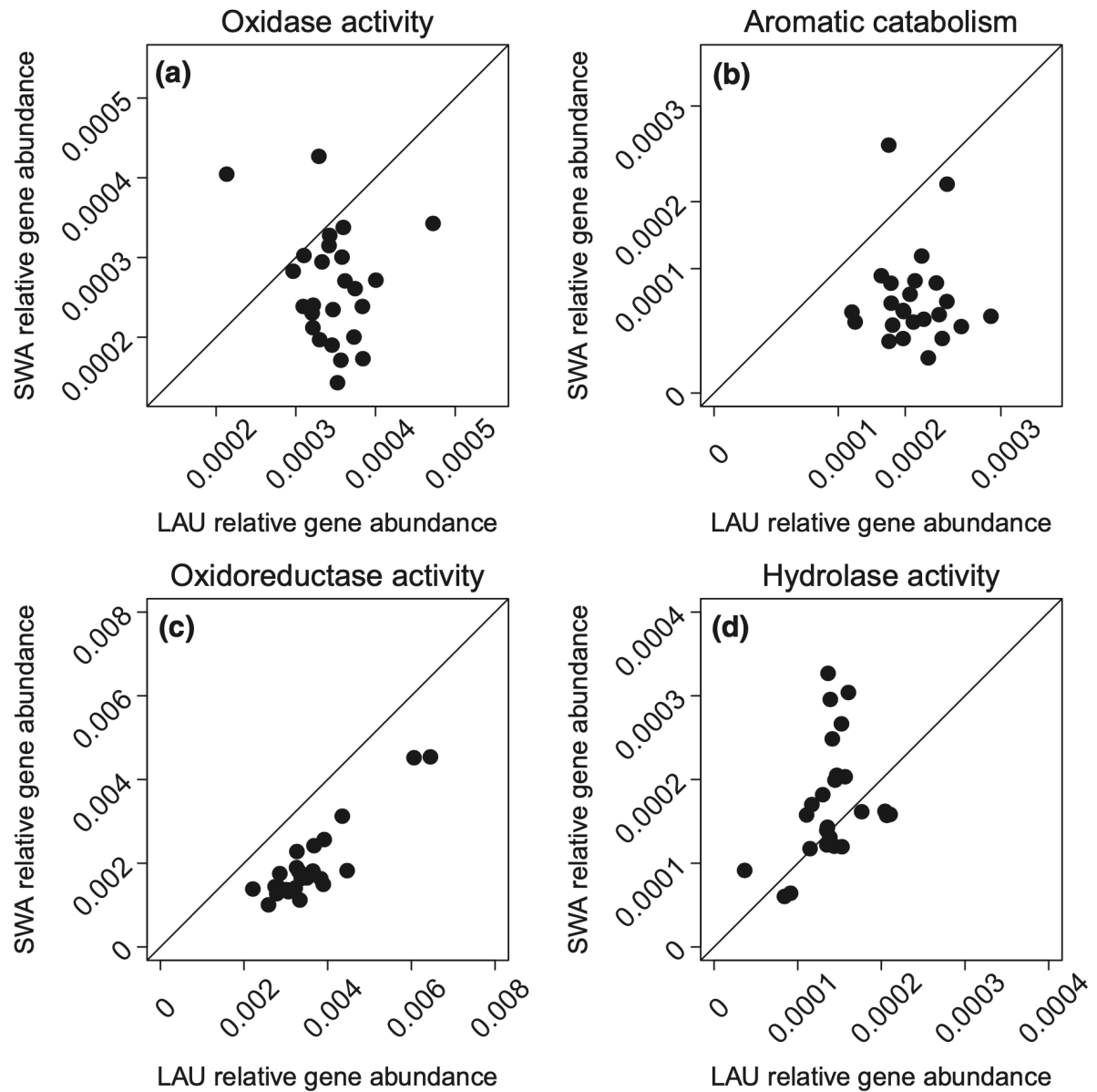


Figure A.3.5. Functional genes involved in OM degradation were relatively more abundant in the dark than clear lake. Genes considered were those involved in (a) oxidase activity, which includes the sum of peroxidase and catechol, 1-2-dioxygenase gene abundances, (b) aromatic catabolism, (c) oxidoreductase activity, and (d) hydrolase activity, which is the sum of glucosidase, cellobiosidase, and xylosidase gene abundances. The line indicates a 1:1 ratio. Points above or below the line indicate a higher abundance of genes in the clear and dark lake, respectively

A.3.4. Discussion

Our analysis of sediment pore water at the interface between sediments and the overlying water column in a dark and a clear lake shows that environmental conditions interact with community composition to control the functioning of bacterial communities and ultimately their responses to t-OM additions. Despite the same t-OM being added to the experimental sediments in each lake, biogeochemical processing differed. More DOC and CO₂ and less aromatic DOM was produced in the sediment pore water of the clear lake than in the dark lake. The bacterial community in the dark lake had higher relative abundances of functional genes related to t-OM decomposition, enabling greater enzyme activity. A relatively lower abundance of these functional genes limited the bacterial response to aromatic DOM in the clear lake. The overall higher amount of CO₂ production in the clear lake suggested that the bacterial community instead mineralized the photo-oxidized, LMW DOM^{327,328}. Mineralization likely arose because of nutrient limitation relative to the dark lake, where terrestrial nutrient inputs were higher and bacterial production could be sustained at a higher overall level (Figure A.3.2f.;^{285,329}). As future influxes of t-OM increase across boreal lakes^{291,330}, these water clarity-dependent responses suggest CO₂ release may be greater in clear lakes with high levels of photo-oxidation at the sediment surface³³¹. Dark lakes may instead experience a decrease in primary production and shift toward retaining rather than mineralizing terrestrial carbon, such as by burying it in sediment^{332–334}. However, over the longer term, the darkening of clear lakes will reduce light exposure on the sediment surface, increasing OM burial and encouraging bacterial communities to develop in sediment pore water that can utilize this material^{335,336}.

The ability for bacteria in the dark lake sediments to break down increasing fractions of terrestrially derived organic matter was possible because they had the genes to produce hydrolyzing and oxidizing enzymes^{279,336}. Although this may have allowed the dark lake bacteria to access nutrients and bioavailable growth-promoting amino acids associated with t-OM, the metabolic cost of producing enzymes likely resulted in less growth^{290,329,337,338}. Additionally, bacteria in the dark lake had a limited availability of LMW substrates from photosynthetically-derived and photo-oxidized OM^{339,340}. The availability of LMW substrates was further limited by the rate at which extracellular enzymes could oxidize and hydrolyze HMW t-OM. As there was no resulting pool of excess LMW substrates to facilitate metabolic

cycling^{277,327}, the sediment bacterial community in the dark lake consequently did not produce excess CO₂ in addition to the respiration required for basal metabolic function, and instead produced extracellular enzymes to degrade DOM with a greater terrestrial signature^{273,286,311,341}.

In the clear lake, bacterial metabolic cycling of a LMW DOC pool and direct photo-mineralization at the sediment surface likely accounted for higher CO₂ production. Previous work has shown that sunlight exposure photo-degrades aromatic DOM and leads to higher concentrations of LMW compounds^{340,342,343}. This process could lead to more bioavailable carbon and elevated CO₂ production^{331,344}. However, growth of sediment bacteria in the clear lake may have been limited by the lower availability of N and P relative to C (per mass of DOC) (Figure A.3.2d-e.;^{266,283,285,345}, despite the available LMW compounds. This nutrient-limitation can explain why communities would have metabolically cycled C instead of allocating it to biomass, thus producing more CO₂ than the dark lake^{266,285,327,346–348}. Therefore, increasing t-OM influx may have varied effects on in-lake carbon cycling³⁴⁴. Photo-mineralization can also produce up to 90% of CO₂ in aquatic systems, and may have accounted for some of the CO₂ observed in the clear lake pore water²⁷⁹. This high rate of photo-oxidation in the clear lake as opposed to the dark lake also altered the mesocosm pore water DOM quality, which may have shifted the composition of the clear lake bacterial community to taxa that were better able to degrade photoproducts, whereas the dark lake community may have been pre-disposed to degrade t-OM^{279,336,349,350}.

Our results add to the growing evidence that terrestrially derived OM is not recalcitrant^{270,338,351–353}. Rather, t-OM can undergo photo-induced transformations and be degraded by EEAs, leading to CO₂ production in lake sediments³⁵⁴. Although bacterial activity was not directly stimulated by aromatic OM, it was relatively higher in lake sediments dominated by aromatic OM, thereby suggesting that the terrestrially sourced fraction of DOM is relatively bioavailable and has a potentially high turn-over rate^{339,355}. These findings challenge the paradigm that t-OM is highly degraded, recalcitrant, and otherwise unavailable to microbial degradation due to its chemical composition^{336,348,352,356,357}. Consequently, we suggest that t-OM inputs to lakes will not remain unchanged in lake sediments, but will be altered by microorganisms and photo-processes, which may change carbon budgets and/or transfer heterotrophic productivity to higher trophic levels^{352,356,358,359}. More broadly, our results advance previous work by showing how

the fate of t-OM in lake sediments will ultimately depend on local environmental conditions and the bacterial community's genetic potential to degrade different OM qualities.

We found that variation in the functional genes of sediment bacteria and nutrient availability could explain differences in t-OM utilization between two lakes of contrasting water clarity. This finding suggests that future inputs of t-OM, such as those observed across the Boreal region ^{250,255,360}, can have dramatically different outcomes for whole-lake C cycles depending on lake-specific characteristics ^{288,348,361}. In clear lakes, increased inputs of t-OM may induce a positive feedback loop, whereby increased CO₂ production stimulates primary production, leading to additional inputs of LMW compounds from algae ^{362,363}. In contrast, elevated inputs of t-OM in dark lakes could lead to an increase in organic matter burial and an increasingly heterotrophic food web ^{333,358}. Future predictions of how t-OM will impact whole-lake processes clearly need to consider lake-specific characteristics, such as water clarity and nutrient availability.

A.3.5. Footnotes

Data availability: All datasets for this study are included in the article above and the supplementary files available at <https://doi.org/10.1111/gcb.14391>.

Author contributions: A.F., C.O., D.W., E.J.S.E., and A.J.T. participated in the design of the study, experimental work, and wrote the manuscript. All authors gave final approval for publication.

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