ASTRAL

All Atlantic Ocean **S**ustainable, Profi**T**able and **R**esilient **A**quacu**L**ture

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Abbreviations

ABCC	*Associação Brasileira de Criadores de Camarão*
AbHV	Abalone Herpes Virus
AEW	Abalone Effluent Water
AGD	Ameobic Gill Disease
AMVRT	Avian Myeloblastosis Virus Reverse Transcriptase
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variants
AVG	Abalone Viral Ganglioneuritis
BFT	Biofloc system
CNRS	*Centre national de la recherche scientifique*
CONICET	*Consejo Nacional de Investigaciones Científicas y Técnicas*
CPGR	Centre for Proteomic and Genomic Research
ddPCR	Digital Droplet Polymerase Chain Reaction
DFFE	Department of Forestry, Fisheries, and the Environment
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic acid
dPCR	Digital Polymerase Chain Reaction
DVP	Differential pulse voltammetry
ELISA	enzyme-linked immunosorbent assay
eNA	Environmental nucleic acids
HACCP	Hazard Analysis Critial Control Point
HDA	helicase dependent amplification
HRM	high-resolution melt curve
IHNV	Infectious hematopoietic necrosis virus
IMTA	Integrated multi-trophic aquaculture
LAMP	Loop-mediated amplification
LFB	Lateral Flow Biosensor
LSU	Large Subunit
LTA	Low-trophic aquaculture
MLRA	Marine Living Resources Act
NAAT	Nucleic acid amplification technique
NAs	Nucleic Acids
NASBA	Nucleic acid sequence-based amplification

NASF	National Aquatic Animal Health Strategic Framework
NMDS	Non-metric multi-dimensional scaling
OIE	World Organization for Animal Health
OsHV	Oyster Herpes Virus
PCR	Polymerase chain reaction
PD	Pancreas Disease
PERMANOVA	permutational multivariate ANOVA
QCM	Quartz Crystal Microbalance
QIIME2	Quantitative Insights into Microbial Ecology 2 program
qPCR	Quantitative polymerase chain reaction
RAS	Recirculating Aquaculture System
RCA	Rolling ciRcle amplification
RFTM	Ray's fluid thioglycolate culture method
RLE	relative log expression
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Real Time – Polymerase Chain Reaction
SAMS	Scottish Association for Marine Science
SAMS	Scottish Association for Marina Science
SAV	Salmonid alphavirus
SDA	Strand Displacement Amplification
SEAP	*Secretaria Especial de Aqüicultura e Pesca*
SENASA	Servicio Nacional de Sanidad y Calidad Agroalimentaria
SHARP	single-stranded DNA binding protein and helicase assisted rapid PCR
SPIA	single primer isothermal amplification
SPR	Surface Plasmon Resonance
SPS	Application of Sanitary and Phytosanitary
SRS	Salmonid Rickettsial Septicemia
SW	Sea water
UCT	University of Cape Town
VHSV	Viral haemorrhagic septicaemia
WOAH	World Organization for Animal Health
WSN	Wireless Sensor Network
WTO	World Trade Organization
WUD	Winter ulcer disease

1 Summary

This document was created as part of the H2020 All Atlantic Ocean Sustainable, Profitable and Resilient Aquaculture (ASTRAL) project. The aim of this deliverable is to describe the main microbial pathogens that are capable of severely affecting productivity in each of the ASTRAL IMTA Labs, describe the current detection methods in aquaculture facilities and identify emerging technologies to improve early warning systems. To contextualize the main pathogen issues in each IMTA lab, several directives and categorizations of fish health status and pathogen risks were identified and summarized. Subsequently, key pathogens for the aquaculture industry, and the current diagnostics used to detect them, were described based on an extensive literature review and the experience of each IMTA lab. Moreover, identified pathogens and bacterial diversity were provided for land-based IMTA labs located in Brazil and South Africa. Finally, molecular tools, biosensors and predictive models were proposed to improve current monitoring methods of the main species-specific pathogens described in the first part of the deliverable.

The results obtained will be useful to i) be aware of the directives that apply to the health status of cultured organisms in each IMTA laboratory depending on the country; ii) understand which potential pathogens can occur in each geographic region or IMTA system type and the environmental stressors linked to their infections; and iii) find innovative forms of monitoring using new and emerging technologies to tackle pathogens within the system anticipating potential outbreaks.

2 Introduction

Globally, aquaculture has contributed significantly towards food security, sustainable livelihoods, and job creation, and is currently the fastest growing food producing sector, growing since 1970 at an average annual rate of 8.4% (Stentiford et al., 2017; FAO 2020; Bennet et al., 2021). However, with the fast growth of this sector there are associated challenges. While aquaculture offers a solution to the growing food security problem, the sector is at risk from interactions with the environment and from the social and economic settings within which it operates. One of the primary risks and barriers to production and on-going growth of the aquaculture sector is disease. Infectious diseases caused by viral, bacterial and eukaryotic pathogens have been shown to significantly disrupt the aquaculture sector, international trade and ultimately food security. Disease losses in specific sectors have been estimated to exceed 40% of global capacity, with the overall global impact of diseases exceeding 6 billion US dollars per annum (Stentiford et al. 2017). The disease control costs, the desire for freedom from disease and the interests of trade has led to the creation of various international agreements and institutions, of which the most relevant is the World Organisation for Animal Health (WOAH). The WOAH, is an intergovernmental organisation that focuses on transparently disseminating information on animal diseases, improving animal health globally and thus building a safer, healthier and more sustainable world (https://www.woah.org/en/home/).

The WOAH annually publishes the Aquatic Animal Health Code. This establishes standards for the improvement of aquatic animal health worldwide. The Aquatic Code also includes standards for the welfare of farmed fish and the use of antimicrobial agents in aquatic animals. The Aquatic Code is available on the WOAH website at: http://www.oie.int. In addition to the Aquatic Animal Health Code, the WOAH has produced and regularly updates the Aquatic Manual, a Manual of Diagnostic Tests for Aquatic Animals to provide a uniform and efficient approach to the detection of the diseases listed in the Aquatic Animal Health Code (Aquatic Code), so that the requirements for the prevention and control of animal diseases and for health certification in connection with trade in aquatic animals and aquatic animal products can be met. The purpose of this guide is to advise the Competent Authorities in WOAH Member Countries on how to use the Aquatic Code. The standards in the Aquatic Code are based on the most recent scientific and technical information and are adopted by the World Assembly of Delegates. Correctly applied, they protect aquatic animal health during the production and trade in aquatic animals and aquatic animal products as well as the welfare of farmed fish.

The European Union, South Africa, Brazil, and Argentina are amongst the one hundred and eighty member states/countries of the WOAH and are therefore a signatory to its agreements. It is important to note that the WOAH is not a regulatory body, but functions to assist member countries to set and meet conditions for trade that are consistent with the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary (SPS) Measures.

Pathogen or disease lists are essential for health certification, disease surveillance and monitoring, emergency response planning, disease prevention and control of diseases in aquaculture facilities. While national pathogens lists consider all serious diseases of national and regional concern, the WOAH listed diseases are essentially those associated with internationally traded communities. Member countries are required to notify the WOAH when a listed disease occurs in their territory. Conversely, member countries are not required to report diseases of national significance that are of current or potential local significance but have not been included in the list of diseases compiled by the WOAH — because of factors related to their international importance or geographic distribution or current knowledge about them. There is no legal obligation to report these diseases to the WOAH, but any occurrence or suspected occurrence of any of these diseases must generally be reported to the relevant government authorities. Another category of disease is an emerging disease, which is defined by the Aquatic Animal Health Code as "a newly recognised serious disease, the cause of which may or may not yet be established, that has the potential to be spread within and between populations, for example by way of trade in aquatic animals or aquatic animal products". Emerging diseases are difficult to manage, due to lack of information, but the realities of intensive aquaculture and stock movements dictate that any emerging disease should be controlled aggressively rather than conservatively, and appropriate health monitoring procedures should be implemented to detect emerging diseases.

Currently, the monitoring of both well-established and emerging diseases is quite rudimentary, assesed visually through general indicators. Once any symptoms are observed within the cultured organism(s), the identification and/or confirmation of the causal agent is done in the laboratory with traditional methods (histopathology and cultivation-based techniques) or molecular tools (PCR and qPCR). Nevertheless, this routine monitoring and diagnosis is very challenging in aquatic animals due to the variability of water conditions, as turbidity or weather, among others, could obscure the early detection of clinical signs (Evans and Andrew, 2011). Thus, new monitoring techniques are vital to provide an early warning before the disease is spread within the cultivated stock and to minimize mortalities and impacts on productivity.

To address this limitation, a review of the key pathogens within each IMTA laboratory by geographic region and the emerging tools that can potentially be used in the aquaculture environments, are described in the following sections of this deliverable. The description of emerging tools is based not only on the pathogen detection efficacy, but also on the cost and applicability of the technology in the field. Morever, an evaluation of how real bacterial data captured from land-based IMTA systems can be used as an emerging tools for monitored the risk of outbreaks is assessed in this report.

3 IMTA systems description and regulation

The ASTRAL project includes four active IMTA labs: two in Europe (Ireland and Scotland), one in Africa (South Africa) and one in South America (Brazil). Moreover, one prospective IMTA lab location is evaluated in Argentina. The IMTA labs include open coastal (Ireland, Scotland), partially recirculating land-based (South Africa) and 100% recirculating land-based (Brazil) systems focusing on production of fish, mollusk, echinoderm, crustacean and macroalgae species. The description and directives applicable to each ASTRAL IMTA lab are described below.

3.1 Ireland & Scotland - Open coastal systems

3.1.1 System description

The **IMTA lab Ireland** is an open water coastal marine system located off the west coast of Ireland, farming a combination of trophic species in differing structures on one production site (Figure 1). The location of the site in the Connemara area of Ireland is relatively sheltered, with no significant fetch or swell, and waves are produced by local wind conditions. It is subjected to runoff from rainfall, reducing salinity and increasing turbidity with high particulate loading. Due to runoff, salinity ranges from 24 to 35 ppt, but is typically greater than 32 ppt. Tidal range is approximately 5 m and water temperature ranges from 5 to 18 ^{o}C. Key to the cultivation of species in an IMTA scenario is the species interactions or established trophic linkage. The species at the IMTA lab Ireland are categorized as: fed, extractive and novel species.

1. Fed species: Feed is inputted to the system for Atlantic salmon (*Salmo salar*) and lumpfish (*Cyclopterus lumpus*). As a result, the fish release waste/nutrients into the surrounding water.
2. Extractive species: There are two types of extractive species. The filter feeders; Native oyster (*Ostrea edulis*), filter out particulate waste from the water as food, reusing the waste from the fish and from the environment. Dissolved 'waste' is extracted by seaweeds *Saccharina latissima* and *Alaria esculenta* by absorbing dissolved minerals and carbon, reusing the 'waste' from the fish and shellfish entering the environment.
3. Novel species: Spiny sea urchins (*Paracentrotus lividus*) are fed with the seaweeds grown in the IMTA process. They are incorporated into the food chain by manual feeding, this process enhances the circularity.

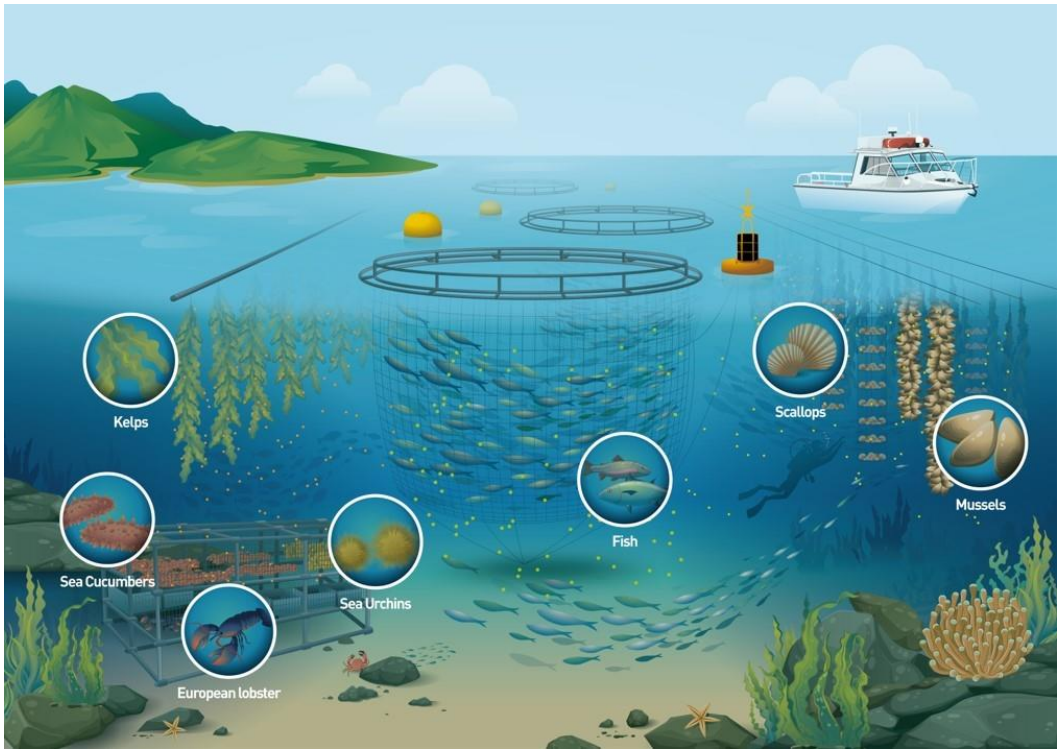

Figure 1. Open coastal IMTA system from Ireland and its produced species. This species at the IMTA lab Ireland are categorized as: fed (Atlantic salmon, lumpfish), extractive (native oysters, seaweed) and novel species (Spiny sea urchin). Other species that were tested in firstly pilot studies are Sea cucumbers, European lobster and mussels.

The IMTA lab in Scotland is an open coastal aquaculture site located 200 m off the mainland coast in the Firth of Lorn - Loch Linnhe estuarine system, West coast of Scotland (site name: Port-a-Bhuiltin [PaB]; 56° 29.176 N, 5° 28.315 W). The site consists of a one-hectare submerged tension grid, moderately exposed to average surface current speeds of 10 cm s^{-1} and tidal range up to 4.3m. Sea surface temperatures range from 6 to 15 °C and salinity from 24 to 33 PSU throughout the year.

PaB has been operated as an experimental seaweed cultivation site by the Scottish Association for Marine Science (SAMS) since 2014. The site is licensed by Marine Scotland for the cultivation of seaweeds and, following an amendment in 2020, also for co-cultivation with selected shellfish species for experimental non-commercial use (Figure 2). Thus, PaB has transitioned from a seaweed monoculture site to a low-trophic aquaculture (LTA) site to exploit the logistical and ecological synergies between seaweed and shellfish aquaculture. As a research site, cultivated species and production scales vary from year to year depending on various research project interests and requirements, but has focused mainly on the cultivation of brown seaweeds (*Alaria esculenta*, *Saccharina latissima*) and native oysters (*Ostrea edulis*). Under ASTRAL, additional LTA species are trialled, including brown (*Laminaria digitata*), red (*Palmaria palmata*) and green (*Ulva* spp.) seaweeds as well as king scallops (*Pecten maximus*) and sea urchins (*Echinus esculentus*).

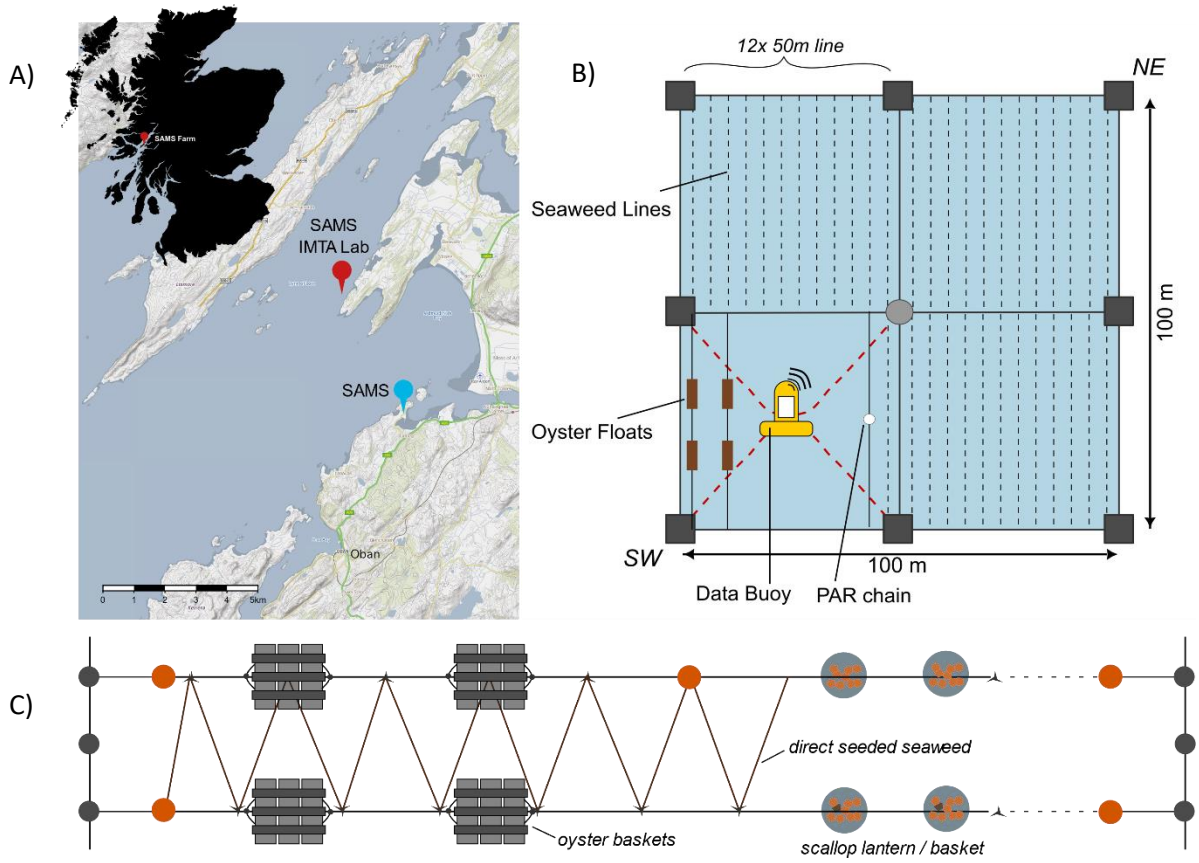

Figure 2. SAMS IMTA lab Port-a-Bhuiltin location and site set-up for co-cultivation of various seaweed and shellfish species. A) Geographic location of the Scotland IMTA lab. B) General scheme of 1ha site, divided in 4 quadrants with one of them used to develop oyster growth. Data buoy and PAR chain are used to monitor several physical parameters at two water depths (1.5 and 3 metres) C) Zoom into the parcel with Oyster baskets, showing how these are placed within the system. Orange and black dots represent buoys to hold the system at right depth, brown lines the seaweeds.

3.1.2 Directives and regulations

There are several European directives and regulations governing the production of aquatic organisms to ensure high health and welfare standards for organisms, produce and consumers.

- Council Directive 90/667/EEC laying down the veterinary rules for the disposal and processing of animal waste, for its placing on the market and for the prevention of pathogens in feedstuffs of animal or fish origin and amending Directive 90/425/EEC. 1990-11-27
- Council Directive 91/174/EEC laying down zootechnical and pedigree requirements for the marketing of pure-bred animals and amending Directives 77/504/EEC and 90/425/EEC. 1991-03-25
- Council Directive 91/496/EEC laying down the principles governing the organization of veterinary checks on animals entering the Community from third countries and amending Directives 89/662/EEC, 90/425/EEC and 90/675/EEC. 1991-09-24

- Council Directive 91/628/EEC on the protection of animals during transport and amending Directives 90/425/EEC and 91/496/EEC. 1991-11-19
- Council Directive 92/118/EEC laying down animal health and public health requirements governing trade in and imports into the Community of products not subject to the said requirements laid down in specific Community rules referred to in Annex A (I) to Directive 89/662/EEC and, with regards to pathogens, to Directive 90/425/EEC. 1992-12-17
- Directives 90/425/EEC and 92/118/EEC with regards to health requirements for animal by-products. 2002-10-21
- Directive 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. 24-10-2006.

Note: The Aquatic Animal Health and Alien Species in Aquaculture (Amendment etc.) (EU Exit) Regulations 2019 ensure that EU legislation in the fields of aquatic animal health and alien and locally absent species in aquaculture will continue to be operable after the UK leaves the EU. Thereby, this statutory instrument does not introduce any policy changes.

Animal health is also legislated in order to control the presence and spread of disease. European Council Directive 2006/88/EC of 24 October 2006 is focused on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals. Annex IV of the directive outlines diseases listed as exotic and non-exotic of which their pathological presence is notifiable to a Fish Health Regulatory body. Article 43 of the Directive also covers diseases which are not specifically listed in Annex IV but are significant diseases which are widespread in certain parts of the European Community but are absent from other parts. These diseases can cause significant economic losses at a local level.

3.2 South Africa – Partially recirculating land-based IMTA

3.2.1 System description

Buffeljags abalone is a commercial aquafarm run by Viking Aquaculture (https://www.vikingaquaculture.co.za/abalone/farming/). It is ca. 200 km east of Cape Town, situated on a pristine stretch of coastline near the remote settlement of Buffeljags on the Cape south coast (34°45’14.7“ S 19°36’51.9” E). The farm is one of the first large commercial abalone farms in South Africa to consistently recirculate 50% of their seawater by making use of the bioremediation capacity of *Ulva*. The farm currently has seven modular abalone-*Ulva* IMTA systems, called platforms, which are each composed of four clusters that each consist of one *Ulva* paddle-raceway and several abalone tanks (6 rows each made of 7 abalone raceway tanks) (Figure 3). Effluent water from the abalone tanks flows into the *Ulva* paddle-raceway and 50% of this bioremediated water is then recirculated back to

the abalone tanks (Figure 3C). Abalone are grown in baskets suspended in sturdy glass fibre tanks. Seawater is circulated through the ponds, ensuring a constant supply of cool, aerated water for the growing abalone. The average water temperature for the Atlantic Ocean coastal environment near Buffeljags over the last 20-years, where the farm extracts its seawater, is 15-16°C. Animals are fed on a combination diet consisting of freshly harvested kelp (*Ecklonia maxima*), farm produced (IMTA) *Ulva lacinulata* and formulated feed. The farm produces ca. 450 tons abalone and ca. 600-700 tons of *Ulva* annually.

Species cultivated at the site include: South African abalone (*Haliotis midae*), referred to locally as perlemoen; sea lettuce (*Ulva lacinulata*); tropical sea urchin (*Tripneustes gratilla*), often referred to as the collector urchin; and the temperate Cape sea urchin (*Parechinus angulosus*).

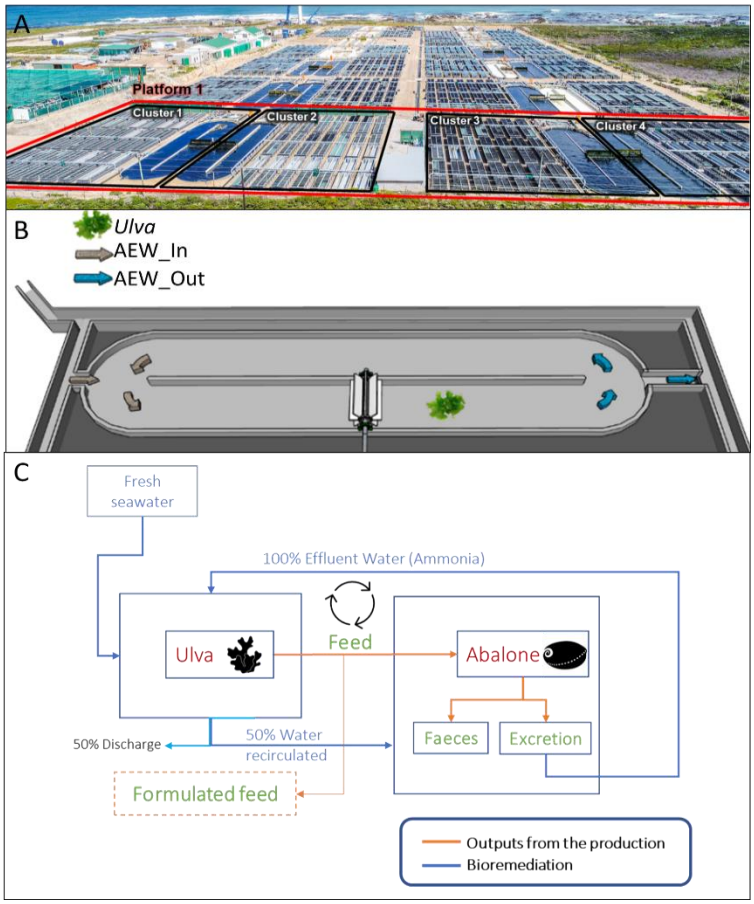

Figure 3. Land-based pump-ashore IMTA system for the production of abalone (*Haliotis midae*) and seaweed (Ulva lacinulata) at Buffeljags abalone. (A) Modular abalone-Ulva IMTA systems, each cluster consisting of one Ulva paddle-raceway and several (42) abalone tanks. (B) Schematic of Ulva raceway receiving effluent water from the abalone tanks (AEW_In) and bioremediated water leaving these tanks (AEW_Out). (C) Schematic of the IMTA process at Buffeljags Abalone, where the effluent water leaving the abalone tanks is bioremediated by the *Ulva* before being mixed with 50% fresh seawater and returned to the abalone raceways.

3.2.2 Directives and regulation

The South African Department of Forestry, Fisheries, and the Environment (DFFE) has been entrusted to lead the development of sustainable aquaculture in South Africa and has been designated as the lead agency for the promotion, development and marketing of aquaculture and aquaculture products. In this regard, the DFFE has developed a National Aquatic Animal Health Strategic Framework (NASF) to provide overarching strategic guidance for the management, control and regulation of aquatic animal health, welfare and disease management in South Africa. The South African legislation and regulations relevant to aquatic animal health include:

- The Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947). This Act aims to “To provide for the appointment of a Registrar of Fertilizers, Farm Feeds and Agricultural Remedies, and for the registration of fertilizers, farm feeds, agricultural remedies, stock remedies, sterilizing plants and pest control operators, and to regulate or prohibit the importation, sale, acquisition, disposal or use of fertilizers, farm feeds, agricultural remedies and stock remedies, and to provide for the designation of technical advisers and analysts, and to provide for matters incidental thereto.”
- The Animals Protection Act, 1962 (Act No 71 of 1962) and Societies for the Prevention of Cruelty to Animals Act, 1993 (Act No 169 of 1993). These Acts cover all aspects of cruelty to animals and the prevention thereof, including “being the owner of any animal, deliberately or negligently keeps such animal in a dirty or parasitic condition or allows it to become infested with external parasites or fails to render or procure veterinary or other medical treatment or attention which he or she is able to render or procure for any such animal in need of such treatment or attention, whether through disease, injury, delivery of young or any other cause, or fails to destroy or cause to be destroyed any such animal which is so seriously injured or diseased or in such a physical condition that to prolong its life would be cruel and would cause such animal unnecessary suffering.”
- The Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965) and Medicines and Related Substances Control Amendment Act, 1997 (Act No 90 of 1997). These Acts aim to "To provide for the registration of medicines intended for human and for animal use, and for the registration of medical devices, and for the establishment of a Medicines Control Council, for the control of medicines, scheduled substances and medical devices, for the control of manufacturers, wholesalers and distributors of medicines and medical devices, and for the control of persons who may compound and dispense medicines, and for matters incidental thereto."
- The Veterinary and Paraveterinary Professions Act, 1982 (Act No 19 of 1982). This Act aims "To provide for the establishment, powers and functions of the South African Veterinary

Council, for the registration of persons practicing veterinary professions and paraveterinary professions, and for control over the practicing of veterinary professions and paraveterinary professions, and for matters connected therewith."

- The Animal Diseases Act, 1984 (Act No 35 of 1984) and Animal Disease Regulations. This Act and the regulations aim “To provide for the control of animal diseases and parasites, for measures to promote animal health, and for matters connected therewith.”
- The Meat Safety Act, 2000 (Act No 40 of 2000). This Act aims “To provide for measures to promote meat safety and the safety of animal products; to establish and maintain essential national standards in respect of abattoirs; to regulate the importation and exportation of meat; to establish meat safety schemes; and to provide for matters connected herewith.”
- Other relevant legislation pertaining to animal health and welfare includes:
  - A list of all legislation which may impact on veterinarians can be found at the Faculty of Veterinary Science library (site http://www.ais.up.ac.za/vet/vetacts.htm)
  - Animal disease legislation and controls can be found at http://www.doa.agric.za/ under Division of Veterinary Services.
  - More information is also available at the South African Veterinary Council site, http://www.savc.co.za/, including the rules pertaining to the veterinary profession.

In addition to the above, protocols and standards have been developed specifically for aquaculture, addressing issues such as disease-free zones and stock movements, emergency response and preparedness, quarantine, listing of diseases, prevention of suffering, disposal of mortalities, decontamination, biosecurity, operation of harvesting vessels and processing facilities, and compliance and enforcement. The specific conditions are outlined in the various types of permits required to engage in marine aquaculture. A “right to engage in marine aquaculture” must be obtained from the DFFE when undertaking any commercial marine aquaculture activity, in terms of section 18 of the Marine Living Resources Act, 1998 (Act No. 18 of 1998) (MLRA). Permission to exercise such a “right” is granted by means of an annual permit, and rights holders have to abide by the specific regulations outlined in the various permits governing, for example, hatcheries, grow-out, broodstock collection, transport, processing, and the import and export of marine aquaculture species and products. A “right to engage in marine aquaculture” is valid for 15 years, whereas marine aquaculture permits are renewable and valid for a period of 12 months.

3.3 Brazil – Biofloc recirculation land-based IMTA

3.3.1 System description

The Brazilian inshore Recirculating Aquaculture System (RAS) IMTA lab operates in closed system that stimulates the production of microbial aggregates (Biofloc system or BFT). This intensive production technology located at the IMTA lab Brazil in the Rio Grande do Sul, utilizes the action of microorganisms aggregated in flocs which present multiple functions in the system, being a key agent in the control of nitrogen compounds, especially the toxic ammonium and nitrite. Moreover, it could be used as food source for shrimp, tilapia and oysters, reducing the amount of formulated feed used for shrimps and fishes. The presence of a great variety of microorganisms in the BFT allows these microorganisms to colonize the digestive system of the organisms that consume the biofloc, reducing some risks of infections and diseases. This technology allows super-intensive production (high density) in small spaces with minimal, or no water renewal, reducing potentially the aquaculture waste production to almost zero.

The Marine Aquaculture Centre is located 300 m away from the shoreline and has 9 experimental shrimp ponds (600 m^2 each), 3 greenhouses for research with shrimp production in bioflocs, 1 pilot commercial size greenhouse for shrimp production (2 tanks with 237 m^2 each) and 1 multi-trophic greenhouse (Figure 4; 6 systems with 3 tanks each). In addition, the laboratory has a shrimp maturation sector (6 tanks with 10 tons each), shrimp hatchery sector with commercial size tanks (8 tanks), sectors of live food production (Artemia and phytoplankton) and water quality laboratory. Usually, the experiments in the IMTA greenhouse with biofloc formation are being stimulated by organic fertilization (molasses).

The choice of species to be used and their biomass ratios are key factors for maintaining the system's water quality. Thus, it is not necessary to change the water and it can be used for several production cycles. Species cultivated at the site include: white shrimp (*Litopenaeus vannamei*), sea lettuce (*Ulva fasciata*) and sea asparagus (*Salicornia neei*); native oysters (*Crassostrea gasar*), and the tilapia (*Oreochromis niloticus*).

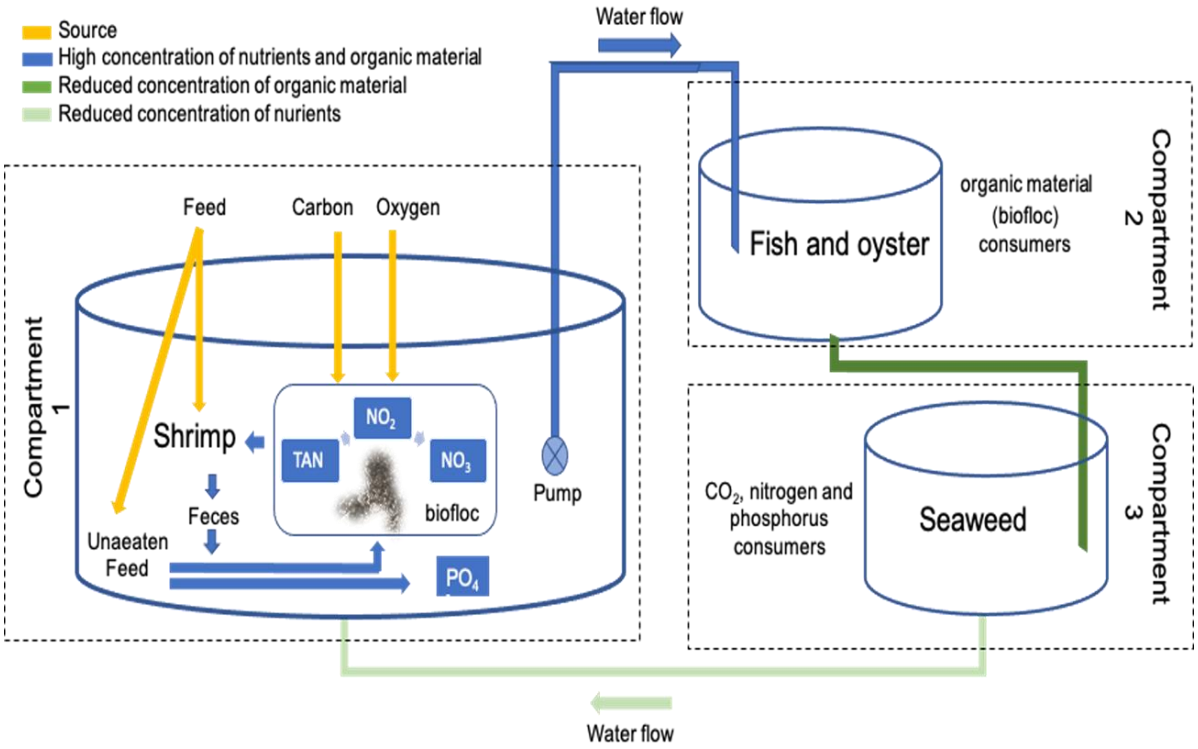

Figure 4. Biofloc Technology in land-based pump-inshore IMTA system for the production of shrimp (Compartment 1), tilapia and oyster (Compartment 2) and seaweed-Ulva (Compartment 3).

3.3.2 Directives and regulation

The Special Secretary for Aquaculture and Fisheries (*Secretaria Especial de Aqüicultura e Pesca – SEAP*) is the main authority for the management and development of fisheries and aquaculture in Brazil. SEAP Normative Instruction No.3 of 2004 regards aquaculture as the cultivation, breeding or holding in captivity, with commercial purposes, of organisms whose life cycle develops, in natural conditions, totally or partially in an aquatic environment. Within all the aquaculture sectors, the shrimp sector is the best organized industry of the aquaculture sector in Brazil. Thus, the Brazilian Shrimp Growers Association (*ABCC - Associação Brasileira de Criadores de Camarão*) has prepared four codes for good management practices, concerning shrimp farm management, shrimp feed production, shrimp hatchery, and processing plants. The latter mainly reinforces Hazard Analysis Critical Control Point (HACCP) standards, whereas the former addresses the following issues:

- Sustainable and responsible shrimp aquaculture management (*Código de Conduta para Desenvolvimento Sustentável e Responsável da Carcinicultura Brasileira*) (June 2004)
- Shrimp feed production (*Código de Conduta e de Boas Práticas de Fabricação para Fabricantes de Rações para Camarão*) (January 2004)
- Marine shrimp larviculture (*Código de Conduta e de Boas Práticas de Manejo para Laboratórios de Larvicultura de Camarão Marinho*) (May 2004)

3.4 Argentina – Prospective IMTA lab

3.4.1 System description

The prospective IMTA Lab, to be carried out by CONICET (*Consejo Nacional de Investigaciones Científicas y Técnicas*), is intended to be designed around Port Almanza, a small artisanal fishing village located 70 km from Ushuaia. This area was selected because the National Service of Food Health and Quality (SENASA) recognizes two class "A" zones for bivalve mollusks production in this area: ARTF 001-Punta Paraná and ARTF 002-Bahía Brown by Resolution No. 433/10, which is the highest category granted to the quality of the environment for mussel production. Here, several research programs evaluating physiological and ecological issues of fish, crustaceans, echinoderms, and mussels of the Beagle Channel have been carried out.

The personal expertise, aquaculture know-how and market study were taken into account to finally set a list of species that could make up an IMTA system in Argentina. Finally, the organisms selected were: Blue mussel (*Mytilus chilensis*), Red sea urchin (*Loxechinus albus*), Small puyen or whitebait (*Galaxias maculatus*), and the halophyte Salicornia or samphire (*Salicornia magellanica*).

3.4.2 Directives and regulation

The Argentina aquaculture is a grown sector that the health organisation SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria) controls under the Disease aquatic animals programs. This program develops as aquaculture progresses and follows the World Organization for Animal Health (WOAH) mandatory reporting standards. The SENASA resolution Nº 153/21 creates the Notification and Communication System for Animal Diseases and Health Events, including those affecting aquatic animals (fish, bivalves and crustaceans). This result highlights the obligation to control and report several diseases and syndromes in Argentina. Formal notification channels for producers are also established. By means of resolution Nº 375/2013, several areas of Argentina are declared as free diseases zone of salmonids and no salmonids. Furthermore, surveillance of several mollusks (oysters) and crustaceans has been carried out, declaring the absence of diseases in these groups. The resolution Nº 153/21 follows several articles in terms to provide aquaculture good standards and food security (free from diseases):

- Article 4 and 6 of lay Nº 4.959: “any owner or person who in any way oversees the care or assistance of animals attacked by contagious diseases or suspected of having them, has the contagious diseases or suspected of having them, is obliged to immediately declare this fact to the authority immediately this fact to the authority determined by the sanitary regulations. Both the declaration of the event and isolation are mandatory for animals dead or presumed dead of contagious diseases.”

- Article 1 and 3 of law Nº 27.233: “animal health is declared to be of national interest, as well as the prevention, control and eradication of diseases that affect the national silvo-agricultural production and fauna. To ensure and be responsible for the health, safety, hygiene and quality of its production shall be the primary and unavoidable responsibility of any natural or legal person linked to the primary production and unavoidable responsibility of any natural or juridical person linked to the production, procurement or industrialization of products, by-products and derivatives of forest-agricultural and fishery origin, whose activity is subject to the control of the enforcement authority of said law.”
- The resolution Nº 422 from 20th August 2003 established “the obligation of systems for epidemiological surveillance and continuous epidemiological monitoring, risk analysis, sanitary risk analysis, sanitary emergencies and a regulatory system that covers all aspects of disease protection and control.”

4 Diversity, early warnings, and current monitoring methods of potential pathogens by IMTA labs

4.1 Potential diseases and pathogens

4.1.1 Open coastal system

4.1.1.1 Atlantic salmon and Lumpfish

For the IMTA lab in Ireland, the fed species are considered the more sensitive members in the system. The maintenance of a high health status and high welfare standard for culturing salmonids demands frequent monitoring and implementation of a veterinary health plan. Changes in physical appearance (scale loss, parasites, injury, deformities), behavior (swimming and shoaling, increased respiration, jumping) or changes in feeding response must be recorded and reported to management. To monitor for the presence of pathogens, normal fish behavior, appearance and the stock must be routinely monitored for signs of disease. While the Atlantic salmon can be affected by a range of viral and bacterial pathogens, this is also true for Lumpfish, which are utilized as a cleaner fish species for the biological control of sea lice on Atlantic salmon farms. Albeit very few diseases in Lumpfish have been investigated, here several potential pathogens for both fish have been identified.

Bacteria

- *Aeromonas salmonicida*

A. salmonicida is a Gram-negative bacteria with capacity of proliferate in both Atlantic salmon and lumpfish, causing furunculosis disease (Mitchell, Scholz and Gutierrez, 2022). Optimum temperature

for *A. salmonicida* is 22^{o}C to 25^{o}C and furunculosis can occur when water temperatures exceed 16^{o}C (Charette, 2021). Disease outbreaks are rare in both Salmon and Lumpfish, with vaccination providing protection for salmon. Nonetheless, increasing trend of clinical disease in vaccinated it suggest a potential issue for Ireland and other countries (Mitchell, Scholz and Gutierrez, 2022). Moreover, Initial concerns of disease transfer, particularly furunculosis, between wild wrasse and farmed fish has stagnated the use of cleaner fish as a biological control by the industry during the late 1990's (Bolton-Warberg, 2017). Clinical presentation of the diseases is typical for present visible furuncles in skin and severe hemorrhaging in swim bladder (Mitchell, Scholz and Gutierrez, 2022). Confirmation is usually carried out by bacteriological culture and biochemical testing, histopathology, or molecular methods (Marine Scotland Directorate).

- *Rickettsial like - organisms*

This is a diverse collection of obligately intracellular Gram-negative bacteria which include the genera *Rickettsiae*, *Ehrlichia*, *Orientia*, and *Coxiella*. One of the known diseases in Atlantic salmon is the Salmonid Rickettsial Septicemia (SRS). The main clinical signs and pathology include marked ascites, severe multifocal liver necrosis, appetite loss, and elevated mortality. This infection is considered potentially significant in terms of salmonids and lumpfish health and biosecurity. It occurs mainly in Chile, but has also been observed, albeit to a much lesser extent, in Norway, Ireland, Canada and the UK. As an example, *Piscirickettsia salmonis* infection has been identified in Lumpfish farmed as cleanerfish in Ireland (Marcos-Lopez et al 2017). *P. salmonis* was by far the most significant driver of antibiotic use in salmon in Ireland, accounting for approximately 80% of antibiotics used in 2022 (Mitchell, Scholz and Gutierrez, 2022). Clinical SRS was often preceded by harmful algae blooms or jellyfish swarms, which complicated categorization of mortalities. Co-infections with *A. salmonicida, N. perurans* and viral cardiomyopathies also occurred. Signs included lethargy, skin lesions of varying presentation, gill pallor, exophthalmia and, in chronic cases, darkening of the skin (Mitchell, Scholz and Gutierrez, 2022). Current monitoring methods include qPCR from localized lesions (Mitchell, Scholz and Gutierrez, 2022).

- *Tenacibaculum spp*

The *Tenacibaculum* genus englobe Gram-negative aerobic bacteria, such as *T. maritimun* or *T. dicentrachi*, which can cause tenacibaculosis or bacterial stomatitis/erosive dermatitis (Santos et al, 2019). This genus has a global distribution, but can be triggered by stressors, including smoltification, handling or exposure to harmful algae, making it significant for aquaculture species, including Atlantic Salmon. Tenacibaculosis was widespread in 2022, most notably following exposure *to Muggiaea atlantica* and other harmful jellyfish species (Mitchell, Scholz and Gutierrez, 2022). Clinical presentation includes skin ulcers, mouth erosion, tail rot, frayed fins and lethargic behaviour. Diagnosis of tenacibaculosis is frequently made by the presence of filamentous bacteria on histology,

but species distinction is not possible (Mitchell, Scholz and Gutierrez, 2022). Being particular species the causal agent of this diseases, specie-specific qPCR will be required to improve the accuracy of pathogen detection. Tenacibaculosis can be treated with antibiotics, disinfectants, and manipulation of husbandry conditions, such as temperature and salinity.

- *Vibrio spp.*

Vibriosis is a Gram-negative bacterial infection caused by *Vibrio* species such as *V. anguillarum* (*Vibrio anguillarum* serotype O1 – *V. anguillarum* biotype 2) and *V. salmonicida* in Atlantic salmon (Kashulin et al, 2017). Clinical symptoms include septicemia, necrosis and inflammation of tissues (Bjelland, 2012). Diagnostic methods include histo- and clinical pathology, often further supported by molecular diagnostics (PCR, 16S ribosomal DNA sequencing). Disease outbreaks in open water systems appear to be rare and treatable with anti-biotics, thus vaccination and early treatment should prevent mortalities and large stock loss.

- *Moritella viscosa*

Several specific strains of *M. viscosa* are considered the main causative agent of winter ulcer disease (WUD) (Løvoll, 2009). This Gram-negative bacterium is considered the primarily disease problem in salmonid sea water aquaculture during cold periods. If it is also true that the improvement of vaccine efficacy has led to a decreasing trend since 2019, single cases of *Moritella sp.* variants has been reported to be less sensitive to available vaccines. Nonetheless, this is not a currently issue in Ireland. The disease is initially characterised by localised swelling of the skin, followed by the development of skin lesions. Currently the histopathology techniques have been widely described and used in Europe (MacKinnon *et al* 2020). Moreover, several in vitro assays suggest that salmons are susceptible to *M. viscosa* isolates from lumpfish, thus the use of lumpfish more often during the winter season can be consider as a risk (Thorbjorg *et al* 2018).

Viruses

- *Novirhabdovirus*

Several genotypes of the genus *Novirhabdovirus* and Family *Rhabdoviridae* can cause diseases in a multitude of marine and freshwater fish species. The *Salmonid novirhabdovirus* (commonly known as infectious hematopoietic necrosis virus [IHNV]) and *Viral haemorrhagic septicaemia virus* (VHSV) are negative single-stranded RNA virus listed as notifiable diseases by WOAH. This disease is well-known by its devastation in Europe marine and freshwater environments (Wolf, K., 1988), but also its expansion in North America (Schlotfeldt H.J. et al, 1991) or Baltic Sea was confirmed (Brunson, True and Yancey, 1989). The primary portal for entry of these viruses has been considered to be the gills, but tissues of the digestive system may become also infected (Dixon et al, 2016). Clinical signs in salmonids are hemorrhages at the base of fins, severely pale gills, periocular hemorrhage, lethargy,

and abnormal to erratic swimming (Yasutake W.T, 1975). In terms of detection, histopathologic examination of muscle, liver, spleen or kidney from diseased fish has offered invaluable insights into the pathogenesis of VHSV (Yasutake, 1975). Nonetheless, according to the OIE diagnosis confirmation of VHSV can be completed through a variety of serologic techniques (plaque neutralization test, immunoblot, fluorescent antibody test, enzyme linked immunosorbent assay and cDNA qPCR analysis (Kim and Faisal, 2011). General good standards in fish husbandry, stocking densities, reduction in physical stressors, such as handling, can prevent this pathogen outbreak in fish.

- *Salmonid alphavirus (SAV)*

The pancreas disease (PD) is caused by the single-stranded and positive-sense RNA virus, *Salmonid alphavirus* (SAV) (Ruane *et al*., 2008). Diagnosis on marine sites is based on clinical signs, gross pathology and laboratory diagnostic tools. Clinical signs include a loss of appetite, lethargy, and an increased number of feacal casts in the cages (Ruane et al., 2008). Moreover, regular voluntary monitoring for SAV infection is carried out prior to the disease risk periods on most salmon farms using a combination of qPCR (Hodneland and Endresen, 2006), serology (Graham et al., 2003), clinical biomarkers such as CPK (muscle damage) and histopathology of affected organs (pancreas, heart and skeletal muscle) (Ferguson, H.W. *et al*, 1986; McLoughlin, M.F., *et al*, 2002). Non-lethal blood sampling enables early detection of infection. Fish are usually viraemic for two to three weeks post initial infection and detecting virus in the serum on qPCR indicates a very recent infection on site, which alerts the farm to modify feeding, reduce handling and avoid oral sea lice treatments. Vaccination, good fish husbandry practices and selective breeding for PD-resistant fish has also contributed to reducing the incidence of PD (Mitchell, Scholz and Gutierrez, 2022)

Parasites

- Sea lice (*Lepeoptheirus salmonis*, *Caligus spp*.)

One of the major challenges to the salmon industry is sea lice, which is costly in terms of both treatment and loss of fish (Costello, 2009). The global salmonid industry and the yearly financial loss caused to farmers globally could run as high as €880 million (INTRAFISH Sea lice report, 2019). There are several species of sea lice, which are naturally occurring seawater parasites. They can infect the salmon skin and if not controlled they can cause lesions and secondary infection. *Lepeoptheirus salmonis* and *Caligus spp*. are the most prevalent natural parasites of wild and farmed sea-caged Atlantic salmon. Presentation of infection with *L. salmonis* is the observation of whitish spots on the dorsal regions, notably the head, neck and base of the dorsal fins. At higher parasite levels these spots become skin lesions and then large open wounds. The subsequent extensive skin damage results in death through osmoregulatory failure or secondary bacterial infection (Marine Institute, 2017). Sea lice are controlled through good husbandry and management practices along with statutory

monitoring programmes in place in most jurisdictions. The use of lice prevention barriers (e.g. skirts), cleaner fish (wrasse species and lumpfish, which eat the lice off the salmon), mechanical removal systems, freshwater bathing and when necessary licensed medicines reduce the infestation of lice (MOWI, 2020). Current monitoring methods for this pathogen are mainly based on daily fish observation and the use of oral/bath medicines as SLICE ® or Salmosan® (Mitchel S., Scholz F. & Gutierrez C., 2023)

- *Neoparamoeba perurans*

The single celled protozoan parasite *N. perurans* is the causal agent of Ameobic Gill Disease (AGD), an endemic disease in Ireland where outbreaks occurred on all marine sites during 2022. *N. perurans* is a free-living, facultative marine amphizoic amoeba, ubiquitous in the marine environment and found in high concentrations in a wide variety of habitats (Oldham *et al.*, 2016). Clinical signs of infection include lethargy, anorexia, congregation at the water surface and increased ventilation rate, increased mucus on the gills with white multifocal patches of swollen tissue. Fish may swim close to the surface and breathe rapidly (Oldham et al., 2016). Preliminary diagnosis of the infection is often done through scoring of white mucoid patches present on gills of infected fish (Rodger, 2014). Further diagnostic methods include: Histopathology, real-time PCR (qPCR) and Sequencing. Higher stocking densities and heavily fouled pens or pens with lower water exchange experience more cases of clinically significant AGD. Co-infections with other pathogens and exposure to harmful phytoplankton/jellyfish blooms occurred in some sites which makes accurate categorization of gill related mortalities difficult. Currently, there are two commercially utilized treatments for AGD: freshwater or hydrogen peroxide bathing (Oldham et al., 2016).

4.1.1.2 Molluscan Shellfish

Many commercially valuable molluscan shellfish species such as mussels, oysters, scallops and clams are susceptible to stress and diseases (Zannella et al., 2017). Their effective filter-feeding capacity allows them to accumulate large concentrations of microorganisms in their tissue; some of which are harmful to other animals including humans. In addition, some pathogens are also able to infect bivalve molluscs. The World Organization for Animal Health (OIE/WOAH) lists seven notifiable mollusc diseases which often spread through national and international trade with sometimes devastating effects on both wild and cultivated stocks (Laing, 2009). The following examples will focus on selected pathogens most common and relevant for European shellfish aquaculture production.

Bacteria

- *Vibrio spp.*

Vibrio spp. are Gram negative rod-shaped bacteria that naturally occur in coastal waters, and which can concentrate inside shellfish and other prevailing seafood. Several *Vibrio* species have been

associated with high larval mortalities in most molluscan shellfish species and especially in bivalve hatchery cultures (Bower, 2009). Pathogenic *Vibrio* spp. infect the soft-tissues which become necrotized as well as triggering the production of exotoxins which in combination causes larval vibriosis (bacillary necrosis) - a rapid and severe disease with up to 100% mortality within a few hours of infection. Diagnostic methods in Europe coastal IMTA laboratories was already provided in section 4.1.1.1. Disease control requires thorough management of the culture system including identifying potential sources of pathogenic *Vibrio spp*. in the seawater, algal cultures and bivalve broodstock (Bower, 2009).

Bivalve mollusc juveniles deployed at the Irish and Scottish IMTA labs have been acquired from local commercial hatcheries respectively which follow their operational microhygiene and biosecurity protocols. As the Scottish IMTA lab is on-growing for research purposes only, i.e the stock is not entering the table market, routine microbial hygiene monitoring is not a mandatory license requirement. However, deployed shellfish stocks (*O. edulis*, *P. maximus*) are regularly assessed for visual signs of distress and any mortalities recorded.

Viruses

- *Oyster Herpes Virus* (OsHV)

OsHV is a specific type of herpes found only in oysters and has a widespread global distribution (Smolowitz, 2021). It is primarily a disease of the Pacific oyster (*Crassostrea gigas;* assignment to new genus *Magallana gigas* under debate, Bayne et al. 2017); a species originally endemic to only a few locations but widely introduced for aquaculture purposes, also due to its lower susceptibility to protist infection (see below for *B. ostreae* in European flat oysters). There are two variants of OsHV – OsHV and OsHV-1 µVar - with the latter being considered more pathogenic, causing acute morbidity and mortality in juvenile oysters but also affecting larval and adult stages. Infected animals show no visible signs of distress except slow growth and sudden death. As for other herpes viruses, specimens may well be infected but not diseased. Diagnostic methods include histological examination for inclusion bodies in the tissue cells, further supported by molecular identification of the respective OsHV variant. Management and treatment methods are often limited to reducing and controlling spat and stock translocations, reducing oyster densities and considering grow-out in cooler waters (temperatures >21°C were found to promote disease development).

For the Scottish IMTA lab a decision was made following the license amendment to a co-cultivation site, that any species grown-out at SAMS IMTA lab is native and local to the area and is of interest and value for restorative and/or commercial aquaculture production. As such, SAMS IMTA lab is not cultivating Pacific oysters and therefore not testing for OsHV presence at site.

Protists

- *Bonamia ostreae*

B. ostreae is a protist parasitic organism infesting European flat oysters (*O. edulis*) and causing Bonamiosis (Bonamia). Also known as microcell disease, hemocytic parasitosis or hemocyte disease, Bonamia is a lethal infection of the hemocytes and sometimes accompanied by yellow discoloration of the soft-tissue as well as lesions on gill and mantle tissues. With direct transmission between oysters and mortality rates exceeding 80%, *B. ostreae* has had a significant negative impact on flat oyster production throughout its European distribution range. Examination of stained tissue sections (histopathology) is the traditional and current method used for screening. This method however is destructive and requires a large sample size (e.g. >100 individuals per screening requested by Marine Scotland, UK) and has been further suggested to not be fully reliable during the first weeks to months of infection. More recently, PCR, sequencing and description by trans electron microscopy (TEM) are increasingly recommended.

The Sottish IMTA lab is located at the periphery of a listed Bonamia restriction zone i.e. *B. ostreae* presence has been confirmed at a nearby commercial oyster farm. Within the ASTRAL project, SAMS compared the growth performance and survival rate between oysters grown subtidally at PaB and intertidally at the monoculture site, observing significant mortalities at the latter and thus suspecting *B. ostreae* presence. A novel qPCR protocol developed by the Roslin Institute, University of Edinburgh UK, for a rapid and non-destructive *B. ostreae* presence-absence testing did not confirm any presence of this parasitic protist at either site, and thus suggesting that high mortalities were rather resulting from temperature stress in the intertidal during hotter summer months.

4.1.1.3 Seaweed

Seaweeds can be grouped into 3 classes, namely the Rhodophyta (red seaweeds), Phaeophyceae (Brown seaweeds) and Chlorophyta (Green seaweeds). In Europe, seaweed aquaculture is not yet widely established and as such comparatively little research has been conducted on potential diseases in temperate seaweed species. The EU H2020 project GeniAlg (Genetic diversity exploitation for innovative macroalgal biorefinery; grant ID: 727892), initiated a survey of pathogens in wild European populations of seaweed and developed the online service 'My Seaweed looks weird' to accelerate the discovery and description of seaweed pathogens. In addition, the project conducted extensive monitoring of known pathogens on kelp species cultivated at SAMS commercial seaweed nursery and SAMS seaweed farm (now SAMS IMTA lab) (Perrineau et al., 2020).

Overall, seaweed pathogens are prevalent in the natural environment and highly diverse, including endophytic and epiphytic algal species, Oomycetes, fungi, algal-lysing bacteria, viruses and cyanobacteria, manifesting as cell enlargement and proliferation in host tissues (Behera et al., 2022; Correa & Flores, 1995; Neill et al., 2008; Ward et al., 2020, 2022). Effects of common infectious disease are often visible to the naked eye, including discoloration, necrosis (or formation of holes), bleaching,

black spots, malformation and outgrowths of infected fronds (Neill et al., 2008). A general consensus for monitoring any algal pathogens is the implementation of a biosecurity plan which sets out routine monitoring of critical environmental parameters (temperature, salinity, pH, oxygen) and any known pathogens during all steps of the algal production chain. For example, a drop in oxygen can coincide with a bacterial bloom, or an increase in pH can result from too dense biomass and thus indicate more frequent water exchanges to be required. Algal disease diagnostics have mainly relied on the histological examination of symptoms. Microscopic observation is usually performed with differential interference contrast (DIC) microscopy and/or by fluorescence microscopy (Perrineau *et al.*, 2020). Over the last decade however, more rapid and reliable real-time PCR (qPCR) assays have been developed for many known algal pathogens allowing for kelp pathogen diagnostics and surveys of wild and farmed populations as well as during the nursery stage and at sea.

The following sections provide more specific information of known pathogens affecting mainly brown seaweed species, i.e. kelp, such as the sugar kelp *Saccharina latissima*.

Fungal-like organisms (oomycetes)

Oomycetes are a group of fungus-like eukaryotic microorganisms that include some of the most devastating pathogens to plants and algae. A total of 15 species have been reported to infect marine algae, most of which displaying host specificity to one class of algae but some infecting all classes from filamentous green to brown algae (Li *et al.*, 2010). For example, *Eurychasma dicksonii* has been identified in 45 species of brown seaweeds whereby infected host cells will be stimulated to abnormal growth in the early infection stage (Li *et al.*, 2010; Perrineau *et al.*, 2020). Infections have also been described in commercial seaweeds occurring in the North Sea (M. Bernard, 2018) and include red rot disease, with symptoms starting with an initial bleaching of the tissue that progresses at later stages to lesions and holes, and finally death of the infected host. The detection of this pathogen in the field is carried out with pathogen specific molecular detection test, as qPCR (Gachon et al., 2009; Perrineau et al., 2020)

Fungal-like organisms (Chytridiomycetes)

Next to Oomycota (*Oomycetes*), the Chytridiomycota (*Chytrids*) contain the most common pathogens observed in marine algal species. Chytrids are aerobic zoosporic fungi present in freshwater, brackish and marine habitats and possess a broad host range including other fungi, algae, plants and amphibians. The most prevailing pathogenic chytrid, *Chytridium polysiphoniae*, was observed in 23 species of brown algae with Chytridium development being strongly host as well as temperature dependent (reduced virulence above 15 °C) (Müller *et al.*, 1999). Apart from molecular techniques, detection is usually performed using fluorescent microscopy while focusing on the chitin component of Chytrid cell walls.

Endophites

- *Laminarionema and Laminariocolax*

These are filamentous brown algae endophytes in the family Chordariaceae, which causes various symptoms including galls and severe thallus deformations that may cause a loss of biomass or lower the market value of the crop significantly. *Laminarionema elsbetiae* has a very high prevalence in wild populations of European *S. latissimi* (M. S. Bernard et al., 2019a). It has been isolated from *S. latissima* in Scotland, France and Helgoland (Ellertsdottir & Peters, 1997). *Laminariocolax* has been isolated from *Laminaria hyperborea* and *S. latissima* in Brittany, Scotland and Kiel (M. S. Bernard et al., 2019b). Different clades of *Laminariocolax* have been identified infecting a broad range of hosts, such as *Macrocystis pyrifera, Lessonia berteroana, Laminaria hyperborea, S. latissima* and *S. nigripes* (M. S. Bernard et al., 2019a). Molecular qPCR-based quantification tests have been developed for the main brown algae endophytes *Laminarionema elsbetiae* (CNRS, Roscoff France) and more recently for the *Laminariacolax* genus (SAMS, UK) (Perrineau et al., 2020).

4.1.2 Land-based

4.1.2.1 Sea urchin

Sea urchin diseases can be complex and have overlapping phenotypic characteristics, and therefore only three sea urchin diseases have been named, including bald sea urchin disease, vibriosis and paramoebiosis (Sweet 2020). These diseases are mostly associated with bacterial pathogens, as very little is known on the impact of fungi, oomycetes or viruses on sea urchins.

Bacteria

- *Vibrio spp*

In sea urchins, microbial consortia can be sensitive to stressors, including animal handling, grading, sudden environmental changes (e.g., system failures) or poor food quality. These stressors could result in a state of microbial dysbiosis and a reduction in beneficial microbes that may facilitate infection(s) by opportunistic pathogens(s), including members of the *Vibrio* genus, causing vibriosis that may ultimately lead to mortalities (Brink et al. 2019). Vibriosis disease in sea urchin is characterized by spine loss and bald spots on the body (test) surface where the spines used to be. Additionally, previous mechanical abrasions, followed by opportunistic bacterial infection is thought to cause bald sea urchin disease. Several *Vibrio* species (*V. anguillarum, V. parahaemolyticus and V. splendis*) and other specific bacteria, including *Exiguobacterium sp.* and *Aeromonas salmonicida,* are known to be capable of inducing this disease in experimental infections (Becker et al. 2007, Grech et al. 2022). However, it is more likely that the lesions are caused by opportunistic bacteria residing in the immediate surroundings of the urchins (Becker et al. 2008; Brink et al. 2019). This notion is supported by the fact that more than one bacterial species is often isolated from lesions and the same bacteria is rarely

isolated from lesions in a population of infected animals. Sea urchins can however recover from this disease when the infected area is small, by mounting an inflammatory-like reaction (Maes and Jangoux, 1984) and when isolated from the remainder of the population (as wounded individuals are often cannibalized by healthy animals). Daily animal health monitoring is used to avoid mortalities from these diseases as animals can be quarantined if infections are observed.

Parasites

- *Paramoeba invadiens*

Infection by the amoeba *P. invadens* has been reported elsewhere for sea urchins, but to the best of our knowledge has not been observed for the sea urchins in the South African IMTA lab (*Tripneustes gratilla* and *Parechinus angulosus*). This parasite causes paramoebiosis, which leads to necrosis and a reddish-brown discoloration of the body surface, along with tube feet muscle degeneration, immobility, and reduced feeding. The parasite can be identified using histological approaches or through culture-based techniques, using malt yeast and non-nutrient agar. No method of control has been identified yet.

4.1.2.2 Abalone

There are currently three diseases of abalone listed by the WOAH, namely infection with abalone viral ganglioneuritis (AVG), infection with *Perkinsus olseni* and infection with *Xenohaliotis californiensis*. None of these diseases are known to occur in South Africa. These pathogens are routinely tested for as part of a targeted surveillance programme to act as an early detection system and to demonstrate the absence of infection with these disease agents in farmed populations. *X. californiensis* is a Rickettsiales-like bacterium and is the causative agent of withering syndrome (Crosson et al. 2020), whereas *P. olseni* is a protozoan parasite capable of infecting a variety of molluscan species and AVG is the causative agent of abalone herpes virus (AbHV) (Corbeil et al. 2012).

Disease agents that have been identified from the commercial abalone species, *Haliotis midae*, include a range of opportunistic *Vibrio* species, such as *V. anguillarum* (Macey and Coyne, 2005) and *V. alginolyticus* (Dixon et al., 1991); the oomycetes *Haliphthoros milfordensis* (Hatai 1982) and *Halioticida noduliformans* (Macey et al., 2011; Greeff et al., 2012); polychaetes, such as the sabellid worm *Terebrasabella heterouncinata* (Gray and Kaiser 2007; Ruck and Cook, 1998) and several *Boccardia* and *Diploydora spp*. (Boonzaaier et al., 2014); a sessile peritrichous ciliate (*Mantoscyphidia midae*) (Botes et al., 2001); as well as gut and digestive gland protozoa (Mouton and Gummow 2011) (Table 1). None of these parasites have however caused large scale mortalities on commercial abalone farms, but can adversely impact production, sales and ultimately farm profits. Infestations with these parasites are routinely monitored for, during grading and cleaning events on the farm through visual observation of shells (worm tunnels in shells, broken shells, lack of new ridges, shiny shells) and

controlled through strict adherence to biosecurity, helping to limit spread of the parasite within and between facilities, and good husbandry/sanitation practices, particularly on farms utilizing formulated feed — which has been shown to increase prevalence of worms as uneaten feed provides a food source for the worms.

Table 1 Abalone pathogens identified by the South African Department of Forestry, and the Environment as part of the health management procedures for South African abalone produced for export.

Category	**Disease / Parasite**	**Distribution**	**Zone**	**Reference**
Viruses	No viruses have been identified as yet from South African farmed abalone that cause disease	N/A	N/A	N/A
Bacteria	*Rickettsia*-like prokaryotes	*Haliotis midae*- Farmed	South Coast	(Mouton & Gummow 2011, Horwitz et al. 2016)
	Vibrio anguillarum	*Haliotis midae*- Farmed	South Coast	(Macey & Coyne 2005)
	Vibrio alginolyticus *Clostridium lituseberense*	*Haliotis midae*- animals housed in a recirculating system	South Coast	(Dixon et al. 1991)
Fungi and Oomycetes	*Halioticida noduliformans*	*Haliotis midae*- Farmed	South Coast	(Macey et al. 2011, Greeff et al. 2012)
Protozoa	Digestive gland protozoa	*Haliotis midae*- Farmed	South Coast	(Mouton & Gummow 2011)
	Gut protozoa	*Haliotis midae*- Farmed	South Coast	(Mouton & Gummow 2011)
Ciliates - Peritrichia	*Mantoscyphidia spadiceae*	*Haliotis spadicea* - Wild	South Coast	(Botes et al. 2001)
	Mantoscyphidia midae	*Haliotis midae* – Wild and Farmed	South Coast	(Botes et al. 2001)
	Caliperia sp	*Haliotis spadicea* - Wild *Haliotis mide – Wild*	South Coast	(Botes 1999)
Platyhelminthes: Trematoda	Digenean	*Haliotis spadicea* - Wild	South Coast	(Botes 1999)
Polychaetes	*Terebrasabella heterouncinata*	*Haliotis midae* – Farmed	West Coast	(Gray & Kaiser 2007)

	Boccardia polybranchia	*Haliotis mide – Wild*	South Coast	(Boonzaaier et al. 2014)
	Boccardia proboscidea	*Haliotis midae* – Farmed	South Coast	(Boonzaaier et al. 2014)
	Boccardia pseudonatrix	*Haliotis midae* – Farmed	South and East Coast	(Boonzaaier et al. 2014)
	Diploydora armata	*Haliotis mide – Wild*	South Coast	(Boonzaaier et al. 2014)
	Dipolydora cf caeca	*Haliotis mide – Wild*	South Coast	(Boonzaaier et al. 2014)
	Dipolydora capensis	*Haliotis midae* – Wild and Farmed	South Coast	(Boonzaaier et al. 2014)
	Diploydora cf giardi	*Haliotis mide – Wild*	South Coast	(Boonzaaier et al. 2014)
	Dipolydora normalis	*Haliotis midae* – Wild and Farmed	South and East Coast	(Boonzaaier et al. 2014)
	Diploydora keulderae	*Haliotis midae* – Wild and Farmed	South Coast	(Boonzaaier et al. 2014)
	Polydora hoplura	*Haliotis midae* – Wild and Farmed	South Coast	(Boonzaaier et al. 2014)
	Pseudopolydora dayii	*Haliotis midae* – Wild and Farmed	South Coast	(Boonzaaier et al. 2014)

Bacteria

- *Vibrio spp.*

Several *Vibrio spp.* can cause disease in a variety of abalone species as well as in humans consuming raw or uncooked abalone products, including *V. harveyi, V. alginolyticus, V. carchariae*, and *V. parahaemolyticus* (Dixon et al., 1991; Elston et al., 1983; Anguiano-Beltrán et al. 1998; Nishimori et al. 1998; Nicolas et al. 2002; Cai et al., 2007; Sawabe et al., 2007; Lee et al., 2016). Bacteria belonging to the genus *Vibrio* are ubiquitous in the marine environment and most aquaculture systems and are thus frequently isolated from abalone and other marine organisms. Lee at al. (2016), for example, identified a total of 66 *Vibrio* species from *H. discus* hanai during a field survey, with *V. tasmaniensis*, *V. splendidus, V. hemicentroti*, *V. lentus*, and *V. pomeroyi* amongst the most abundant of the species. Members of the *Vibrio* genus are well known for causing opportunistic infections in immunocompromised animals and can cause systematic infection of soft tissues, tissue necrosis and death. Monitoring systems for mortalities and signs of infection, as well as making use of strict biosecurity practices can limit the impact of this production disease.

- *Xenohaliotis californiensis*

X. californiensis is an intracellular rickettsial bacterium and the causative agent of withering syndrome disease in wild and farmed abalones, *Haliotis* spp. (Friedman, et al. 2000; Moore et al. 2000). The bacterium has been reported to infect a variety of *Haliotis spp*., including black abalones (*H. cracherodii*), white abalones (*H. sorenseni*), red abalones *(H. rufescens*), pink abalones (*H. corrugata*), green abalones (*H. fulgens*), the small abalone (*H. diversicolor supertexta*) (Wetchateng, 2008), and the European abalone (Balseiro et al., 2006), both in the wild or in culture facilities. The South African abalone, *H. midae*, is not specifically described as a susceptible species by the WOAH for this disease agent. However, the definition of a host species for this disease does not exclude *H. midae* and it is entirely reasonable, based on existing knowledge, to treat it as potentially susceptible. Consequently, this disease agent is routinely monitored for to demonstrate freedom from this disease for export purposes. Clinical signs of infection include shrinking of the foot muscle (pedal atrophy), molted digestive gland, anorexia, weakness, and lethargy. Infections may persist for long periods without the development of clinical signs of the disease when the host is maintained at cool water temperatures (e.g., 15°C), and exposure to elevated seawater temperatures (e.g., >17°C for red, black and white abalones) typically results in clinical manifestation of the disease (Friedman et al., 2003). Histological approaches (presence of basophilic, oval intra-cytoplasmic bacterial inclusions in digestive epithelia) can confirm the presence of these bacteria in the abalone gut epithelium. When used in conjunction with histology, PCR and sequencing (or *in situ* hybridization) can be used for confirmation of *X. californiensis*. Detailed procedures for the detection and identification of this pathogen are outlined in

the WOAH Aquatic Manual (Manual of Diagnostic Tests for Aquatic Animals). As a WOAH listed disease, this disease requires immediate reporting. Preventative measures include routine animal health monitoring and health assessments before moving animals.

Fungal-like organisms (oomycetes)

- *Halioticida noduliformans*

One of the first major disease problems documented on South African abalone farms occurred around 2006, when there was an outbreak of abalone tubercle mycosis caused by the oomycete *H. noduliformans*. The disease was associated with significant pathology and mortality (up to 90% mortality among spat and up to 30% mortality among older animals) of cultured *H. midae* (Macey et al. 2011). The oomycete infection is characterized by multifocal areas of necrosis of the epithelium, underlying muscle fibers and connective tissues of the foot, epipodium, and mantle. The oomycete has also been detected in other abalone species, including *H. rufescens* Swainson, imported from the Republic of Chile and the United Mexican States, and *H. sieboldii* collected at Nagasaki, Japan — that were maintained together in holding tanks at a facility in Japan (Muraosa et al. 2009). Closely related organisms to this oomycete, all members of the *Peronomycetes*, have also been shown to cause disease in abalone as well as other commercially important marine species, including shrimps, crabs and fish, and have had devastating effects on commercial aquaculture operations (Atami et al., 2009; Chukanhom et al., 2003; Kitancharoen et al., 1995; Leaño,2002; Muraosa et al., 2009; Roza and Hatai, 1999). *H. noduliformans* is capable of growing at a wide range of temperatures (10-25 °C), but optimal growth was observed between 20-25 °C and no growth was observed at 5 °C and 30 °C (Macey et al., 2011). Abalone tubercle mycosis can be identified by visual inspection (clinical signs described above) and histological examination can be used to confirm the presence of fungal hyphae. However, this is only possible in animals exhibiting clinical signs of the disease. More recently, a real-time quantitative PCR (qPCR) method was developed to allow for more rapid and reliable diagnosis of this disease (Greeff et al., 2012), and the diagnostic is now routinely used for monitoring and confirmation of this pathogen on South African abalone farms. Infection with *H. noduliformans* (Abalone tubercle mycosis) must be reported to the DFFE under current permit conditions. The PCR primers for *H. noduliformans* specifically amplify a region of the large subunit (LSU) rRNA gene of the pathogen. This diagnostic tool, in conjunction with the other methods for diagnosis, have greatly improved management and containment of this pathogen. Abalone tubercle mycosis in S. Africa is currently regarded as a production disease and effective containment has been achieved by destocking of affected raceways/facilities, sterilization of equipment, removal, and decontamination of biological filter material (when present) and adherence to suitable fallowing periods. Moreover, strict adherence to biosecurity, optimal husbandry practices, and early detection and subsequent management are

regarded as effective means to reduce the occurrence of this pathogen, as is the case for other production diseases.

Viruses

- *Haliotid herpesvirus 1 (HaHV-1)*

Haliotid herpesvirus 1 (HaHV-1), formerly called A*balone herpesvirus (*AbHV*)*, is a member of the genus *Aurivirus* and Family Malacoherpesviridae, and known to be the aetiological agent of the Abalone Viral Ganglioneuritis (AVG) disease. Herpesviruses associated with abalone disease have been reported from Chinese Taipei and Australia, and recent genome sequencing has revealed a number of genotypic variants of the virus exist in Australia (Cowley et al., 2011). Species known to be susceptible to AVG include the greenlip abalone (*Haliotis laevigata*), blacklip abalone (*H. rubra*) and hybrids of these two species. AVG has never been detected in South African *H. midae* and as is the case for *Xenohaliotis californiensis*, *H. midae* is not specifically described as a susceptible host species by the WOAH for this disease agent. However, the definition of a host species for this disease does not exclude *H. midae* and it is entirely reasonable, based on existing knowledge, to treat it as potentially susceptible. As such, AVG is routinely monitored for in South Africa as part of the targeted surveillance program to demonstrate absence of infection with this disease agent. Clinical signs of infection include swollen and protruding mouth parts, curling and/or blistering of the foot muscle. Affected abalone demonstrating clinical signs are likely to die within 1 day of showing these signs. Ganglioneuritis is observed in sections of neural tissue by light microscopy and confirmation of the presence of AbHV is obtained by quantitative polymerase chain reaction (qPCR) and/or *in-situ* hybridization (Crane et al., 2009). The spread of this disease agent is limited by the implementation of good biosecurity practices on farms.

Parasites

- *Perkinsus olseni*

P. olseni is a highly infectious protozoan that results in brown pustules (approximately 8mm in diameter) filled with a brown, creamy fluid in the foot and mantle of abalone. *P. olseni* has an extremely wide host range, including the clams *Anadara trapezia*, *Austrovenus stutchburyi*, *Ruditapes decussatus*, *R. philippinarum*, *Tridacna maxima*, *T. crocea*, *Protothaca jedoensis* and *Pitar rostrata* (Goggin & Lester, 1995; Villalba et al., 2004; Cremonte et al., 2005; Park et al., 2006; Sheppard & Phillips, 2008); oysters *Magallana ariakensis*, and *C. sikamea* (Villalba et al., 2004); pearl oysters *Pinctada margaritifera*, *P. martensii*, and *P. fucata* (Goggin & Lester, 1995; Sanil et al., 2010); and abalone *Haliotis rubra*, *H. laevigata*, *H. scalaris*, and *H. cyclobates* (Goggin & Lester, 1995). As with the other WOAH listed diseases in South Africa, the local abalone *H. midae* is not specifically described as a susceptible species by the WOAH for this disease agent. However, *P. olseni* is routinely monitored

for in South Africa as part of the targeted surveillance program to demonstrate absence of infection with this disease agent. In countries where it occurs, it has been documented to spread through direct contact between animals. The annual cycle of *P. olseni* is controlled by temperature, with infection intensity peaking as temperature increases to about 15°C, remaining high at temperatures of 19–21°C, and declining with temperatures of 9–10°C (Villalba et al., 2005). Clinical signs are dead animals and gross pathology includes thin, watery tissue, pale digestive gland and nodules in the mantle and gills of some hosts; but these signs are not specific to infection with this pathogen. Various early detection methods exist, including assays (whole body burden assay), histology, as well as genus- and species-specific PCR assays. The tests recommended by the WOAH for targeted surveillance of infection with *P.s olseni* are PCR and Ray's fluid thioglycolate culture method (RFTM).

- *Spionidae*

The commercially exploited abalone species in South Africa, *Haliotis midae*, is infested by a number of spionids, including indigenous *Dipolydora capensis* (Day 1955) and *Polydora hoplura* Claparède 1870, and by non-indigenous Boccardia proboscidea Hartman 1940, which were first reported in South Africa in 2004 (Simon et al., 2006; Simon and Booth, 2007). These annelid worms negatively impact production, and increase production costs, by reducing flesh condition of cultured molluscs through delayed growth rates and increased mortality (Boonzaaier et al. 2014). In the study by Boonzaaier et al. (2014), a total of 14 polydorid species were identified from farmed (14 farms sampled) and wild abalone collected along the South African coast. *Boccardia proboscidea* and *D. capensis* were shown to infest the most abalone per site and were recorded on the greatest number of farms. Of the 14 species recorded, 46% were associated both with wild and farmed abalone, and of these the indigenous *D. capensis* was encountered most frequently. These data suggest that polydorids move between the wild and farms and possibly between farms. As with the sabellid polychaetes, the polydorid polychaetes bore holes in abalone shells, resulting in shell deformaties, brittle shells and slow growth. These shell boring parasites are routinely monitored for on farms during grading events (approximately once every 3 months by looking for worm holes in shells, broken shells and lack of new ridges) and daily cleaning/feeding events on the farms. Currently, methods of control include strict adherence to biosecurity, helping to limit spread of the parasite within and between facilities, and good husbandry/sanitation practices, particularly on farms utilizing formulated feed — which has been shown to increase prevalence of worms as uneaten feed provides a food source for the worms.

- *Terebrasabella heterouncinata*

The sabellid *T. heterouncinata* (5mm or smaller) infects various gastropods, including abalone (Ruck and Cook, 1998). Besides occurring on *H. midae* in South Africa, *T. heterouncinata* is believed to occur in most abalone culture facilities in California, USA and Baja California, Mexico, and has also more recently been documented from cultured *Haliotis rufescens* in Iceland and from an abalone aquaculture facility in southern Chile (Moreno et al. 2006). Susceptible abalone species include *H. rufescens*, *H. fulgens*, *H. corrugata*, *H. midae* and other species of abalone as well as other species of marine gastropods, including snails and limpets (Kuris and Culver 1999). The short reproductive cycle of 3 – 4 months to sexual maturity and rapid reproduction thereafter results in rapid increases in their abundance in abalone culture facilities (Simon et al. 2005), which is further exacerbated by high abalone stocking densities and poor hygiene and water quality in tanks (Ruck and Cook 1998, Sales and Britz 2000; Simon et al. 2004). Detrimental effects include significantly reduced growth rates, grossly deformed shells with the absence of respiratory aperture (gill pore) formation on the leading edge of the shell, decreased meat yields, increased mortality due to the inability of the abalone to right themselves when dislodged from their substrate, and reduced marketability. Infested abalone are characteristic by a dome shaped shell, with damage and deformation as described previously. Regular monitoring of shells can inform management of this pest (Infected shells have characteristic white tunnels along the edge of the shells where worms live). Currently methods of control include strict adherence to biosecurity, helping to limit spread of the parasite within and between facilities, and good husbandry/sanitation practices, particularly on farms utilizing formulated feed — which has been shown to increase prevalence of worms as uneaten feed provides a food source for the worms.

4.1.2.3 Seaweeds

There is growing evidence that *Ulva*, when grown in integrated systems with abalone, can have a modulatory effect on the microbiome of the seawater in the system as well as the abalone digestive tract, when effluent grown *Ulva* is fed back to abalone as a supplementary feed (de Jager 2019; Brand et al., unpublished). In South Africa, several land-based abalone (*H. midae*) farms practice integrated multi-tropic aquaculture (IMTA) by growing *Ulva lacinulata* in D-Shaped paddle-raceway system using abalone effluent. This practice also allows for the water to be recirculated back to abalone raceways. *Ulva* spp. presently do not have any listed pathogenic agents and do not act as a sink for harmful bacteria, but rather reduce the abundance of opportunistic pathogens, such as *Vibrio* spp., in abalone-*Ulva* IMTA systems (de Jager 2019; Califano et al. 2020). Though limited information on *Ulva* diseases exists, *Ulva* spp. have a known epiphyte, *Myrionema strangulans,* that can impact aquaculture production. The monitoring and continued characterisation of the microbial communities associated with *Ulva*, as well as careful approaches to moving material between farms, will remain important to

prevent future production diseases impacting both the seaweed as well as other animals, such as abalone, in the production systems.

Epiphytes

- *Myrionema strangulans*

Various *Ulva* species can be colonised by the epiphyte *M. strangulans*, which can be identified by brown spots that form on the *Ulva* fronds. Although these epiphytes do not pose a major risk to the operation of aquaculture facilities, they compete with *Ulva* for nutrients obtained from the water column and make the *Ulva* prone to fragmentation (Buschmann and Gomez 1993; Siniscalchi et al. 2012). The presence of *M. strangulans* also results in a shading effect, which together with the competition for nutrients, results in slow growth of the seaweed, thereby reducing productivity.

4.1.2.4 Biofloc - White shrimp - Tilapia

As these bioflocs are based in microbial production, a complete food chain based on bacterial grazing can grow in the system, such as flagellates, ciliates, nematodes, copepods and amoeboid forms (Biddanda and Pomeroy, 1988). Albeit the presence of a well stablished microbial community keeps the system more stable and less susceptible to diseases, the Brazil IMTA system has been confronted by some pathogens outbreaks. Thus, a routine use of probiotic bacteria in the biofloc based IMTA, mostly of bacteria belonging to the genus *Bacillus*, strengthens the immune system of all organisms in the system. The high recirculation rates in the system make that the entire will be susceptible to the same pathogens. Below are provided the main diseases that can appear in Biofloc IMTA labs:

Bacteria

- *Vibrio spp.*

Vibrio spp. is a well described potential pathogen genera that has been mentioned during the entire document. Particularly, in biofloc shrimp culture, *Vibrio spp.* infections are capable of reduce production in several proportions and can infect about 70 to 80% of individuals (Chandrakala and Priya, 2017). Particularly, *V. harveyi* a Gram-negative luminous bacterium is one of the aetiologic agents of mass mortalities of *Penaeus monodon* larval rearing systems (Chandrakala and Priya, 2017). Albeit the exoskeleton provides an effective physical barrier to pathogens, outbreaks may occur when environmental factors trigger rapid multiplication of bacteria already tolerated at low levels with shrimp blood or by bacterial penetration of host barriers (Chandrakala and Priya, 2017). Several predispositions factors that could lead to disease outbreaks in shrimp culture are poor soil and water quality, high stocking density with limited water exchange facilities, accumulation of unutilized feed, inadequate aeration, presence of virulent pathogen in high counts (Chandrakala and Priya, 2017). Monitoring methods for *Vibrio* species in Land-based IMTA lab is controlling the predisposition factors

mentioned and clinical signs within the system: necrosis of appendages, expanded chromatophores, empty gut and absence of faecal strands.

- *Cyanobacteria*

Cyanobacteria is a blue-green algae that usually dominate phytoplankton community in aquaculture systems, with several physiological mechanisms that allow them to outcompete other types of phytoplankton for sunlight (Paerl and Tucker, 1995). Nonetheless, certain species of this group are undesirable due to their production of odorous compounds that can accumulate in the flesh of fish and subsequently result in an "off-flavor" and unmarketable product (Schrader, Green and Perschbacher, 2011). The rapid exhaustion of oxygen in the water and release of the cyanobacterial toxin can cause huge mortalities in shrimps and fish (Gao et al. 2017). Moreover, high concentrations of bacteria from Cyanobacteria phylum have caused mortality due to gill occlusion in shrimps and fish near to Brazil IMTA lab facilities. Although diseases caused by this bacterium group was never registered within the Biofloc IMTA system, probably due to the reduction of light penetration into the water column that causes the biofloc.

4.1.3 Dynamics of opportunistic bacterial pathogens and communities observed from real microbial data in the ASTRAL IMTA land-based systems

The microbial diversity of the partially recirculating land-based systems in South Africa and the 100% recirculating biofloc IMTA system in Brazil has been studied. The use of real microbial data from IMTA labs aims mainly to identify the presence of pathogens. In addition, a brief description of the communities dynamics within the system is provided to increase the understanding of the microorganisms that dominate each part of the systems, which is intended to be another monitoring tool for the prevention of pathogen outbreaks.

4.1.3.1 Samplings

For the partially recirculating (50% recirculation) land-based system in South Africa, *Ulva* and seawater samples were collected from two types of *Ulva* paddle-raceway systems at Buffeljags Abalone Farm. One system consisted of tanks that received seawater directly from the adjacent coastline, hereafter referred to as the seawater (SW) or non-IMTA *Ulva* paddle-raceway system. The other systems comprised paddle-raceways receiving abalone effluent water, with 50% recirculation between the abalone and *Ulva* raceways, referred to as abalone effluent water (AEW) or IMTA paddle-raceway systems (see Figure 3, section 4.2.1). *Ulva* samples were collected from within each paddle-raceway, whereas the water samples were collected at the inlet (representative of effluent water from the abalone tanks) and outlet (representative of water bio-remediated by *Ulva*) of each *Ulva* paddle-raceway. One SW *Ulva* paddle-raceway (only one exists on the farm) and 4 AEW *Ulva* paddle-raceways

were sampled in autumn (n = 1), winter (n = 1), spring (n = 1) and summer (n = 1). Water samples (500 mL, in triplicate) were filtered through 0.22 µm filter membranes to concentrate microbial cells. A total of 64 samples (including controls) were collected and pre-processed prior to DNA extractions.

For the biofloc recirculating land-based system in Brazil, biofloc, shrimp gut, fish gills/gut and seaweed samples were collected from two different types of biofloc dominance (heterotrophic x chemoautotrophic) IMTA systems. The system dominated by heterotrophic biofloc was obtained by organic fertilization and the chemoautotrophic by inorganic fertilization. Both systems consisted of three compartments, where the inlet water achieved a 100% of re-circularity (see Figure 4, section 4.2.2). First of all, the water is introduced into the system thought compartment 1, where the shrimp is fed. In compartment 1, the oxygen, carbon and uneaten feed transforms into biofloc (microorganisms aggregated in flocs). Then, water from this tank (compartment) is transferred by a pump system to compartment 2, where tilapia and oyster participate in the organic matter consumption and biofloc stabilization. Subsequently, the water with less organic matter concentration passed to the seaweed tanks, transforming the nitrogen compounds into seaweed biomass. In this way, water quality is maintained, and the same water can be used for several production cycles. All samples were collected from triplicate systems, with triplicates of each sample type. After three samplings (T0 – day0, T1 – day45 and T2 – day90), a total of 195 samples were processed for next generation sequencing (NGS) analysis.

4.1.3.2 Sample processing and raw sequencing processing

Samples from both experiments were processed independently but followed standard practices in bacterial communities' analysis. Microbial DNA was isolated using a QIAamp® DNA Micro Kit (Qiagen, Cat. No. 56304) for the South African samples or ZymoBIOMICS DNA Miniprep kit (Zymo Research, Irvine, Canada) for Brazilian samples, following the manufacturer's instructions. Sequencing of the hyper-variable regions V4 (South Africa) or V3-V4 (Brazil) was carried out with the following forward and reverse primers: V4 – 515F/806R (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA/5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT); V3-V4 – 341F/805R (5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG/5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC). The underlined sequences are the adaptor overhang nucleotide sequences used in the Illumina 16S metagenomics workflow. Both hypervariable regions (South Africa: 360-370 base pairs, Brazil: approx. 450 base pairs) of the 16S rRNA gene was amplified. PCR products were normalized to equal concentrations and sequenced using an Illumina® MiSeq sequencing instrument. Sequencing for South Africa samples was carried out at the Centre for Proteomic and Genomic Research (CPGR) in South Africa (https://www.cpgr.org.za/) using a MiSeq

Reagent v2 Kit (500 cycles) with 2 x 250 paired end reads. Meanwhile, Brazil samples were sequenced in Genome Québec Inc. (Centre d'expertise et de services Génome Québec, Montréal (Québec), Canada) with 2 x 300 paired end reads.

Demultiplexed paired-end FASTQ files were imported into the Quantitative Insights into Microbial Ecology 2 program (QIIME2- 2020.11). The Divisive Amplicon Denoising Algorithm 2 (DADA2) software package (Callahan et al. 2016) in QIIME2, was used to quality filter, trim, de-noise, and merge reads. Taxonomy was assigned to amplicon sequence variants (ASVs) using the SILVA database (Quast et al. 2013). The raw sequence processing of South Africa samples was performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team (hpc.uct.ac.za).

4.1.3.3 Microbiota analysis

All data analysis was conducted in MicrobiomeAnalyst (Dhariwal et al. 2017) or directly in R software (Version 1.4.1717) using the R packages vegan (Dixon 2003) and phyloseq (McMurdie and Holmes 2013). Briefly, a marginally filtered dataset, based on ASVs, was used to calculate and estimate overall taxonomic abundance and diversity metrics. Uneven sequencing depth, under-sampling, and data sparsity were all corrected for using the relative log expression (RLE) transformation (Hawinkel 2015), prior to conducting between sample differential abundance assessments (DESeq2; Love et al. 2014). Particularly, for the differential abundance assessment of the Brazil dataset, a centered log-ratio (CLR) and ANOVA approach (Gloor 2017) was used instead of the previously mentioned transformation.

4.1.3.4 Identified opportunistic pathogens and other bacteria

In the land-based abalone-*Ulva* farm in South Africa, the relative abundance of specific bacteria changed as the seawater moved from the inlets to the outlets of each of the *Ulva* paddle raceway systems (Figure 5). For example, the abundance of bacteria belonging to the genus *Pseudoalteromonas* and *Vibrio* decreased from 15% to 12% and 19% to 15%, respectively, from the inlet to the outlet of the AEW systems *(*DESeq2; $P < 0.05$). The reduction in the relative abundance of *Vibrio* spp. in the IMTA system is beneficial, as members of this genus have been shown to cause disease in a variety of molluscs, including abalone. A higher *Vibrio* abundance (DESeq2; $P < 0.05$) was observed for water samples collected in autumn and summer (data not shown), across both the non-IMTA and IMTA systems, suggesting that members of this genus should be more closely monitored during these seasons. Therefore, the development of monitoring tools, such as bacterial profiling using 16S sequencing technology or whole genome sequencing, is useful to manage disease risks for abalone farming. This is particularly important for the IMTA system, as a higher *Vibrio* abundance was observed in this system when compared to the non-IMTA system (Figure 5), likely as a result of the higher nutrient availability in this system. Notably, the *Vibrio* was consistently in low abundance in *Ulva*

samples, regardless of the season that the samples were collected in. The reduction in the abundance of opportunistic pathogens observed in the abalone-*Ulva* IMTA systems in this study, as water moves from the inlets to the outlets of the paddle raceway, has also been observed in other IMTA systems cultivating *Ulva* (Califano et al. 2020). Since *Ulva* has a positive modulatory effect on the microbiome and controls the abundance and composition of microorganisms growing on it, it could be used as a functional feed or formulated feed ingredient for aquaculture species to reduce potential pathogenic bacteria.

Figure 5. Relative ASV abundance at (A) phylum and (B) genus level across the 60 samples from the South African lab. The 30 most abundant ASVs are depicted and the remaining ASVs were grouped as "Others". SW=seawater or non-IMTA system; AEW=abalone effluent water or IMTA system.

Further taxonomic abundance profiling revealed the presence of diverse bacterial communities associated with the samples collected from Buffeljags Abalone Farm, with 380 ASVs identified at genus

level and some distinct differences between the groups. Most notably, there are a lower number of unique bacteria associated with *Ulva* in comparison to the seawater sampled in the raceways, supporting the notion that *Ulva* only allows specific taxa to colonise the seaweed (Figure 5). Bacteria belonging to the genera *Vibrio*, *Pseudoalteromonas* and *Acinetobacter*, and families Flavobacteriaceae, Arcobacteraceae and Rhodobacteraceae were more prevalent in the seawater samples collected from the *Ulva* raceways (inlet and outlets), whereas bacteria belonging to the genera *Granulosicoccus* and *Maribacter*, and families Saprospiraceae and Rhodobacteraceae were more prevalent on the *Ulva* samples growing in the raceways (both IMTA and non-IMTA) (Figure 5). Some of the bacteria found to be associated with *Ulva* in the present study, such as species within the Class gammaproteobacteria (e.g., *Marinobacter* sp.) and Family Rhodobacteraceae (*Roseovarius* sp.), have previously been shown to be beneficial to the growth, morphogenesis and development of *Ulva* (Weiss *et al*., 2017) and could be contributing towards the health of *Ulva* growing vegetatively in the IMTA.

Non-metric multi-dimensional scaling (NMDS) plots revealed that the water and *Ulva* samples are different in terms of beta diversity (Figure 6), with some separation observed for water samples collected from the IMTA (AEW) and non-IMTA (SW) (PERMANOVA; $P < 0.001$). Distinct clustering was also evident for the *Ulva* samples collected from the IMTA and non-IMTA systems, indicative of unique microbial communities associated with the *Ulva* grown in the two systems. The microbiome associated with the inlet and outlet water samples collected from each system showed a degree of overlap, however the bacterial community profile of the outlet samples shifted towards the profile of the *Ulva* samples, supporting that *Ulva* has a modulatory effect on the microbiome as the water moves from the inlet to the outlet of the paddle raceway systems which was observed in both the IMTA and non-IMTA systems. Therefore, it is likely that integrated abalone-*Ulva* IMTA contributes towards the maintenance of system- and animal health through the known reduction of nutrients (nitrogen) (Robertson-Andersson et al., 2008; Bolton et al., 2016) and opportunistic pathogenic microorganisms in the system.

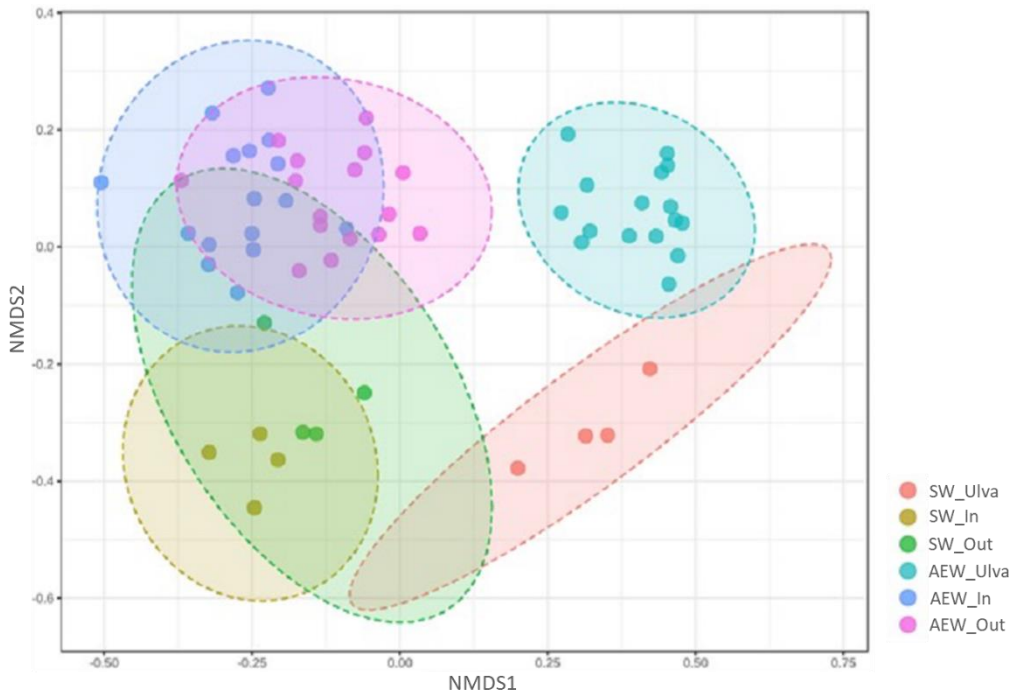

Figure 6. Non-metric multidimensional scaling (NMDS), computed from Bray–Curtis distance matrix and grouped by sample (inlet, outlet and Ulva) and system type (SW=seawater/non-IMTA system; AEW=abalone effluent water/IMTA system) for the South African lab.

In terms of **Brazil IMTA lab**, the taxonomic representation of the 30 most abundant phyla within the system showed that the bacterial communities present in the three compartments changed under both diets compared to the initial timepoint (Figure 7A). In detail, the AVSs not identified at genus level represented a majority of the microbial community in all samples studied (Biofloc: 42 – 48%; Shrimp gut: 19 – 42%; Fish gills: 40 – 45%; Fish gut: 32 – 66%; Seaweed: 31 – 39%) (Figure 7B). The lack of taxonomy resolution at genus level is a factor that should be considered when this type of data is processed, re-evaluating the taxonomic database and how these ASVs have been identified. Albeit these ASVs avoid us to have complete information at a deeper taxonomic level, several differences in terms of abundance of the identified genera has been observed.

From all the pathogens described in previous section (4.1.2) only the opportunistic pathogenic genus *Vibrio* was found within the 30 most abundant ones. In the compartment 1, composed by biofloc and shrimp, *Vibrio* abundance decreased from the initial sampling point (Biofloc = 5%, Shrimp Gut = 4.2%) to the middle and the end of the experiment under both fertilizations (Inorganic T1-T2: Biofloc = 0.06 - 0.04%, Shrimp gut = 0.24 – 0.08%; Organic T1-T2: Biofloc = 0.04 - 0.07%, Shrimp Gut = 0.48 – 0.17%). In the case of the compartment 2, statistically significant reduction compared to the initial point were only observed in the case of the *Vibrio* genus in the tilapia gills bacterial communities (Initial: 1.4%; Inorganic T1-T2: 0.5 – 0.2 %, Organic T1-T2: 1 – 0.3%), meanwhile the abundance in the tilapia gut was marginal over the entire experiment (Initial: 0.5 %; Inorganic T1-T2: 0.7 – 0.5%, Organic T1-T2: 0.7 –

0.4%). Regarding the compartment 3, *Vibrio* was detected, but with a low relative abundance and no significant change in abundance within the bacterial communities present in the Ulva tissues was observed (Initial: 0.03%; Inorganic T1-T2: 0.08 – 0.03%, Organic T1-T2: 0.1 – 0.02%).

In addition, other described pathogenic genera were identified as *Tenacibaculum* and *Coxiella* (Ricketsia like organisms) but are not showed in the Figure 7B due to their low abundance in almost all the samples. Only the biofloc bacterial communities at the initial of the experiment showed a relevant presence of *Coxiella* genus (2.5 %), that was significantly reduced under both fertilization at T1 and T2 (Inorganic T1-T2: 0.18 – 0.07%; Organic T1-T2: = 0.08 - 0.08%). Compared between fertilizers, none of the three pathogenic genera were found to have significantly different abundances within each of the sample type studied. Nevertheless, the genera *Mycobacterium,* increased its relative abundance in the inorganic fertilization compared to the organic at T1 (ANOVA, $P = 0.05$) and T2 (ANOVA, $P < 0.05$). Thus, further studies of the *Mycobacterium* presence in biofloc systems and how the fertilizations affect this genus should be done, as several strains belonged to this genus are described by its high pathogenicity (e.g. *Mycobacterium tuberculosis*).

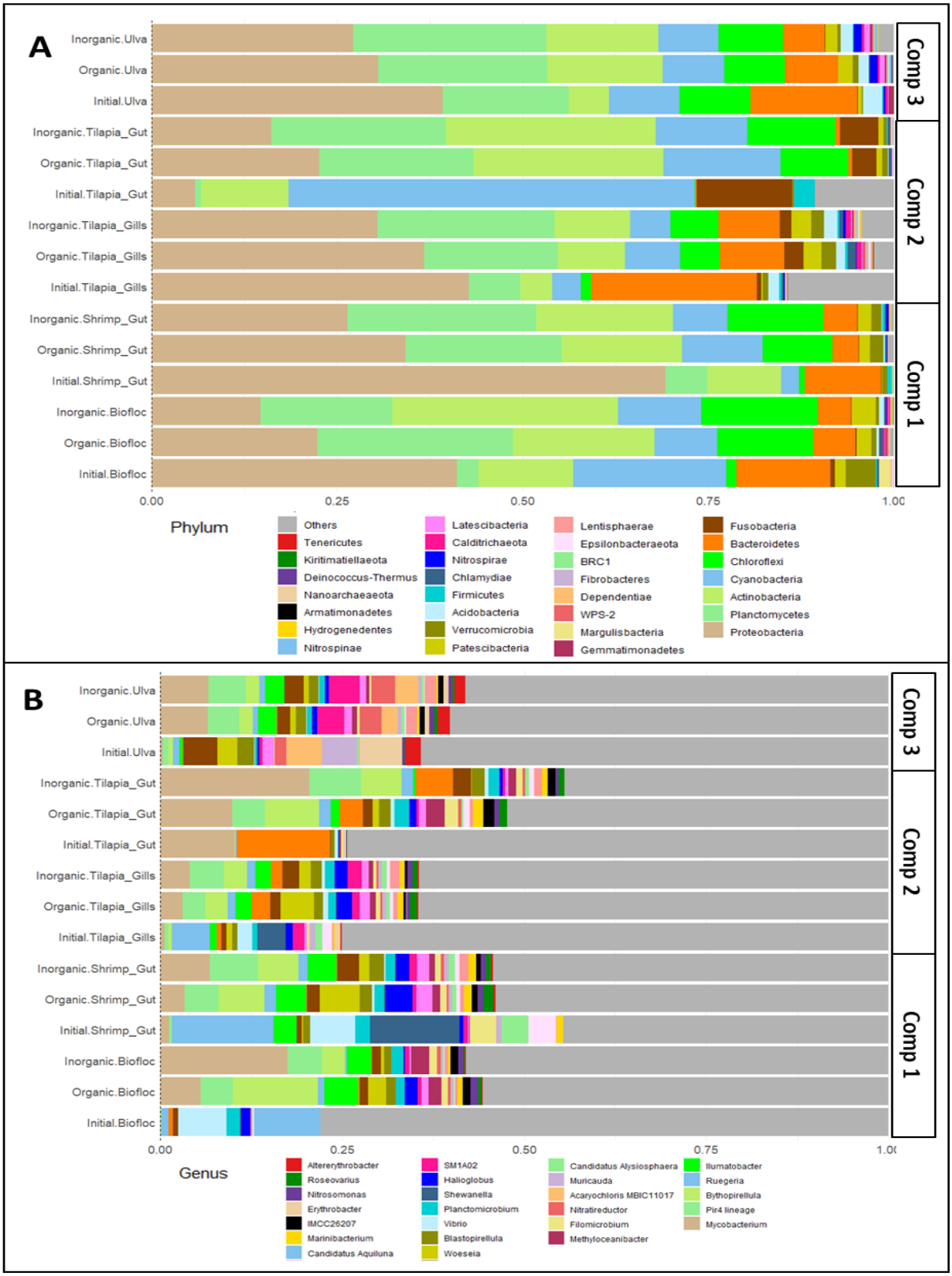

Figure 7. Bar graph showing the mean relative ASV abundance at (A) phylum and (B) genus level for the Brazilian lab across the different conditions included in this study. Within each bar, different colours were assigned to the 30 most abundant phyla or genera, grouping the remaining ones in the category "Other". Comp1 =compartment 1, Comp 2 = compartment 2, Comp 3 = compartment 3.

The beta diversity results of bacterial communities just before the experiment started (initial point) appeared clearly differentiated by sample type studied in the Non-metric multidimensional analyses NMDS (Figure 8, PERMANOVA, $P < 0.001$). Albeit the bacterial communities under both types of diet are still significantly different comparing all sample types (PERMANOVA, Organic: $P < 0.001$; Inorganic: $P < 0.001$), a clear overlapped between sites was observed (Figure 8B). Meanwhile the bacterial communities from biofloc, shrimp gut and tilapia gut showed a similar disposition in the NMDS graph, Ulva and tilapia gills bacterial communities showed a slightly different disposition compared to the previous ones. Dividing by timepoint, the biofloc bacterial communities showed no statistical differences when compared to the tilapia gut under both treatments (T1 and T2). This statement is also true for the comparison between biofloc and shrimp gut under organic fertilization, but differences were observed at T2 in the inorganic one (pairwise PERMANOVA: $P < 0.05$). Moreover, clear similar evolution was observed in the shrimp gut and tilapia gut microbial communities under both fertilizations, with no differences among them (pairwise PERMANOVA: $P > 0.05$). The Ulva associated bacterial communities end up being more similar to fish gut and shrimp gut under the organic fertilizer (pairwise PERMANOVA: $P > 0.05$), while the inorganic fertilizer showed more similarities compared to biofloc and fish gills (pairwise PERMANOVA: $P > 0.05$).

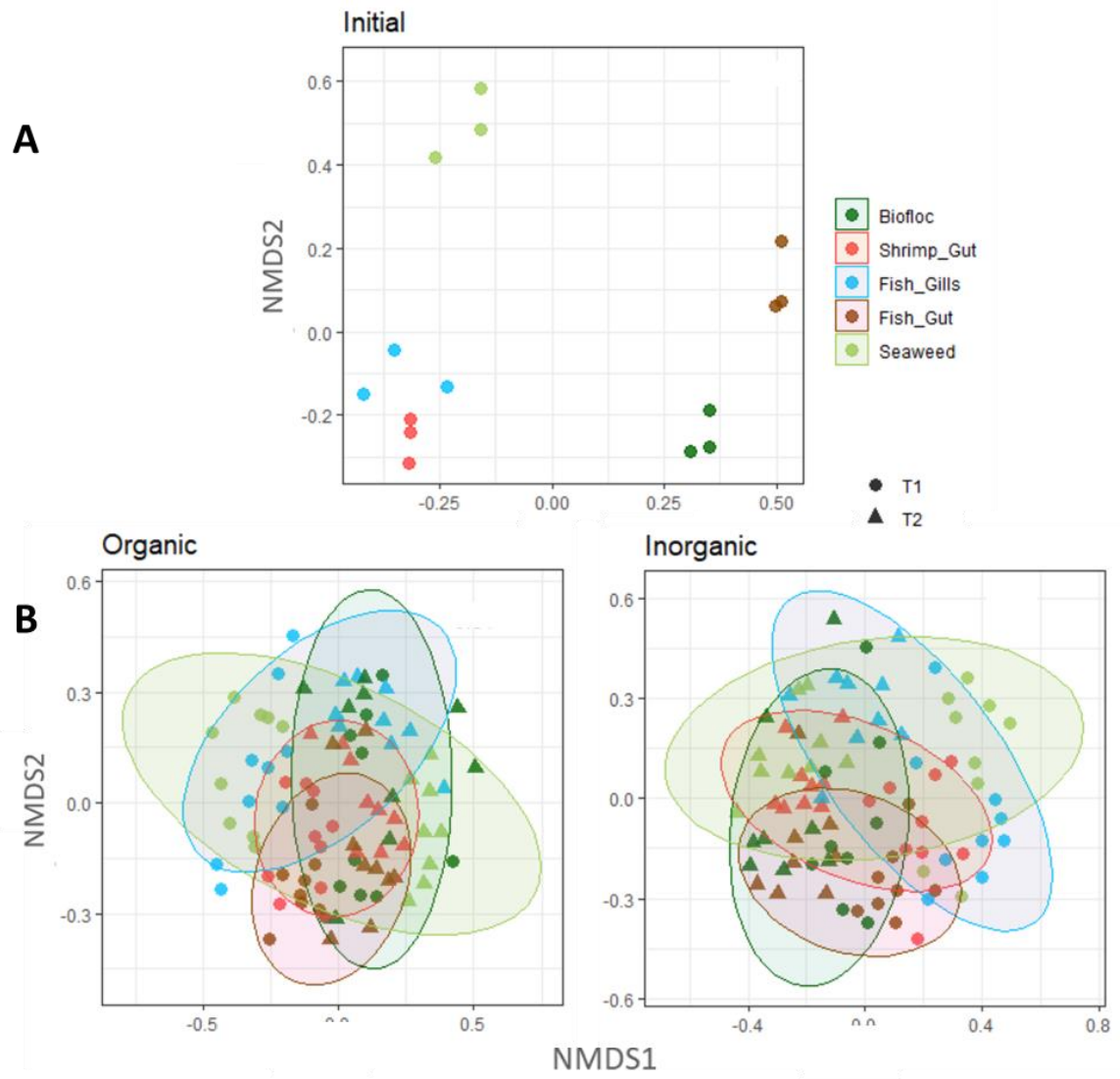

Figure 8. Non-metric multidimensional scaling (NMDS), computed from Bray–Curtis distance matrix, divided by each diet type for the Brazil IMTA lab. The two timepoints after the beginning of the experiment are shown in different shapes: circles for 45 days (T1) and triangles for 90 days (T2). Each condition was coloured differently.

4.1.3.5 Future directions for microbiome assessments

South African IMTA lab

Our results have shown that an abalone-*Ulva* IMTA improves water quality and the bacterial microbiome through the modulatory action of *Ulva* and its associated microorganisms. This practice and the use of effluent grown *Ulva* as a supplementary feed for abalone does not appear to pose a biosecurity risk to the South African IMTA lab — particularly since each abalone cluster linked to a single *Ulva* raceway is independent (and treated as a separate epidemiological unit) from the other abalone-*Ulva* clusters on the farm and can be isolated in the event of a disease outbreak without impacting production on the remainder of the farm. Future studies will investigate the fungal and

oomycete communities within the non-IMTA and IMTA systems to characterize the dynamics of these microorganisms in conjunction with the bacterial microbiome. The impact of increasing recirculation rates (75% and 100%) on the bacteriome, mycobiome and virome will also be assessed to ensure that these practices do not compromise system- and animal health. All data collected in these microbiome studies will be used to construct a database that can be used for future system monitoring, as these data represent an apparently healthy integrated abalone-*Ulva* IMTA system and will be used to identify a system, abalone or *Ulva* in a state of dysbiosis.

Brazil IMTA lab

Bacterial diversity results of the Biofloc IMTA lab system showed a significant evolution during the aquaculture production. All the opportunistic pathogens identified showed a significant decrease with time or a marginally abundance in all the samples analyzed, suggesting the good microbiological status of this system. Nevertheless, the increase of ASVs relative abundance belonged to *Mycobacterium* should be further study by other more precise molecular tools or classical microbiological methods, in order to define the potential pathogenicity of this microbes. Next steps will consist of building a robust database altogether with the microbiome data from the IMTA South Africa laboratory, in order to provide information on healthy IMTA systems, which could lead to future monitoring of metabarcoding and machine learning implementation in the aquaculture industry.

4.1.4 Prospective IMTA in Argentina

The total fishery production fluctuates in Argentina between 1997 and 2017, and the aquaculture production has been marginal throughout this period (FAO, 2022). Consequently, there is very little literature on pathogens within the Patagonian aquaculture systems of the species described in section 3.4.1. Thus, we have selected the blue mussel (*Mytilus chilensis*) and the red sea urchin (*Loxechinus albus*), which were the most described.

4.1.4.1 Blue mussel

By contrast in other parts of the world, knowledge about molluscan bivalves diseases from South America is very scarce. Even more pronounced is the case of Argentina, where only a few studies with field data were identified.

Bacteria

- *Rickettsia-like* organisms

Rickettsia-like organisms have been reported in marine bivalve mollusks from Argentina, with no observable clinical symptoms (Vázquez N et al. 2017). These intracellular prokaryotic microorganisms were found infecting both epithelial cells of digestive tubule and intestine in several bivalve species (*A. mactroides, A. tehuelchus, M. platensis, O. puelchana, P. rudis, P. abbreviata, E. macha*) on the

Argentinean coasts (Vázquez N et al. 2017). These pathogens are commonly distinguished by using transmission electron microscopy from epithelium samples.

Protozoa

- *Perkinsus olseni*

P. olseni is widely recognized as a serious pathogen for several molluscs, mainly present in Asian waters. Nevertheless, its presence was recently detected in the tissue of culture mussels cultivated in the Beagle channel (Vázquez et al., 2022). The expansion of *P. olseni* has occurred worldwide, due to its low host specificity with a wide range of marine mollusks for hosts (Villalba et al., 2011) and high environmental tolerance (Casas et al., 2002). Nonetheless, the absence of this parasite may be related to the colder water (<20°C), unfavourable for its development, since proliferation of all *Perkinsus* species occurred in temperatures over 20°C (Vázquez, 2017). The identity of the parasite was determined through a species-specific PCR and histological, as usually shows a severe haemocytic infiltration and an encapsulation of the trophozoites as host defense response (Vázquez, 2017)

- *Bonamia exitosa*

B. exitosa belongs to the haplosporidian protozoan parasites, which infect haemocytes of several molluscs species, inducing physiological disorders and eventually causing the death of the animal (Vázquez, 2017). This specie has been identified and blamed for mass events in North and South America, Australia, New Zealand and the Mediterranean Sea (Vázquez, 2017). There are not shown specific clinical signs for this disease in molluscs, being characteristic the haemocytic infiltration in all the connective tissue observation through microscopy (WOAH, 2022). This disease is currently detected by histopathology techniques.

4.1.4.2 Red sea urchin

The red sea urchin is widely used along the Chilean coast, but not much information about specific disease outbreaks have been documented in South America regions. Apart from the several pathgoens of sea urchins that have already been described in the section 4.1.2.1. (Land-based: sea urchin), the potential pathogen Pea Crab *Pnnaxodes chilensis* was identified in this review. Although this crustacen was considered to maintain a symbiotic relationship with the red sea urchin, very recently studies have alarmed about a potential parasitims (Jaramillo et al. 2023).

4.2 Environment and warning signs for early detection by each IMTA lab

Within open water and land-based IMTA systems there are several environmental parameters and warning signs exhibited by the organisms that could indicate a risk of a disease outbreak (Table 2). Water temperature is one of the parameters identified by the different IMTA labs which can be strongly associated with a pathogen outbreak. Most pathogens identified in the IMTA labs can cause diseases in the aquaculture organism when the water temperature is between 16ºC and 25ºC, with the months of spring identified as the most susceptible period. Moreover, animal handling and sudden changes in the system can lead to a dysbalanced in the cultured organisms-microbiome relationship, leading to opportunistic pathogen infection(s). These environmental stressors could favor an infection, which could be perceived in the system with several warning signs. In the case of fish, ulcers, mouth erosion, lethargy, whitish spots, loss of appetite and respiratory distress, among others, are the most common signs of disbalance in the system.

Table 2. Table summary of the most problematic pathogens selected by each IMTA lab, providing environmental stressors and warning signs of each disease.

IMTA lab	Pathogen	Type	Seasonality and environmental stressors	Organism affected / Warning signs
Scotland/Ireland (Open water)	*Aeromonas salmonicida*	Bacteria	Water temperatures exceed 16^{o}C	Atlantic salmon & lumpfish / ulcers, mouth erosion, darkening of the skin, abdominal swelling, lethargy, loss of appetite, respiratory distress, and surface swimming.
	Tenacibaculum	Bacteria	Smoltification, handling or exposure to harmful algae	
	Vibrio species	Bacteria	Spring and autumn	
	Moritella viscosa	Bacteria	winter	
	Novirhabdoviruses	Virus	Summer. Ambient water temperature >16ºC, poor water circulation	Atlantic salmon & lumpfish / No external signs
	C. elongates/L. salmonis	Copepods (sea lice)	All year. March – May most abundant.	Atlantic salmon & lumpfish / Whitish spots on the dorsal 0.3 – 0.5 ovigerous lice per fish (March – May).

				2.0 ovigerous lice per fish (June – February)
	Bonamia ostreae	Protist	Infection detection all year. Highest prevalence of infection and resulting disease in Spring (Europe) - potential impacts of seawater temperature and salinity. Infections more frequent in adults (>2yrs of age)	European flat oyster (*O. edulis*) / Reduced growth rate; sudden and high mortalities.
	Oyster Herpes Virus (OsHV, OsHV-1 µVar)	Virus	Higher susceptibility in juveniles and above 21 °C water temperature	Pacific oyster (*Crassostrea gigas*, *Magallana gigas*) / No external signs
	Chytridium polysiphoniae	Chytridiomycetes	Host and temperature dependent (reduced virulence above 15ºC)	Brown algae / Bleaching of the tissue
South Africa (Land-based)	*Vibrio species*	Bacteria	Animal handling, sudden environmental changes, poor food quality	Sea urchin & abalone / Sea urchin spine loss and bald spots on the body (test) surface, abalone tissue necrosis
	Xenohalitis californiensis (Rickettsia)	Bacteria	Elevated seawater temperatures (e.g., >17°C)	Abalone / Shrinking of the foot muscle, molted digestive gland, anorexia, weakness, and lethargy.
	Halioticida noduliformans	Oomycete	Optimal growth was observed between 20-25 °C. No growth was observed at 5 °C and 30 °C	Abalone /

				Multifocal areas of necrosis of the epithelium
	Abalone herpesvirus	Virus	Not season-dependent, effect of temperature, salinity and dissolved oxygen on viral replication unknown	Abalone / Swollen and protruding mouth parts, curling and/or blistering of the foot muscle
	Paramoeba invadiens	Amoeba	Optimal growth observed at 15-20 °C	Sea urchin / Necrosis and reddish-brown discoloration of body surface, tube feet muscle degeneration, immobility, reduced feeding
	Perkinsus olseni	Protozoan	Spreads through direct contact between animals. Increased infection intensity >15-20 °C	Abalone / Brown pustules filled with brown, creamy fluid in the foot and mantle
	Spionidae	Polichaetes	Exacerbated by poor hygiene and use of formulated feeds	Abalone / Shell deformities, brittle shells, and slow growth
	Terebrasabella heterouncinata	Sabellid	Increase in numbers with high abalone stocking density, exacerbated by poor hygiene/water quality	Abalone/ Shell deformities, gill pore deformities, dome-shaped shell, inability to right themselves
Brazil (Land-based)	*Streptococcus*	Bacteria	Rainy season, water temperatures persistently high and low salinity values for lengthy periods of time.	Tilapia & Shrimp / Lethargic, anorectic, and accumulate at the pond edges, near its surface. Histopathological lessions

	Cyanobacteria	Bacteria	High luminosity in the water column and high water temperature	Tilapia & Shrimp / Massive deaths of shrimp
	Vibrio species	Bacteria	Animal handling, sudden environmental changes, poor food quality	Tilapia & Shrimp / Massive deaths
Argentina	*Perkinsus olseni*	Protozoan	Proliferation occurred in temperatures > 20ºC	Blue mussel / Inflammatory response, tissue damages and abnormal morphologies
	Areospora rohanae	Cilliate	Aggregations during early stages of king crabs would enhance the pathogen transmission	Southern king crab / Hypertrophy of colonised host cells

4.3 Current monitoring technologies

In general, current surveillance technologies are reduced to a daily observation that evaluates the warning signs described in the previous section. Following this observation, if an anomaly is observed, pathogen presence confirmation must be carried out using several tools. Table 3 summarizes the current methods used to monitor and detect pathogens in the system, that have been already mentioned during the previous sections.

Table 3. Table summary of the current methods for monitoring and detection of most problematic pathogens

Pathogen	Type	Current monitoring method	Pathogen detection
Aeromonas salmonicida	Bacteria	Daily system observation	Bacteriological culture and biochemical testing, histopathology or molecular methods
Tenacibaculum	Bacteria	Daily system observation	PCR
Vibrio species	Bacteria	Daily system observation	PCR
Moritella viscosa	Bacteria	Fish observation during winter	Histopathology

Xenohalitis californiensis (Rickettsia)	Bacteria	Routinely tested for as part of a targeted surveillance programme	histology, PCR
Streptococcus	Bacteria	Daily system observation	PCR
Cyanobacteria	Bacteria	Daily system observation	PCR
Abalone herpesvirus	Virus	Routinely tested for as part of a targeted surveillance programme	qPCR and/or in-situ hybridization
Novirhabdoviruses	Virus	Daily system observation	Lateral flow biosensors
Oyster Herpes Virus	Virus	Daily system observation	PCR
Spionidae	Polichaetes	Daily system observation	PCR
Paramoeva invadiens	Protozoan	Daily system observation	Histology or culture-based techniques (malt yeast, non-nutrient agar)
Perkinsus olseni	Protozoan	Routinely tested for as part of a targeted surveillance programme	Whole body burden assay, histology, PCR and Ray's fluid thioglycolate culture method (RFTM).
Areospora rohanae	Cilliate	Daily system observation	Histopathology
Bonamia ostreae	Protist	Routine testing at *O. edulis* cultivation sites (e.g. Marine Scotland)	Histopathology
Halioticida noduliformans	Oomycete	Daily system observation	PCR, histology
Chytridium polysiphoniae	Chytridiomycetes	Daily system observation	PCR, fluorescent microscopy (chitin observation)
Terebrasabella heterouncinata	Sabellid	Daily system observation	Naked eye
C. elongates/L. salmonis	Copepods (sea lice)	Daily system observation	Microscopy

5 Review of emerging technologies for pathogen monitoring in aquaculture

Emerging technologies, such as molecular techniques, biosensors, remote sensing, and artificial intelligence-based predictive tools have great potential to transform disease surveillance in the aquaculture setting (MacAulay et al., 2022). While traditional methods, such as visual diagnosis, histopathology and cultivation-based techniques are indispensable to combat infection, they are not always well suited for early diagnosis that is essential for taking preventative measures and to prevent further spread of the disease and loss of stock. Emerging technologies can facilitate a more pro-active monitoring approach as they are able to identify potentially infective pathogens with high specificity and sensitivity before an outbreak occurs (Figure 9). To identify highly sensitive, highly specific, rapid, and cost-effective surveillance approaches for the IMTA-lab-specific priority pathogens, we reviewed the literature for molecular tools, biosensors, and predictive modeling approaches. First, we briefly introduce these three methods and show they can potentially work together, then provide an in-depth description of each in three subsequent sections (5.2, 5.3, and 5.4).

5.1 Introduction

Molecular tools can be defined very broadly as analytical approaches for the identification and quantification of informational macromolecules, i.e., DNA, RNA, and proteins. By far DNA and RNA or collectively termed nucleic acids (NAs) are the most common analytes (MacAulay et al., 2022). Molecular techniques provide an array of tools for accurate identification, sensitive quantification, and discovery of new pathogens. Since it has been recognized that the major limitations of these tools arise from logistical challenges, reliance on benchtop instrumentation and expensive reagents, significant effort has been put into developing portable, field compatible and more cost-efficient solutions. Biosensors are sensing analytical devices capable of converting biological responses into electrical signals. Recently, they have taken a central role, as biosensors are simple and easy to work with and capable of detecting low concentrations of specific pathogens. Biosensors provide fast, real-time automated monitoring of the environment, which is very helpful for the detection and prevention of pathogens and diseases. Molecular tools and biosensors do overlap in a sense that the latter can also target informational macromolecules as analyte, however, biosensors are more versatile, as they can detect and quantify whole cells, virions as well as small molecules such as toxins and can be used to measure other water quality parameters (Bhalla et al., 2016). The two approaches are often employed in combination, to achieve detection of low levels of analyte without compromising assay time and ease of use. Such synergy is achieved when biosensors are used for the detection of amplified nucleic acid analytes. With molecular tools and biosensors, pathogen surveillance, reflecting the current

disease status, can be achieved. However, to obtain disease outbreak predictions, early warning systems need to integrate these various streams of surveillance data with trained models to forecast future disease status. With the help of data from molecular tools, biosensors, and other contextual measurements (observations), predictive machine learning models can be built (trained) considering parameters from all aspects of the host-pathogen-environment epidemiological triad. This is essential, since the interplay between host, pathogen and environment does ultimately determine actual disease manifestation, thus the probability of an upcoming disease outbreak. The following sections will provide an in-depth description of molecular tools, biosensors and predictive models.

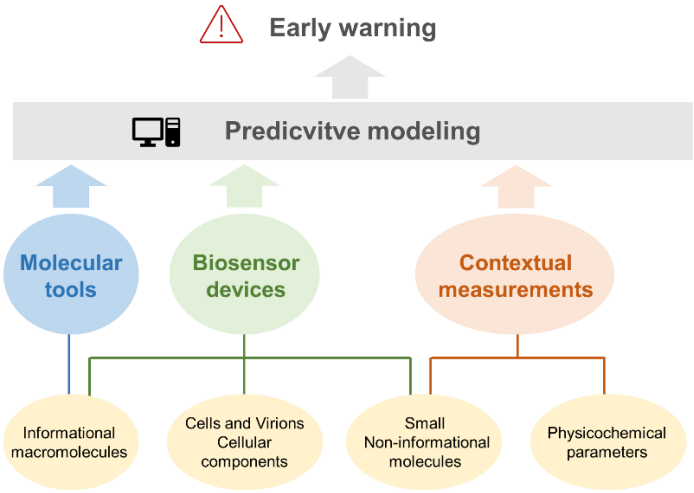

Figure 9. Concept of utilizing data from various tools to build predictive models which can alert when early warning thresholds are being approached.

5.2 Molecular tools

Most molecular tools employed in the detection and quantification of viruses, bacteria, fungi and various parasites rely on some form of nucleic acid amplification technique (NAAT) (Oliveira et al., 2021). Amplification selectively enriches NA fragments of the target organism from the NA pool obtained during a DNA/RNA extraction step. Depending on the thermal conditions required during this step, NAATs can be divided in two major groups. The first comprises of different polymerase chain reaction (PCR) approaches relying on thermal cycling (repeated cycles of different temperatures), while the second group includes isothermal amplification methods relying on constant and generally lower temperatures than PCR (isothermal NAATs). The resulting amplicons can be analyzed either through (1) endpoint detection techniques or (2) through techniques allowing continuous readout. In the first case, the amplification is first completed, then a signal is measured, while in the second case, a signal is continuously measured throughout the amplification reaction. Detection methods range from naked-eye observations of color or turbidity change, agarose gel electrophoresis and enzyme-linked immunosorbent assay (ELISA) or biosensors for endpoint reactions, to real-time image analysis

of fluorescent signals in continuously monitored reactions. In addition, high-resolution melt curve (HRM) analysis of real-time PCR products is emerging as a highly multiplexable and highly specific method for differentiating between species and for confirmation of pathogens (Edwards et al., 2018). Various sequencing approaches can also be viewed as detection methods, including Sanger sequencing of highly target specie-specific endpoint amplifications, metabarcoding of amplicons generated with broad-range primers as well as amplification free shotgun sequencing. The complexity of the data types resulting from these different analysis approaches, respectively, ranges from (1) simple presence-absence type data, through (2) quantitative information, *i.e.*, concentration of the target in the tested sample to (3) species composition matrices generated through advanced bioinformatics pipelines. Lastly, molecular tools can also be categorized depending on the source of the nucleic acid used in the analysis. On the one hand, disease detection and identification can rely on nucleic acids obtained invasively from infected tissue samples (viral or bacterial NA from tissue of diseased individuals) and directly from the disease-causing agent (NA from the infecting parasite). On the other hand, an emerging alternative approach is to use environmental nucleic acids (eNA) collected non-invasively from matrices surrounding the individuals. The latter eNA-based approach increases the range of considerations one needs to take when relying on molecular tools for pathogen detection. The following subsections will first provide a general overview of NAATs, which are at the heart of most molecular tools (5.2.1). This is followed by general considerations for selecting molecular tools, to be taken into account regardless of the NA source (5.2.2). Finally, the last subsection (5.2.3) will be dedicated to specific considerations for and examples of using environmental nucleic acids as NA source for pathogen surveillance.

5.2.1 Overview of NAATs

Polymerase chain reaction, **PCR**, and its derivatives, *i.e.*, quantitative (or real-time) PCR (**qPCR** or **RT-PCR**) and digital PCR (**dPCR**) or droplet digital PCR (**ddPCR**) are the gold standard NAATs due to their capacity to reach very high analytical sensitivity (<1 copies/µl reaction) and specificity. PCR and qPCR assays are also the tools often already implemented in routine monitoring practices or disease diagnostics at aquaculture sites. Moreover, they are increasingly being adopted by the World Organization for Animal Health (WOAH) as confirmation of specific pathogens. To amplify the target fragment through PCR, repeated cycles of thermal denaturation (heat-induced denaturation of double-stranded DNA), annealing of primers and polymerase-assisted elongation of a complementary strand are carried out at temperatures optimized for the target, primers, and the DNA polymerase in the reaction. Once sufficient genetic information about a disease-causing agent is available, target-specific oligonucleotide probes, *i.e.*, primers can be designed, and a PCR assay developed and validated. RNA targets can also be analyzed following a reverse transcription step, which can be

performed in the same reaction mix as the PCR itself. For PCR, only thermally stable *Taq* polymerases are suitable due to the repeated exposure to 95 °C. The fidelity of the enzyme is essential when the amplification products are to be further processed through sequencing, in other cases, polymerase properties such as inhibitor resistance may be more important to consider. In fact, PCR's sensitivity to inhibitors is often cited as a major drawback when it comes to working with crude NA extracts obtainable through portable (field-compatible) methods. PCR needs to be carried out in a thermocycler, an instrument with fast and precise temperature control, whereby the quality of the temperature control is key to PCR's success. This requirement for investment in costly instrumentation and extensive training of personnel is also often mentioned as a downside of PCR. These constraints are particularly true for qPCR and digital PCR techniques, especially for multichannel (multi-color) instruments that can enable multiplex detection, *i.e.*, quantification of several targets in a single run. Nevertheless, when it comes to analytical sensitivity, as a rule of thumb, a 10-fold lower detection limit can be attained by qPCR compared to traditional PCR and another 10-fold lower limit may be achieved with digital PCR approaches. Except for the parasitic polychaetes belonging to *Spionidae*, at least one qPCR assay has been published for all IMTA-lab-selected pathogens with publicly available sequence information. For polychaetes, a multilocus barcoding (PCR and sequencing) approach has been suggested for accurate species identification (Williams et al., 2017). Multiplex droplet digital PCR was demonstrated to be a useful, high-throughput and sensitive tool for the salmonid aquaculture industry through a 1-year trail in a commercial fish disease surveillance program in New Zealand and its utility was also tested in a Norwegian land-based setting (Lewin et al., 2020; von Ammon et al., 2022). Although the capital investment is highest for digital and droplet digital PCR instruments, their working principle enables calibration curve-free absolute quantification, which in turn reduces running costs, facilitates higher throughput, and simplifies the interpretation of results. Nevertheless, the highest throughput still can be achieved using qPCR devices, which are often used in the clinical setting. When it comes to solutions to field applications, several developments have been made in recent years, including portable and more affordable qPCR machines, such as the Biomeme Franklin™. This handheld instrument was successfully used for rapid and on-site quantification of *Flavobacterium psychrophilum* (Nguyen et al., 2018). Ready-to-use kits and user-friendly mobile apps accompanying such machines make it possible to perform qPCR by less experienced users and opening possibilities for point-of-care/need/use/encounter applications. Prior cross-validation of benchtop and portable solutions is recommended to ensure robust results (Voelker et al., 2022). Another cost-reducing development in the field of qPCR has been the development of micro/nano-fluidic approaches that enable larger number of samples to be analyzed simultaneously as well as a substantially greater number of assays to be run on each (A. L. Bass et al., 2023). It has been shown that this type of

approach requires very stringent primer selection due to the "one-size-fits-all" reaction conditions (most critically, annealing temperature) (Crane, van Dorst, Hose, King, & Ferrari, 2018).

Nucleic acid sequence-based amplification, **NASBA**, was one of the earliest isothermal NAATs developed as a sensitive RNA amplification method that can be carried out at ~40 °C (Compton, 1991). NASBA relies on the activity of a T7 RNA polymerase, which performs continuous transcription of double-stranded DNA templates containing an *in vitro* incorporated T7 promoter. Like PCR, NASBA requires only one forward and one reverse primer, of which one must contain the T7 RNA polymerase promoter sequence. The traditional NASBA rection mix is composed of 3 different enzymes: avian myeloblastosis virus reverse transcriptase (AMVRT), RNase H and T7 RNA polymerase. The reverse transcriptase is necessary to generate cDNA containing the T7 RNA polymerase promoter, while the RNase H degrades the RNA strand of the DNA:RNA duplex resulting from cDNA synthesis. Similar to PCR, an initial denaturation step at 65 °C is often included in NABSA protocols to disrupt RNA secondary structures. This, however, necessitates the reaction to be carried out in two steps, due to the thermal lability of the RNA polymerase. Recently, an improved, truly isothermal and single step procedure has been proposed, recommending the inclusion of single-strand binding proteins in the NASBA mix (Nai et al., 2022). The approach was successfully tested on *Macrobrachium rosenbergii* nodavirus (Lin et al., 2023). NASBA can be employed with DNA targets as well and can be combined with CRISPR-cleavage (NASBACC) for detection systems where strain-level distinction between targets is necessary (Pardee et al., 2016).

Recombinase polymerase amplification, **RPA**, one of the most popular emerging isothermal NAATs enables exponential amplification without the need for thermal denaturing or annealing (Stringer et al., 2018). Like PCR, it generates single-size amplicons and requires one forward and one reverse primer flanking the DNA region of interest. Often, already established PCR primers can be used in RPA assays without further optimization which is a great advantage. Unlike PCR, in RPA the primers interact directly with the double-stranded DNA once they formed a complex (recombination filaments) with recombinase proteins. The recombinase-primer complexes create an opening in the double-helix structure by binding to the complementary sites. This loop is then stabilized by single-strand binding proteins. Next, a strand displacing polymerase extends the 3'-end of the primers along the target sequence. Since the denaturing and annealing steps are omitted, the reactions can be completed in under 20 minutes using a constant temperature of 37-42°C. Commercial master mixes (TwistAmp) are available together with lateral flow immunochromatographic assay detection, at a cost of approximately 15 EUR/sample. RPA assays, real-time (so called exo-RPA) and coupled with lateral flow dip stick detection, have been developed for some of the selected pathogens: *A. salmonicida* (Zhou et al., 2022), *V. alginolyticus* (Dong et al., 2020; Wang et al., 2021) and *V. parahaemolyticus* (Geng et al.,

2019). A thorough comparison of RPA with both PCR and the rest of the isothermal methods have been summarized by Tan et al., highlighting that RPA is indeed the fastest method, least sensitive to temperature variations and capable of detecting trace levels of DNA material (Tan et al., 2022). Based on its earlier success with viral and protozoan pathogens (White Spot Syndrome Virus and *Perkinsus beihaiensis*), its easy adaptability from existing qPCR assays (despite the current lack of primer design software for RPA), and many recent developments, there is a great potential for this tool to become much more widely used as a diagnostic and monitoring tool also in aquaculture settings (Sullivan et al., 2019; Tan et al., 2022; Wu et al., 2019).

Loop-mediated amplification, **LAMP**, is one of the most established and most accepted NAATs in point-of-care disease diagnostics. This is reflected in the fact that among the 18 IMTA-lab-selected pathogens with existing qPCR assays 7 also had a LAMP assay available (*A. salmonicida*, *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *N. perurans*, *P. olseni*, Abalone herpesvirus). LAMP uses a polymerase with strong strand displacement capacity to carry out an auto-cycling strand displacement DNA synthesis with the help of 4 different primers. All 4 primers are required for the initial, template-generating stage, and only 2 of them are needed for the later, cycling synthesis stage(Notomi et al., 2000). Since the target sequence must be recognized by 6 different sequences initially, LAMP is expected to exhibit very high analytical specificity. The reaction takes place at a constant 65°C temperature and can produce 10^9 copies of a single template within 1 hour. Despite being isothermal, an initial denaturation step at 95°C and another heating step at 80°C is necessary to initiate and terminate the reaction, respectively. While potentially very simple to perform, LAMP can suffer from low analytical sensitivity (100-fold higher detection limit compared to qPCR) as well as low diagnostic sensitivity, such as high false negative rates (>10%) (Phusantisampan et al., 2022). In contrast to PCR, NASBA and RPA, LAMP generates multiple amplicons with different sizes because once the initial dumbbell-shaped template is formed, all 4 primers could bind to it in any combination and initiate the DNA synthesis. The major advantage of LAMP is being significantly less sensitive to contaminants and inhibitors thus more suitable for analysis of crude DNA/RNA extracts generated with simple field-compatible approaches (Oliveira et al., 2021).

There are many isothermal methods (reviewed in Glökler et al., 2021; Oliveira et al., 2021) with yet-to-be explored potential in point-of-care diagnosis, including: (1) strand displacement amplification, **SDA**, which utilizes the nature of polymerase to repair DNA breakage and thus relies on the simultaneous activity of a nicking enzyme and a polymerase, (2) rolling circle amplification, **RCA**, which mimics the rolling circle replication of plasmids and relies on the generation of a circular target in the initial phase, (3) helicase dependent amplification, **HDA**, which employs a helicase enzyme to separate the strands of duplex DNA as opposed to heat while still relying on a pair of primers and a polymerase

for the elongation, as well as innovative approaches like (4) single primer isothermal amplification, **SPIA**, which requires a single chimeric DNA/RNA primer, DNA polymerase, RNase H, and blocker in the reaction mix (Q. Yang et al., 2021) and (5) single-stranded DNA binding protein and helicase assisted rapid PCR, **SHARP**, which is a modified version of HDA (Gavrilov et al., 2022). All share the advantages over PCR, including the simplicity of instrumentation required (sometimes as simple as a thermos bottle) as well as avoiding the heating of template DNA to high temperature (95 °C) repeatedly, thus avoiding its degradation. As a result, isothermal methods can generate higher amplicon concentrations than conventional PCR, which can be easier to detect with simple techniques and biosensors. In general, they offer a great potential for lower-cost, faster analysis, and for automation of all processes into sample-to-result solutions in lab-on-chip formats. For this reason, it is likely that future *in situ* real-time molecular monitoring tools will be based on some form of isothermal methods. The following subsections include examples of how these NAATs have been implemented in the detection of the target pathogens selected with the help of ASTRAL IMTA labs. The first subsection is dedicated to general considerations relevant for method selection regardless of the sample material serving as the source of NA. The second subsection is then dedicated to considerations specific to eNA-based approaches.

5.2.2 General considerations for selecting molecular tools

A major and sometimes limiting prerequisite for designing molecular tools is the lack of appropriate sequence information about the target pathogen. Among the selected pathogens, many had only one or two partial sequences of common marker genes (ribosomal RNA or cytochrome oxidase) available in the NCBI Nucleotide database and no sequence records were available for the microsporidian *Areospora rohanae* (despite a publication with 16S sequences to be submitted) and the sessile ciliates, *Mantoscyphidia spadicea*, *Mantoscyphidia midae* and *Caliperia* (Bojko et al., 2022; Stentiford et al., 2014). In these cases, barcoding or genome sequencing of the pathogen is necessary as a first step.

Purpose of the method: Surveillance can be carried out to obtain information regarding the presence of a particular pathogen or a group, to track the abundance of a target and to identify newly emerging pathogens. Depending on the exact surveillance goal, different approaches will be best suited, and the analytical sensitivity and analytical specificity required will also be different. Isothermal amplification methods, coupled with naked-eye observation or lateral flow dipstick detection appear to be an optimal compromise between ease of use and sensitivity. Such simple and low-cost tests can be performed on a regular basis as part of the routine monitoring practices carried out at the farms (water quality measurements) to obtain presence absence information. For increased specificity, they can be combined with emerging CRISPR-tools albeit at a cost for the extra reagents (still cheaper than qPCR yet equally specific) (H. Yang et al., 2023). For tracking longitudinal trends in the levels of certain

pathogens, quantitative approaches, such as qPCR, real-time LAMP or real-time RPA, are better suited, albeit at the cost of increased complexity. For rapid and accurate profiling of pathogen genotypes, amplification products of real-time PCR can be subjected to high resolution melt curve analysis. Lastly, to capture emerging new pathogens, a non-targeted sequencing-based analysis is required. Viruses tend to evolve fast and new variants, undetected by the established methods, may arise quickly.

Choice of target sequence: Most commonly, phylogenetic marker genes (well-established molecular clocks) such as small or large subunit ribosomal RNA genes (genomic or mitochondrial) and mitochondrial genes, such as cytochrome oxidase 1 or cytochrome oxidase *b* are chosen for assay design. This can work well for some targets, while it remains insufficient for others, such highly similar *Vibrio* species, that differ very little in their 16S rDNA sequence. For metazoans, it can be challenging to identify a single marker that is alone suitable for species-specific detection, and several marker genes may need to be amplified simultaneously for reliable identification, as has been the case with spionid polychaetes (Williams et al., 2017). A recent study on spionid polychaetes showed that barcoding of the 16S and and the 18S markers is a suitable tool for genus level identification of larval stages, however, in some cases, adults and larvae had distinct sequences (Abe and Sato-Okoshi, 2021). In case of *Vibrio* species (*V. parahaemolyticus*, *anguillarium*, *alginolyticus*, *harveyi*, *campbelli*) virulence and virulence-related genes are chosen as targets for assay design as only these can provide sufficient species-level resolution (Loo et al., 2022). Besides taxonomic resolving power, the intracellular abundance of the target gene may be an important factor to consider when it comes to assay sensitivity. Targets that are located on plasmids are often found in multiple copies, and in some groups, particularly protozoa, ribosomal RNA genes are present in very high copy numbers within a single genome. Choosing such a naturally enriched target gene can increase assay sensitivity several-folds.

5.2.3 Specific consideration for using environmental nucleic acids (eDNA/eRNA) for early warning

Surveillance and earlier detection of aquatic pathogens is key to mitigate outbreak risk and prevention of mass mortalities. This may be achieved by testing the rearing environment (or intake water) with sensitive and robust tools for capturing signals of target organisms at the lowest possible concentration. While approaches using environmental nucleic acids (eNA) are very promising for a variety of monitoring purposes, it remains an outstanding question whether and to what extent *in situ* molecular tools using eDNA/eRNA can be used as effective means for real-time prediction and management of pathogen risks in the aquaculture setting (Bass et al., 2023).

Environmental DNA/RNA approaches need to consider both the ecology of the pathogen and the ecology of the targeted nucleic acid for designing an appropriate sampling strategy and downstream analysis. The promise of eDNA/eRNA-based monitoring is to potentially capture genetic information

from all organisms present in a given environment at a given time in a non-invasive manner. This is, however, complicated by many factors. eNA can be present in an environment as (1) dissolved or free NA released from any damaged and lysed cell, (2) viral particles, (3) particle-attached cell-free NA, and cellular NA including that of (4) living unicellular organisms, *i.e.*, prokaryotes and protozoans and (5) NA contained in shed cells of multicellular organisms (or very small multicellular organisms captured during sampling). This is important because capturing one form or the other may require very different sample collection and processing approaches. Another major constraint is the typical low concentration of targets in relatively large volumes of environmental samples that also contain a great diversity of non-target nucleic acids. In addition, different environmental matrices can have a variety of different inhibitors that may interfere with downstream analysis and therefore be more or less suitable for certain diagnostic purposes. The sampling strategy is therefore of utmost importance in eNA-based monitoring, beginning with the choice of the sample matrix. In the aquatic environments associated with different IMTA setups, various environmental matrices can serve as sources of eNA such as (1) rearing water in open or closed system, (2) production units' surfaces, *e.g.*, tank walls or net pens (3) animal surfaces*, e.g.*, gill or skin swabs, mucus and (4) settleable solids, *e.g.*, sediments and bioflocs. Which sample matrix and collection method is ideal for early detection of a pathogen depends on the organism's lifecycle, lifestyle and size. The sampling strategy, *i.e.*, spatial-temporal design, sample size, level of replication and the sample collection method needs to be optimized to maximize the likelihood of capturing the eNA form expected for the selected target (*e.g.*, filtration though 0.22 µm pore size filters is commonly the best choice for bacterial targets that are intended to be captured as whole cells). This can be a very challenging task for open water systems, where all eNA signal is highly diluted and affected by local and large-scale transport patterns (Buchwald et al., 2018; Fuller et al., 2022; Shea et al., 2022). In all cases, the use of statistical tools, such as occupancy modeling is strongly recommended to be employed already at the sampling strategy decision stage, as the number of field replicates and the number of samples from each of the replicates were shown to correlate with increased detection probability, more so than estimated biomass or volume per sample (Willoughby et al., 2016).

Pathogens with planktonic lifestyle, forms, stages (viruses, bacteria, protozoa, spores of fungi and oomycetes, larvae of metazoans etc.) represent good candidates for non-invasive emerging sampling methodologies, such as eDNA and eRNA collection. For example, the three well-established transmissible infective life stages of *Perkinsus olseni*, *i.e.*, trophozoite (5-15 µm), hypnospore (50-90 µm), and zoospore (2-5 µm) have all been detected in sediment and pelagic samples with a characteristic seasonal pattern (Ríos-Castro et al., 2022). Other pathogens that were detected in eDNA samples include *Lepeophtheirus salmonis* (Krolicka et al., 2022), *Paramoeba invadens* (Buchwald et al.,

2018), *Neoparamoeba perurans* (Bridle et al., 2010), *Vibrio harveyi* (Mougin et al., 2020), *Xenohaliotis* californiensis (Crosson et al., 2020), *Vibrio alginolyticus* and *V. anguillarium* (Siddique et al., 2019). Diversity and spatiotemporal distribution of *Paramoeba* species has been explored with success near an aquaculture facility in Tromsø, Norway using metabarcoding of sediment and seawater samples (Mertz, 2020). Viruses require tailored sampling approaches (*e.g.*, precipitation rather than filtration) due to their small size and lack of cellular structure, nevertheless, can successfully be detected in eNA samples as exemplified by Iridovirus (Kawato et al., 2021), Nervous Necrosis Virus (Krishnan et al., 2021), White Spot Syndrome Virus (Kim et al., 2022), and Tilapia Lake Virus (Taengphu et al., 2022). Viral pathogens may require different sampling approach depending on their size, presence, or absence of an envelope, and genome type (DNA vs RNA, single stranded or double stranded). Bacteria are the most often explored group of organisms in eNA samples with standard procedures established. *Xenohaliotis californiensis* has been quantified in seawater near and far from abalone farms, however, eDNA levels did not correlate with tissue levels and were also not indicative of infection levels (Fuller et al., 2022). *Aeromonas salmonicida subsp. salmonicida* was shown to survive in seawater for up to 10 days (Rose et al., 1990). *Tenacibaculum maritimum* was shown to survive in sterile seawater for more than 5 months (1000 cfu/mL), however, cells proved to be very labile in non-sterile seawater, rendering culturable cells no longer than 5 days (Avendaño-Herrera et al., 2006). Fungi, oomycetes and protozoa include morphologically and phylogenetically diverse groups of unicellular eukaryotes that are generally larger than 1 µm in any one dimension, thus facilitating easy collection from water by filtration. Sessile protozoans, *i.e.*, some pathogenic ciliates typically have a swarmer stage which can be detected in water. Other parasitic protozoans tend to form cysts and oocysts which are released into the environment. Fungi and oomycetes form spores, oospores and zoospores, respectively, which can be detected from the water (Muraosa et al., 2012). Amoebic gill disease-causing *Neoparamoeba* (*N. perurans*) was found to be detectable in various matrices at and near ADG-affected salmon farms (during outbreaks or with history of ADG), but the organism was not found within biofouling organisms, salmon lice, biofilm or sediment at sites not affected by AGD (Hellebø et al., 2017). The same study showed that cleaner fish may withhold the amoeba longer than salmon, as they tested positive for *N. perurans* when salmon tested negative. Metazoans have planktonic larvae stages and can shed detectable levels of eNA which can be recovered in either dissolved form or as cellular NA. This group may represent a more challenging target for eNA-based detection in the water phase, thus alternative sinks of metazoan nucleic acids, such as sludge should be evaluated for a more sensitive approach. Nevertheless, in the open ocean setting, planktonic larvae derived from the benthic polychaetes are one of the most abundant groups of coastal zooplankton, and these planktonic stages last relatively long, facilitating their easier sampling (Abe and Sato-Okoshi, 2021). Larvae can be

collected using plankton nets with large enough mesh sizes to avoid enriching bacteria simultaneously (> 100 µm) (Williams et al., 2017; Spencer et al., 2021).

In addition to determining the diagnostic marker, designing an appropriate assay and validating the assay itself, eNA approaches will require further laboratory experiments and cross-validation field studies to establish the relationship between eNA signals and host infection levels. Some studies have established, under laboratory conditions, the relationship between pathogen levels in rearing water and associated mortalities. For example, *Neoparamoeba perurans* have been shown to induce amoebic gill disease (AGD) at a concentration of 1 amoeba/L seawater (Bridle et al., 2021). Bathing experiments with juvenile red abalone exposed to *Xenohaloites californiensis* established that a 3 hr exposure to a pathogen dose of 2.3×10^3 DNA copies/mL was necessary to induce infection. The same study found that DNA from *X. californiensis* was stable only up to 2 days in seawater (Crosson et al., 2020). Nevertheless, it may be difficult to extrapolate such findings to open water systems, where sometimes no correlation is found between pathogen load and disease severity (Bridle et al., 2010). More of these studies and validation cases are needed to determine eDNA-based early warning thresholds that should trigger mitigation efforts, particularly in open water systems.

A greater emphasis on **statistics and modeling of eDNA data** will be necessary to appropriately integrate the outcomes of molecular tools employed on eDNA samples into predictive early warning systems.

5.3 Biosensors

A molecular biosensor is a device that measures specific biomolecules known as biomarkers, generating signals proportional to the analytes. They can use a biological element such as an antibody, enzyme, cells, or nanoparticles, with an electronic component to generate a measurable signal. This electronic component is then able to detect, record and transmit the necessary information regarding a physiological change or the presence or absence of biological materials in the environments. This results in a signal that can be optical, electronic, or magnetic. The signal is then translated, and a disposable sensor device then displays this into data. Biosensors have a wide range of capabilities and can be applied to different sectors depending on the needs (Bhalla et al., 2016). They can be used in field applications, as they are easy to work with, sensitive and can be automated for remote use. Some applications of biosensors include health diagnosis, environment monitoring, food, and water quality analysis (Naresh and Lee., 2021). Biosensors can be used for the detection of biological contaminants and pathogens, such as bacteria and viruses. Generally, a DNA probe or antibodies are immobilized on the sensor surface to capture target analyte of viral RNA or bacteria cells, respectively (Su et al., 2020). Biosensors have been developed for detection of pathogens related to livestock, poultry, and aquaculture (Vidic et al., 2017; Su et al., 2020).

In aquaculture, sensors provide a fast, real-time automated monitoring of the aquatic environment. Aquaculture is a rapidly growing sector and is becoming increasingly intensive, and sensors are important for monitoring and controlling water quality and animal health, to ensure high quality and productivity (Heerthana and Preetha, 2019). The development and usage of these novel technologies will help improve efficiency and make the system more resilient. There are various challenges that could be more easily approached with the implementation of sensors in combination with other relevant devices, such as water quality *in-situ* monitoring. These sensors can be used for

- Disease detection: Biosensors can detect the presence of specific pathogens or disease-causing agents in the water or organism. They can identify the DNA, RNA, or proteins associated with pathogens, enabling early disease detection and intervention. This would help in the prevention of disease outbreaks (Sharma et al., 2006; Wang et al., 2022).
- Biomarker analysis: Biosensors can analyse specific biomarkers in aquatic organisms to assess their health status. For example, biosensors can measure stress hormones, enzymes, or other indicators of physiological responses in fish or shellfish (Endo and Wu, 2019). By monitoring these biomarkers, early signs of stress or disease, as well as health, could be identified.
- Environmental monitoring: Sensors can be used to monitor the impact of aquaculture operations on the surrounding environment. They can detect and measure contaminants, pollutants, or toxins, providing insights into potential environmental risks and allowing for timely remedial actions (Su et al., 2020).
- Nutrient monitoring: Sensors can measure nutrient concentrations in the water, such as nitrogen, phosphorus, and other essential elements. Monitoring nutrient levels is crucial to prevent excessive nutrient loading, which can lead to water pollution, algal blooms, and ecosystem imbalances. (Johnston, 2018)

Overall, the use of sensors and specifically biosensors in aquaculture provides valuable insights into the aquatic environment, improves health monitoring and disease management in situ, optimizes resource utilization, and enhances the sustainability of aquaculture operations.

There are numerous biosensors to detect pathogens in aquaculture. Biosensors can be classified based on their biological recognition element and their main objective is the fast, affordable, and easy detection. Here we highlight the state of the art of the main biosensors used in aquaculture, their mechanisms, and advantages.

5.3.1 Overview of biosensors commonly employed in aquaculture monitoring.

- Lateral flow biosensors, **LFB**, have been used for the detection of various analytes such as proteins, RNA, DNA, chemical contaminants, and bacteria, as well as virions. LFB are pre-made material strips in which the test results can be seen using the naked eye in the detection area of the strip through the flow of samples. They are considered one of the most promising technologies, because of their simplicity, high sensitivity, and specificity, and potential to be used for field analyses. This method allows the operator to visually detect particles within 30 minutes, without instrumentation usage, and the cost is often under 3 euro. In aquaculture LFB have been used for simple and accurate detection of pathogenic agent salmonid novirhabdovirus of the genus *Novirhabdovirus,* also known as Infectious hematopoietic necrosis virus (IHNV), in various fish species. Moreover, there has been a successful development of LFB combined with aptamer-based isolation that was able to visually detect *Iridoviruses* infections *in-situ*, without the use of RNA extraction and purification (Liu et al., 2021; Toubanaki et al., 2020).
- Differential pulse voltammetry, **DVP**. Voltammetry biosensors work by changing current when applied potential. This has been used to detect bacteria, such as *Vibrio spp*. These portable analyses devices are able to work by referencing electrodes, while difference in peak current is detected between the working and auxiliary electrode. DVP studies have reported high selectivity against *Vibrio spp*., as well good reproducibility. Additionally, when using antibodies or aptamers, it can characterize whole-cell bacteria. Advantages are that this is a fast, low-cost method and requires minimal sample preparation (Schutz et al., 2021).
- Surface plasmon resonance spectroscopy, **SPR**, is a direct optical biosensor, which allows real time monitoring of biomolecular events. In aquaculture systems it is crucial to maintain specific parameters in concentration within limits to avoid adverse conditions that could affect fish growth and survival. Critical parameters include pH, dissolved oxygen, NH_3, NO_2 and NO_3-, amongst others. Surface plasmon resonance imaging is employed in water and can sense NO_3- and NH_4^+. It can sense stress indicators, detect pathogens (viral and bacterial), and the freshness of fish and shellfish by detecting biogenic amines (Quintanilla-Villanueva et al., 2023). In relation to pathogen detection, aptamer based SPR can detect early infection stages of *Vibrio spp.* and Nervous necrosis virus. SPR is used for quality control processes that are carried out through the production chain and in post-production activities. On the other hand, it lacks information studies on the application of its use in oysters, shrimps, and other crustaceans. SPR is introduced in the tanks and then used remotely. The disadvantage in this is that it can be altered by surface alterations in the tank, causing disturbances in the results

and data needed. Moreover, they require sophisticated equipment and careful handling. Nevertheless, the detection of *Vibrio* pathogens is crucial, as vibriosis is often the cause of mass death in cultured fish, shrimps, and shellfish (Steglich et al., 2022; Sielaff et al., 2019; Yan et al 2020).

- Quartz crystal microbalance, **QCM**, biosensors based on quartz crystal microbalance (QCM) are a real-time surface sensitive technique, which monitors the change in mass or the thickness of the layers that adhere to the surface of the quartz crystal. This is achieved by measuring the change in the quartz´s frequency when a voltage is applied. QCM has been used to detect various pathogenic bacteria and viruses, such as *Vibrio harveyi, Aeromonas salmonicida,* as well as viral haemorrhagic septicaemia (VHS), *pathogens that have* produce significant loses in aquaculture in general and more specifically fish farming. This technology supports the use of low-cost and portable instrumentation that provides the ultrasensitive, simple, and fast quantification of pathogenic bacteria. Moreover, it has been used for the detection of fish hormones. This method could provide for online monitoring in aquaculture systems (Messaoud et al., 2022; Buchatip et al., 2010; Pali et al., 2017).
- Potentiometric aptasensing involving magnetic beads, is an aptamer biosensor that has been developed for the detection of *Vibrio alginolyticus*, involving DNA nanostructure-modified magnetic beads. The bacterial cells attach to the DNA aptamer coated with magnetic beads, causing disassembly of the DNA nanostructures on the bead. The change in the charge or DNA concentration on the magnetic beads is detected by an electrode using protamine as an indicator based on the electrostatic interaction between DNA and protamine. This proposed strategy could be used for the detection of other microorganisms, by changing the aptamers in the DNA nanostructures.
- Wireless sensor networks, **WSN**, are a promising option for *in-situ* monitoring of the aquaculture environment. They can control parameters to improve water quality, and in so doing help enhance fish performance and improve the sustainability and production of IMTA systems. WSN are composed of various self-sensors, which can collect, measure, and transmit data in real-time. They allow higher quality and quantity of data, avoiding human error. They work by detecting changes in the tank state. They record parameters such as temperature, turbidity, conductivity, and fish behaviour, using optic and acoustic techniques, amongst other. Although these sensors are in development and have no capacity of predicting presence or absence of pathogens, they could potentially predict a high pathogen risk outbreak. The algorithm would detect abnormal values and send alarms when detected. Novel WSN system proposal are aiming to reduce its costs (Parra et al., 2018).

- AEFishBIT biosensor in fish operculum. This biosensor is used to monitor individual physical activities and respiratory frequencies of fish, as well as swimming activities and metabolic conditions. This method works by tagging the fish, by attaching it to the fish operculum. This way, it can measure the physical activities and the respiratory frequency to help understand behavioural insights. Studying the fish behaviour is a health indicator, as well as an early warning sign of stress and other factors (Kolarevic et al., 2021; Chakraborty et al., 2022).

Aquaculture quality monitoring uses several analytical methods. However, the monitoring process is quite complex and needs different technologies. Although biosensors are widely used in agricultural production, their use in aquaculture is limited. Therefore, developing biosensors would help in water quality monitoring and pathogen detection. Direct detection of cells by biosensors allow to skip the step of pre-processing samples, and can be used on site for field applications, being a fast and cheap alternative useful to detect and predict different diseases. Nevertheless, it is important to keep in mind the sensitivity that these methods may lack in comparison to molecular techniques. Although they are a growing field, biosensors still need to be more studied and developed, to reach the sensitivity of molecular analytical techniques and gain commercial relevance.

5.3.2 Pathogens and biosensors

From the described summary of most problematic pathogens and environmental warning signs at the IMTA labs, potential specific biosensors could be used to target specific pathogens.

- ***Bacteria:*** For bacteria, one of the most problematic pathogens is *Vibrio spp.*, in both land-based and open water IMTA aquaculture systems. As described, *Vibrio spp*. cause high mortality rate in fish as well as shellfish (Sharma et al., 2006). They can occur suddenly and can lead to the whole system infection. Moreover, antibiotic resistance is increasing among *Vibrio spp.*, so the fast detection and prevention of infections is crucial. There have been many biosensors developed to detect *Vibrio*, including **DVP** that has been previously reported to characterise *Vibrio spp*. Nevertheless, it is also important to characterise different genera. **Quartz crystal microbalance** has detected *Vibrio harveyi,* **while potentiometric aptasensing involving magnetic beads** was recorded to detect *Vibrio alginolyticus* and **surface plasmon resonance detected** *Vibrio cholerae O1* (Sharma et al., 2006). In open water, it is also important to detect *Aeromonas salmonicida,* as it is ubiquitous and current antibiotic treatments are inefficient in controlling this bacterium (Ligaj et al., 2014). **QCM** can be implemented in this case. For *Tenacibaculum,* biosensors that control the environment of the surrounding water, such as **wireless sensor network** can be implemented (Messaoud et al., 2022). Temperature, water parameters, stress and other environmental factors in the tank influence the infection severity of this bacterium, so being able to monitor the system could

be used for early detection of potentially dangerous parameters. Furthermore, for *Moritella viscosa,* the individual physical activities of the fish could be used for early detection using **AEFishBIT biosensor in fish operculum**. In land – based systems, *Rickettsia* has previously been detected using **amperometry immune-sensor using a synthetic peptide**, although this technique would require prior processing of the sample. For *Streptococcus*, LAMP biosensors for the detection of specific genes, can be combined with the previously described **lateral-flow biosensor** (Chang et al., 2013). This is an easy, fast way for its detection. Lastly, excessive growth of *Cyanobacteria* leads to the development of toxic blooms, so real-time monitoring of the tank using **Wireless sensor network** would be beneficial.

- ***Virus:*** In open water it is important to monitor genus *Novirhabdovirus,* which causes the infectious hematopoietic necrosis in fish. Several biosensors could be used such as **Lateral flow biosensors** or **QCM**. In land-based IMTA systems, for Viruses such as the *Abalone herpesivirus*, causing infection in abalone, the monitoring of the tank is crucial, as spreading is fast and caused by horizontal transfer. Disinfection of the water and equipment appears to be an effective method to prevent reinfection, although monitoring of the system is crucial to prevent infection via **Wireless sensor network**. (Shyam et al., 2023; Toubanaki et al., 2020; Liu et al., 2020; Buchatop et al., 2010)
- ***Other pathogens:*** in open water, other pathogens such as sea lice, threaten fish in aquaculture systems, as they attach to them and can become lethal**. Light-emitting diode based light trap biosensor** has been used for the monitoring and control of sea lice on fish and in the water. In fact, experiments under laboratory conditions found to catch 70% of larvae and 8% of female adults (Guragain et al. 2021). **AEfish operculum** could be used to monitor fish and provide early detection of the presence of sea lice. For land-based pathogens including ciliates, optical biosensors can be implemented, as they are ubiquitous in water. Therefore, **Wireless sensor network** could work to detect it early thanks to the environmental parameters. Furthermore, to tackle the presence of oomycetes as well as other pathogens, previously mentioned surveillance mechanisms as well as molecular tools can be implemented (Novales et al., 2009).

It is also important to consider that certain biosensors have the potential to be pathogen-specific and can be designed to specifically target the presence of certain pathogens, so they could be placed in different positions of the IMTA systems depending on host and pathogen, as convenient.

5.4 Prediction models

Combining microbiome data with biosensors and molecular tools in aquaculture can provide valuable insights and contribute to the development of species-specific prediction models (Sosnowski et al., 2020). There are different ways in which these data can be combined and utilised. Microbiome

analyses allows the characterization of the microbial communities present in aquaculture (IMTA) systems. These includes fish, water, sediments, and biofilms amongst others. By performing high-throughput sequencing techniques, such as 16S rRNA gene sequencing or metagenomic sequencing, the quantification of microbial taxa and their functional potential can be studied. Moreover, as previously described, biosensors provide real-time data on different parameters, such as pathogen presence, water quality or physiological changes in aquaculture species. Both microbiome and biosensor data can be combined to understand the relationship between microbial communities and specific environmental conditions or pathogen dynamics. Microbiome and biosensor data can be used to develop **species-specific prediction models** that link microbial community composition and function with specific outcomes, such as disease susceptibility, growth performance, or water quality. Machine learning algorithms, such as random forests, support vector machines, or deep learning models, can be employed to build predictive models that relate microbiome and biosensor data to desired outcomes. The models can be trained using known datasets that include microbiome profiles, biosensor measurements, and corresponding phenotypic data for the target aquaculture species. Furthermore, the developed prediction models can be validated using independent datasets to assess their accuracy and generalizability. Once validated, the models can be used to predict outcomes or provide recommendations based on microbiome and biosensor data for specific aquaculture species. For example, the models can predict disease outbreaks, identify optimal feeding regimes, optimize water quality management, or guide the selection of probiotics or other interventions to enhance fish health and productivity. For the development of these models, it is important to consider the microbiome variability between different aquaculture species and systems. Species-specific prediction models should consider these variations. Moreover, the integration of microbiome and biosensor data would require a standardization and quality control to ensure accurate interpretation and results.

Various specific prediction models can be developed in this matter. These include:

Disease Outbreak Prediction: Microbiome data can be combined with biosensor data on water quality, pathogen presence, or fish physiological parameters to develop models that predict disease outbreaks. Machine learning algorithms can be trained to identify specific patterns in microbiome composition, coupled with changes in biosensor measurements and historical disease occurrences, to predict disease outbreaks. These models can assist in implementing interventions, such as targeted treatments, improved water management, to help prevent and mitigate disease outbreaks (Yilmaz et al., 2022).

Growth Performance and Feed Optimization: Microbiome data, biosensor measurements, and feeding parameters can be integrated to develop models that predict fish growth performance and optimize feed formulations. By considering the composition and functional potential of the

microbiome, along with biosensor data on water quality and feeding behaviour, models can estimate the optimal feed type, quantity, and feeding schedule for specific aquaculture species. These predictive models can contribute to improved feed utilization and growth, reducing costs and environmental impacts (Sharma et al., 2021).

Water Quality Management: Microbiome data and biosensor measurements related to water quality parameters (temperature, pH, dissolved oxygen, ammonia, nitrite) can be combined to develop models for water quality management. These models can predict changes in water quality based on microbial community dynamics, enabling proactive interventions to maintain optimal conditions and prevent harmful fluctuations (Bibby et al., 2019).

Probiotic Selection and Efficacy: Microbiome data can be used to identify beneficial microbial taxa associated with improved fish health and performance. Biosensor data, such as immune response markers or disease incidence, can be incorporated to develop models that predict the efficacy of specific probiotic formulations or interventions. These models can help in selecting appropriate probiotics or designing targeted treatments to modulate the microbiome composition and enhance disease resistance, growth, or stress tolerance in aquaculture species (Van Doan et al., 2021).

Environmental Impact Assessment: Microbiome data and biosensor measurements can be utilized to develop models for assessing the environmental impacts of aquaculture operations. By integrating microbiome composition, nutrient cycling, and biosensor data on water quality parameters, models can estimate the ecological footprint of aquaculture systems, including nutrient discharge, carbon footprint, and potential impacts on surrounding ecosystems. These predictive models can support sustainable aquaculture practices by guiding the optimization of feed inputs, waste management strategies, and ecosystem-based approaches (Su et al., 2020).

The specific models will depend on the available data, research objectives, and the target aquaculture species or system. Therefore, the combination of microbiome data with biosensor data enables the development of species-specific prediction models in aquaculture. These models can provide valuable insights into the relationships between microbial communities.

5.5 Next steps for the industry

In the aquaculture sector it is key to improve monitoring and control of the environment by implementing new technologies that can provide information of IMTA lab (or others aquaculture systems) health status in real time, allowing for monitoring of possible pathogen(s) presence. Although molecular tools provide specificity and accuracy when detecting pathogens, biosensors allow for fast, easy detection and monitoring of pathogens in IMTA lab systems. Thus, the surveillance strategies in the aquaculture industry should standardize the combination between molecular tools and biosensors,

providing a more accurate, cost-effective, faster, and easier handling of data when it comes to pathogen detection and identification. The standardization of these methods and subsequent increase of the amount of data generated from the systems, could lead to the implementation of novel machine learning and specie/system-specific predicting models which are needed to anticipate pathogens outbreaks.

6 Conclusions

A high number of potential diseases and pathogens have been identified in the various IMTA systems, some of which have also been listed as notifiable by the WOAH. Most of these pathogens are currently monitored by good manipulation and control of general infestation intensity, taking action once signs of disease have already been identified. Thus, for the early detection and control of the pathogens, not only is it important for the presence/absence of potential pathogens in the system to be known, but it is also important to understand the environment parameters and host susceptibility in which outbreaks can occur. With information compiled in this deliverable, we can conclude the following for each IMTA lab:

- The open coastal IMTA systems (Ireland/Scotland) shows high susceptibility to several bacteria, viruses and parasites, most of them more prevalent in spring/summer when water temperatures increase. Several monitoring techniques have been proposed to enhance early detection and identification of potential pathogens. Firstly, the introduction of biosensors in tagged fish (e.g. AEFishBIT operculum), should be evaluated to obtain real-time data about fish stress/health within the system. Early pathogen identification should then be based on samples obtained from mucosal tissues using field-compatible methods such as portable qPCR machines or Lateral Flow Biosensors (LFB). Based on previous studies, qPCR techniques seems to be optimal for bacterial pathogens (e.g., *A. Salmonicida, Tenacibacilum, Vibrio spp., M. vicosa*), meanwhile LFB are more optimal for rapid detection of viruses (e.g., *Novirhabdoviruses*, *Oyster Herpes Virus*). As for sea lice (*C. elongates/L. Salmonis)*, several traps based on Light-emitting diode biosensors have been used to reduce the sea lice ifestation. Further technologies based on techniques described for other parasites (e.g., qPCR for their adult life stage) toghether with the improvement of eDNA methodologies and machine learning implementation should be considered to tackle this problematic pathogen before an outbreak occurs.

- The partially recirculating land-based IMTA system (South Africa) has not experience any major disease outbreaks, no WOAH notifiable diseases have been identified from the system and

routine surveillance methodologies/ programs are currently implemented. Nevertheless, new and more sophisticated technologies to improve early detection of specific pathogens are recommended, particularly for *Vibrio* species (e.g., *V. anguillarum, V. parahaemolyticus, V. harveyi and V. splendis*), *Haliotid herpesvirus-1* and several parasites (e.g., *Amoeba, Spionids and sabellids)*. Moreover, considering the type of system, eDNA samples from the incoming or effluent seawater can be used to anticipate the entry or presence of specific pathogens in the system. Thus, for this type of sampling, digital qPCR is proposed for virulence related genes (e.g., hemolysin production, gyrase subunit B or malate dehydrogenase) of *Vibrio* species, lateral flow biosensors for *herpesvirus* and multi-locus barcoding for the identification of different parasite species. Moreover, wireless sensors networks are recommended for monitoring water quality parameters and microbiome metabarcoding is proposed to generate continuous data to anticipate system dysbiosis and a possible disease outbreak occurrence.

- The Biofloc recirculating land-based IMTA system (Brazil) is similarly characterized by a low incidence of disease outbreaks, with the biofloc identified as the key variable for the reducing pathogen entry into the system. However, dysbiosis caused by several bacteria, such as *Streptoccoccus*, *Vibrio spp*. and *Cyanobacteria*, has been identified as a primary concern for the system and no standardized surveillance methods are currently in place. Analogous to the South Africa land-based IMTA system, analysis of eDNA samples collected from the biofloc are proposed to monitor the presence or change in abundance (increase) of potential pathogens described for the system. For this, the combination of Wireless biosensor, microbiome metabarcoding and digital qPCR are proposed for improving surveillance of this system.

- The prospective IMTA system in Argentina also present several potential pathogens that will need to be considered previously to implementation. Although the SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria) is improving the notification and communication of disease events, further work should be done to identify the most problematic pathogens impacting the aquaculture industry in the Patagonian area and technologies to monitor the systems. Regarding the pathogens identified, digital qPCR and biosensors (LFB and wireless sensor) are suggested to be implemented in these new IMTA laboratories.

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