Bijay Kumar Behera *Editor*

Biotechnological Tools in Fisheries and Aquatic Health Management

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The Book is dedicated to my Parents and Teachers.

Foreword

It is heartening that the College of Fisheries, Rani Lakshmi Bai Central Agricultural University, Jhansi, Uttar Pradesh, India, is bringing out a book on *Biotechnological Tools in Fisheries and Aquatic Health Management*. It is remarkable that different branches of biotechnology have emerged from few disciplinary micro niches to well-defined dynamic research areas, making substantial impact in the field of fisheries science. The book covers emerging fields of genomics with a special focus on various biotechnological tools applied in fisheries science and aquatic health management. The recent developments in genome editing using CRISPR-Cas9 technology, molecular marker-assisted selective breeding in fish, metagenomics and metatranscriptomics in aquatic environmental health management, soil biotechnology, bioremediation of degraded ecosystems, bioinformatics, nanobiosensor technology applications in fish disease diagnosis and bio-electronics, nanotechnology, role of immunostimulants in aquaculture, etc. have been included in the book. The contributors of this book are from reputed academic and scientific institutions across India. The editor has tried to capture such a wide and dynamic topic in a series of captivating articles highlighting both existing and newly emerging technologies in the field, protocols, methodologies and approaches, advantages, new school of thoughts and potential future prospects with some frontier development of biotechnological research. I am confident that the publication shall be a reference book on the present status, trends and approaches of different biotechnological tools applied in fisheries and aquatic health management. This publication will be beneficial to students and researchers in the field of biotechnology and related areas. I wish to compliment the editor and the contributors for their hard work and painstaking efforts.

Himanshu Pathak

Government of India, Department of Agricultural Research and Education (DARE) and
Indian Council of Agricultural Research (ICAR),
Ministry of Agriculture and Farmers Welfare
New Delhi, India

Preface

The book *Biotechnological Tools in Fisheries and Aquatic Health Management* mainly focuses on genetic improvement, environmental management and bioremediation, biosecurity and disease control, fisheries management and biodiversity conservation. Fish genetic improvement for better growth and disease resistance using genome editing through CRISPR-Cas9 Technology is the need of the hour. Similarly, marker-assisted selective breeding for fish genetic improvement using molecular markers also has tremendous potential for fish production enhancement. Disease outbreaks can have a significant impact on the growth and sustainability of the aquaculture industry. Health management and disease control strategies for aquaculture must take into account the unique features of the aquatic environment and be designed to address these challenges. These biotechnologies can help to improve the health and productivity of farmed fish and other aquatic species, reducing the risk of disease outbreaks and ensuring sustainable growth of the aquaculture industry. Immunoassay and DNA-based diagnostic methods are commonly used in many developing countries for the detection and diagnosis of significant pathogens in aquaculture. Therefore, Nanobiosensor Technology has emerged as a tool for early disease diagnosis. In this book, particular emphasis has been given to recent developments in nanobiosensor technology. Many immunostimulants are being used in aquaculture and fisheries for better fish health management. Aquaculture has faced criticism for its potential negative impacts on the environment, including water pollution, habitat destruction, and the spread of diseases and non-native species to wild populations. In this Omics era, metagenomics studies have much relevance for identifying Novel Antibiotic Resistance Genes (ARGs) from the aquatic environment. The book *Biotechnological Tools in Fisheries and Aquatic Health Management* contains 12 chapters and covers most of the topics related to fish biotechnology and fish health management contributed by our distinguished scientists. The book's objective is to draw the attention of our future budding scientists, researchers and policymakers for exploring and developing "Biotechnological Tools in Fisheries and Aquatic Health Management" in the frontier area for societal benefit.

Jhansi, Uttar Pradesh, India — Bijay Kumar Behera

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The preparation of this book has been guided by several reputed scientists in Biotechnology and Fisheries Science. I am obliged to Dr. Himanshu Pathak, Secretary (DARE) and Director General (ICAR), New Delhi, Dr. Joykrushna Jena, Deputy Director General (Fisheries Science, ICAR), New Delhi, Dr. Ashok Kumar Singh, Vice Chancellor, Rani Lakshmi Bai Central Agricultural University, Jhansi, Padma Shri Dr. Subbanna Ayyappan, former Secretary (DARE) and Director General (ICAR), New Delhi, Dr. Trilochan Mohapatra, former Secretary (DARE) and Director General (ICAR), New Delhi, Prof. Shiva Dhar Singh, former Assistant Director General (Inland Fisheries, ICAR) and Dr. Basanta Kumar Das, Director, ICAR-Central Inland Fisheries Research Institute, Barrackpore, for providing their valuable inputs and support for this book. I thank the anonymous reviewers for their constructive comments that led to a substantial improvement in the quality of this book. I acknowledge all authors who have significantly contributed to this book. I also thank my wife Mrs. Jyotsna Dei and my son Shri Aman Jagannath for their constant motivation for bringing this book to reality. This work would not have been possible without support and enthusiasm from my colleagues, students and researchers, Dr. Ajaya Kumar Rout, Dr. Chirasmita Nayak, Dr. Pranay Kumar Parida, Dr. Vikash Kumar, Dr. Himanshu Sekhar Swain, Dr. Neelesh Kumar, Shri Partha Sarathi Tripathy. Finally, I also thank my publisher and its publishing editor, Springer Nature, for their continuous support and cooperation in the publication of this book.

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Bijay Kumar Behera is the Dean, College of Fisheries, Rani Lakshmi Bai Central Agricultural University, Jhansi, India. Dr. Behera has more than 20 years of research experience in the field of fish biotechnology, microbiology, bioinformatics and nanobiosensor technology. He has received Australian Government-Endeavour Award, Japanese Government-Mombusho MEXT Fellowship, Prof. Har Swarup Award, Krushak Pratibha Award, Scientist of the Year Award, Dr. Hiralal Choudhury Award, Best Scientist Award and Dr. M.-S. Swaminathan Best Indian Fisheries Scientist Award. He is also a Fellow of Zoological Society of India and Inland Fisheries Society of India.

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Recent Developments in Biosensor Technology for Fisheries and Aquaculture

1

Bijay Kumar Behera

Abstract

Aquaculture is the fastest growing sector among the agriculture and allied sectors. However, fisheries and aquaculture face lots of challenges due to biotic (viral, bacterial, fungal and parasitic) and abiotic stresses of various chemical pollutants of organic and inorganic biotoxin which are negatively impacting the growth of aquaculture. Therefore, it became imperative to detect microbes and endocrine-disrupting chemicals (EDCs) in aquatic ecosystems rapidly to mitigate those problems. The conventional techniques (molecular techniques for microbes and high-end equipments like GC-MS (gas chromatography-mass spectrometry), HPLC (high-performance liquid chromatography), ICP-MS (inductively coupled plasma mass spectrometry), etc. for detection of microbes and chemical contaminants are costly and need skilled manpower. The receptor molecules like antibodies, enzymes, aptamers, etc. are very important for development of suitable molecular recognition element (MRE) against a particular analyte for biosensor development. Biosensor can help in detection of pathogenic bacteria and EDCs in less time with low cost and point of use. This chapter highlights the recent developments of biosensor technology which can be used in fisheries and aquaculture.

Keywords

Biosensor · Aquaculture · Aptamer · Pathogens · Endocrine-disrupting chemical

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1.1 Introduction

The world has been continuously addressing the rise in the demand for food as the population is increasing and thus an increase in the need of protein is seen. Fish is a very rich source of easily digestible protein (Mustapha et al. 2021). The world's fish production in 2018 was estimated to be 178 million tons, to which aquaculture contributed 87.5 million tons and capture fisheries contributed 90.3 million tons (FAO 2022). The enormous growth of fisheries and aquaculture has come with the responsibility to manage and develop sustainability. The growth of capture fisheries has become stagnant for the past few years; on the other hand, aquaculture has continuously shown growth, but the higher stocking densities have demanded better management practices and precision farming. Aquaculture has been continually shifting from extensive to semi-intensive farming and to intensive farming. This shift requires optimum water condition monitoring, disease monitoring, feeding efficiency and waste management. All these become very expensive if done through traditional methods as they require time and skilful manpower. Biosensors are essential to bettering the sector's growth because they save on labour costs, time and expensive machinery. It is also helpful for small-scale industries which cannot afford technical experts for the scientific purposes.

Biosensors are sensing systems/devices which can measure biological and chemical reactions, hence generating output signal that is proportional to the analyte concentration. Sensors are developed to substitute the traditional testing procedures which require scientific personnel, specified knowledge and costly equipment, thus representing significant cost and time. Moreover, most of the testing procedures often fail to produce results on-site. Thus, sensors can address several issues in one go.

A biosensor primarily consists of a bioreceptor, a transducer and a processing unit; quantifiable signal gets generated upon the interaction of the target analyte with the bioreceptor. Recent research and developments have focused to design low-cost efficient sensors which can serve the purpose efficiently without counting for higher costs. Alternative bioreceptor molecules like enzymes, cells, aptamers, deoxyribonucleic acid (DNA) and antibodies have been tested for their suitability to different testing conditions, cost-effectiveness and stability (Bhalla et al. 2016).

1.2 Working Principle of Biosensor

By using some of the common approaches, a certain enzyme or preferred biological material is often deactivated and the deactivated biological material is in close proximity to the transducer. The biological object and the analyte work together to create a clear analyte, which in turn produces a calculable electronic reaction. In some instances, the analyte is switched to a component that could be linked to a source of heat, gas discharge, electron ions or hydrogen ions. The transducer in this can affect the connected device by converting it into electrical signals that may be adjusted and calculated (Fig. 1.1).

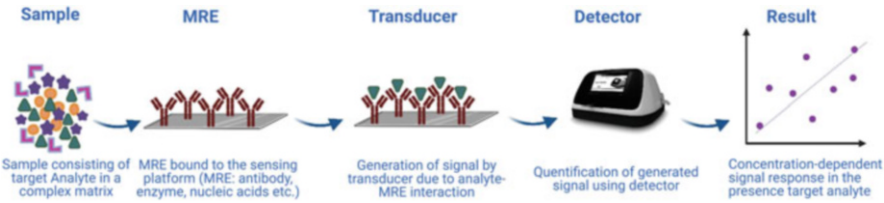

Fig. 1.1 Various components of biosensor

1.3 Main Components of Biosensor

Analyte: The target substance to be detected, for example, a metalloid, arsenic in water.

Bioreceptor: It is a molecule which uniquely recognises analyte. Bioreceptors comprise of antibodies, cells, enzymes and aptamers. Bio-recognition is the method of signal production (in form of charge or mass shift, pH, heat and light, etc.) in response to the contact of the bioreceptor with the analyte.

Transducer: An element known as a transducer converts one type of energy into other form. Transducer's function in a biosensor is to transform a bio-recognition event into a quantifiable signal. Signalisation is the term used to describe this energy conversion process. Most of the transducers generate electrical or optical signals, which are typically proportionate to the number of interactions between analyte and bioreceptor.

Electronics: This section of a biosensor is responsible for processing the transduced signal and getting it ready for display. It is made up of intricate electrical circuitry that carries out signal conditioning tasks like signal amplification and conversion from analogue to digital form. The biosensor's display device then quantifies the signals that have been processed.

Display: The display comprises of a user interpretation system that provides legible figures or curves for the user, like a computer's liquid crystal display or a direct printer. This component frequently consists of a hardware and software combination that produces user-friendly biosensor findings. Depending on the needs of the end user, the output signal on the display may be numerical, visual, tabular or even a picture.

1.4 Types of Biosensors

The biosensors are of different types based on the sensor device and the biological materials used. Depending on the biological element employed in the study or the transduction mechanism used, biosensors are divided into two classes. DNA, enzymes, antibodies, bacteria, tissues, cell receptors, and others are some of the

frequently utilised biological elements or bio-recognition elements. The following and most popular method of categorizing biosensors is based on the kind of physiochemical that is produced as a result of the sensing event or type of transduction employed in sensor. The biosensors are of three types based on the transduction mechanism, and they are mass-based, electrochemical and optical biosensors. Again, a few subclasses exist for each of these categories.

1.4.1 Electrochemical Biosensor

The electrochemical biosensor is often based on an enzymatic catalytic reaction that either generates or consumes electrons. Redox enzymes are the name for these kinds of enzymes. Three electrodes, usually of the working, reference and countertypes, are typically present on the substrate of this biosensor. The target analyte participates in a reaction that occurs on the surface of an active electrode, and this reaction could also be a source of electron transfer across a potential dual layer. At a specific potential, the current may be determined. There are four types of electrochemical biosensors; they are voltammetric, potentiometric, impedimetric and amperometric biosensors.

1.4.1.1 Amperometric Biosensor

A self-contained integrated device called an amperometric biosensor is based on the amount of current produced by oxidation and provides precise quantitative analytical data. Generally speaking, these biosensors are analogous to potentiometric biosensors in terms of response times, energy ranges and sensitivities. The "Clark oxygen" electrode is a basic amperometric biosensor that is rarely used. The operating principle of this biosensor is based on the rate of current flow between the counter electrode and working electrode, which is aided by a redox reaction there. For a variety of applications, including high-throughput pharmaceutical screening, quality control, problem identification and handling and biological checking, selecting analyte centres is crucial.

1.4.1.2 Potentiometric Biosensor

This kind of biosensor responds in a logarithmic manner with a wide active range. These biosensors are frequently finished by monitoring the production of the electrode prototypes, which are laid out on a synthetic substrate and covered with a functioning polymer that is related to an enzyme. They consist of two incredibly powerful and responsive electrodes. They enable the identification of analytes at levels that were previously only possible with HPLC and LC/MS and without precise model creation. Due to the biological detecting component's extreme prudence in choosing the analyte in question, sample preparation for all types of biosensors is typically the least time-consuming. Due to modifications occurring on the outside of the biosensor, a signal will be produced by changes in physical and electrochemical properties in the layer of conducting polymer. Ionic force, hydration, pH and redox reactions—the latter of which is the label for an enzyme

revolving above a substrate—might be responsible for these alterations. The gate terminal in field-effect transistors (FETs) has been replaced with an antibody or enzyme, and because the needed analyte towards the gate terminal modifies the drain to source current, it can also sense very low attention from various analytes. The Ion-selective electrodes are based on membranes, ion-selective FETs, screen-printed electrodes, solid-state devices and modified electrodes through chemical-like metal oxides. Otherwise, electrodeposited polymers like sensitive layers are the main types of potentiometric biosensors.

1.4.1.3 Impedimetric Biosensor

A responsive indicator for a wide range of chemical and physical properties is electrochemical impedance spectroscopy. Currently, there is a developing tendency towards the development of impedimetric biosensors. Impedimetric techniques have been used to study the catalysed reactions of enzymes, receptors, nucleic acids, lectins, antibodies and entire cells as well as to distinguish the design of biosensors.

1.4.1.4 Voltammetric Biosensor

The voltammetric biosensor's carbon glue electrode has been modified with haemoglobin, which has four prostatic heme groups (Fe). This kind of electrode displays a reversible process for oxidizing or reducing haemoglobin (Fe).

1.4.2 Physical Biosensor

Physical biosensors are the most fundamental and often utilised sensors when it comes to classification. The fundamental concepts underlying this classification are also discovered through looking inside human minds. Any detecting device that responds to the physical properties of the medium is referred to as a physical biosensor since the general mechanism behind the intelligence of hearing, sight, and touch is to react on the outer physical stimuli. Piezoelectric biosensors and thermometric biosensors are the two categories under which physical biosensors fall.

1.4.2.1 Piezoelectric Biosensors

These sensors are a group of analytical tools that operate in accordance with the "affinity interaction recording" law. Due to a collecting leap on the surface of a piezoelectric crystal, the platform of a piezoelectric is a sensor element that operates on the law of oscillation transform. These, biosensors with changed surfaces include an antigen or antibody, a polymer that has been molecularly stamped. Nanoparticles are typically used to join the declared detection components.

1.4.2.2 Thermometric Biosensor

The basis of thermometric biosensors is the wide range of biological processes that are linked to the creation of heat. Thermal biosensors are the common name for these sensors. Serum cholesterol is measured or estimated using thermometric biosensor technology. The heat that is produced while cholesterol is oxidised by the enzyme

cholesterol oxidise can be computed. These biosensors can also be used to measure penicillin G, glucose, urea and uric acid.

1.4.3 Optical Biosensor

A device that makes use of the optical measurement principle is the optical biosensor. Both fibre optics and optoelectronic transducers are employed. The words optical and electrode are combined to form the phrase optrode. Like the transducing elements, the main components of these sensors are antibodies and enzymes. Optical biosensors provide for safe, nonelectrical, inaccessible equipment sensing. Additionally, since the comparison signal can often be generated using a light source comparable to the sample sensor, these often do not require reference sensors. Direct optical detection biosensors and labelled optical detection biosensors are the two categories into which optical biosensors fall.

1.4.4 Wearable Biosensors

A wearable biosensor is a digital device that is worn on the body and used in various wearable systems, such as smartwatches, smart shirts and tattoos, to measure things like blood pressure, heart rate, blood sugar levels and others. These days, we can see that the world is getting better thanks to these sensors. Their greater use and simplicity can provide a unique level of insight into a patient's current fitness status. This data accessibility will enable better clinical decisions, which will impact improved health outcomes and more skilful utilisation of the healthcare systems. These sensors may help humans avoid hospitalisation by providing early detection of health events. The potential for these sensors to shorten hospital stays and prevent readmissions will undoubtedly generate interest in the near future.

1.4.5 Enzyme Biosensor

One type of analytical equipment is this sensor, which combines an enzyme with a transducer to produce a signal proportional to the concentration of the target analyte. This signal can also be analysed, stored and amplified for subsequent study.

1.4.6 DNA Biosensor

Nucleic acid identification methods for analysis of easy, quick and affordable detection of pathogenic bacteria and endocrine-disrupting substances in water can serve as the foundation for the development of DNA biosensors. Furthermore, accurate DNA sequence detection is important for a variety of industries, including environmental, medical and food analysis. To improve detection methods for

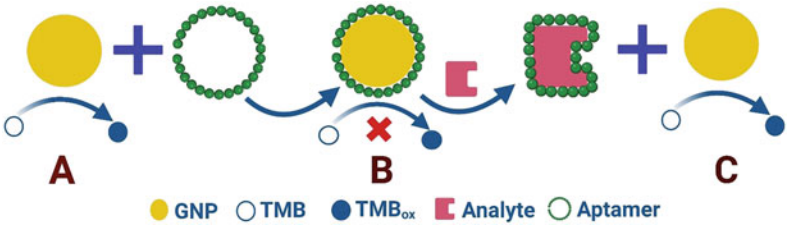

Fig. 1.2 Mechanism of biosensor through nanoparticle-coupled aptasensing (GNP, gold nanoparticles; TMB, 3,3′,5,5′-tetramethylbenzidine; TMBox, oxidised 3,3′,5,5′-tetramethylbenzidine)

aptamer-based biosensors, systematic evolution of ligands by exponential enrichment (SELEX) technologies is used to develop better recognition methods. Nucleic acid layer recognition can be intentionally manufactured and renewed for a number of reasons, unlike antibodies or enzymes. Due to their enormous ability to gather precise data more easily, cheaply and quickly than conventional hybridisation, these sensors and gene chips have significant advantages over that process. Figure 1.2 shows the fundamental workings of the aptamer-based nanobiosensor concept. When a gold nanoparticle (GNP) interacts with the peroxidase substrate, it exhibits peroxidase-like activity (step A). By protecting the GNP surface through the adsorption of target-specific ssDNA aptamers, this peroxidase-like activity is prevented (step B). In the absence of a target analyte, the aptamer undertakes target-responsive structural deviations, followed by desorption from the GNP surface, allowing an aptamer-target binding event. In the presence of target, the aptamer undertakes target-responsive structural deviations and desorption from the GNP surface. The GNP can then return to its initial state and resume target-specific peroxidase-like activity as a result (step C). This tactic is comparable to the competitive inhibition mechanism used by natural enzymes, which exhibit a loss of activity in the presence of an inhibitor and a resumption of activity in the presence of a substrate with a higher binding affinity once the inhibitor has been removed. Despite an increase in the number of these sensors, basic research is still required to advance sensor technology, detecting schemes, analytical tools and methodologies.

1.4.7 Immunosensors

Immunosensors have been discovered based on the fact that antibodies have a high affinity for their specific antigens, such as when they join with poisons or pathogens or communicate with different parts of the host immune system. These kinds of biosensors are built on solid-state affinity ligand devices, where an immunochemical reaction can be coupled to a transducer.

1.4.7.1 Magnetic Biosensors

These sensors are used to monitor alterations in magnetically influenced phenomena or magnetic characteristics. These sensors measure changes in magnetic characteristics, such as changes in coil inductance and resistance, and use superparamagnetic or paramagnetic crystals or particles to detect biological communications.

1.4.7.2 Resonant Biosensors

A bio-element in a resonant biosensor can link to a transducer, such as an acoustic wave. The membrane's mass changes as soon as the analyte molecule is attached to it. Consequently, the final modification to the mass changes the resonance frequency of the transducer. After that, the change in frequency could be gauged.

1.4.7.3 Thermal Detection Biosensor

The temperature changes when a biological reaction takes place using a thermal detection-type biosensor, which exploits one of the fundamental aspects of the reaction, such as heat production or absorption. By employing temperature sensors to connect the molecules of an immobilised enzyme, this sensor can be designed. The thermal reaction of the enzyme can be detected and calibrated in relation to the concentration of the analyte once the analyte and the approaches come into contact. The total heat produced, as opposed to being absorbed, is proportional to molar enthalpy and overall molecule numbers involved in reaction. Enzyme thermistors are a common type of thermistor used to measure temperature. Due to their sensitivity to heat fluctuations, thermistors are perfect in particular applications. Thermal sensors do not require routine calibration like other types of transducers do, and they are insensitive to the sample's electrochemical and optical properties. These sensors are used to find pathogenic bacteria.

1.4.8 Biosensors in Aquaculture

Contrary to terrestrial animal husbandry, it is quite difficult to regularly monitor health and behaviour of fishes in aquaculture. Visual monitoring of fishes and their responses to environmental change and management interventions is challenging. As a result, it is a standard procedure for traditional aquaculture monitoring to choose a random fish and then physically inspect that species away from its native habitat. A historical profile of an individual fish or population's responses to environmental or managerial events is produced by periodical subsampling (Andrewartha et al. 2015). These welfare spot checks require physical handling times that are stress events in and of themselves, which may have an impact on any following measurements or observations. The use of easily measured physicochemical parameters such as temperature, water and quality (oxygen, pH, nitrogen and turbidity) as proxies for fish health is another prevalent approach (Andrewartha et al. 2015). Small biosensors have made it possible to track a range of physiological and/or behavioural traits over an extended period of time without causing any harm

to the animals. These traits include body temperature, heart rate, motility, mollusc valve activity, depth and body temperature. Animals with biosensors have already been created for dairy industry, and same has been developed for aquaculture fish and commercially farmed molluscs (e.g., pacific oyster) (Andrewartha et al. 2015).

1.4.8.1 Ammonium Detection in Aquaculture

Ammonia nitrogen, a crucial indicator of aquaculture water, is the sum of molecular ammonia (NH_3) and ammonium ion (NH_4^+) in water. The majority of the ammonia nitrogen in aquaculture water is obtained from the excrement and faces of aquaculture animals, leftover bait, plankton debris and bottom sludge. When water is anoxic, anaerobic bacteria denitrify organic molecules, nitrate, and nitrite to produce some ammonia nitrogen. In aquatic animals, chronic ammonia nitrogen poisoning results in decreased retarded growth, food intake and tissue damage. Anomalies include hyperactivity, swimming on the water's surface and losing balance, and even death is possible at the same moment (Wang et al. 2021). Screen-printed electrodes are disposable enzyme electrodes created by combining screen printing technology with mediator enzyme electrodes. Its benefits include affordability, good reproducibility, quick response time and minimum sample consumption. The electrode enhanced with nanomaterials has clear benefits in terms of improving sensitivity and selectivity. The first step involves the reversible amination of α-ketoglutarate to L-glutamate by GLDH (glutamate dehydrogenase) in the presence of a cofactor for NADH (nicotinamide adenine dinucleotide) and an NH_4^+ ion. In the second phase, the remaining NADH reacts with the oxidised PMB (PMBox) on the electrode surface to form NAD^+ and reduced PMB (PMBred). In the third stage, the electrochemical reoxidation of PMBred to PMBox results in the transfer of two electrons to the SPEC and the simultaneous loss of a proton. The electrocatalytic oxidation of NADH in steps 2 and 3 produces the signal. Due to the fact that all of the present NADH takes part in the electrocatalytic oxidation reaction, the biosensor performs at its best when there are no NH_4^+ ions present. Because some NADH was consumed in step 1, the electrocatalytic current of the biosensor decreases when there is an NH_4^+ ion present. The decline in current is proportional to concentration of ammonium ion in sample when the substrate concentration and GLDH loading amount remain constant. After a disposable SPEC/AuNPs/PMB-modified electrode was created, the reaction substrate's NADH, α-ketoglutarate and GLDH additions were optimised. The ammonia nitrogen biosensor's reaction temperature is controlled by a thermostat, and its output signal is calibrated in real time using a pH compensation strategy based on piece-wise linear interpolation lookup table method. The optimal storage period for the electrode is 14 days. Strong concerted catalysis, great detection sensitivity and extraordinary anti-interference capacity are all displayed by the modified electrode. Biosensor's linear range is 0.65–300 M. The study revealed that the biosensor and Nessler's method essentially measured low concentration ammonia nitrogen samples in a similar manner (Wang et al. 2021) (Fig. 1.3).

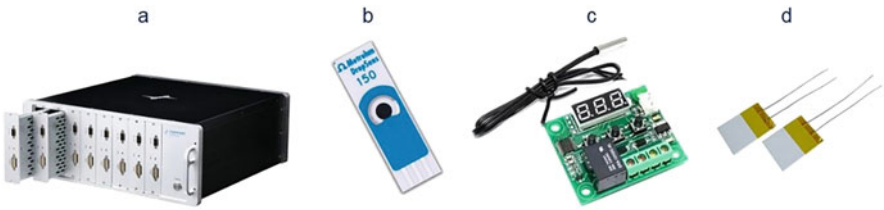

Fig. 1.3 Instrument for experiment. (**a**) Potentiostat, (**b**) screen-printed electrode (SPE), (**c**) thermostat and (**d**) ceramic heating plate

1.4.8.2 Detection of Water Pollutants

Water pollutants could be detected using graphene field-effect transistor (GFET) sensors, which are very useful analytical tools. Water contaminant, 17-estradiol, has been specifically detected in buffer and tap water using a differential aptamer-based graphene biosensor. With or without pH variation interferences, as well as in tap water with unknown interferences present, consistent detection findings can be obtained. These findings show that nonspecific interferences can be efficiently reduced by the differential graphene affinity sensor, allowing for selective water pollutant detection for water quality monitoring (Li et al. 2019).

A developing technology for detecting contaminants in water is aptamer-based biosensors. Due to their toxicity after prolonged exposure, low molecular weight pollutants have garnered attention on a worldwide scale, even at trace amounts in water. Appropriate techniques to find these contaminants in aquatic systems are increasingly in demand. Similar to the interaction between an antigen and an antibody, aptamers have a high affinity and specificity for each target molecules. Highly specific aptamers could be developed using SELEX technique. The development of aptamer and aptamer-based sensors for contaminants in water, including river water, lake water, tap water, saltwater and wastewater, has advanced significantly in recent years (Zhang et al. 2018).

1.4.9 Biosensor for Fish Health Monitoring

1.4.9.1 Biosensor for VHSV Detection

Each year, a fish viral disease, viral haemorrhagic septicaemia (VHS), costs both governmental and private aquaculture projects large sums of money. The causative agent of viral haemorrhagic septicaemia, VHSV (viral haemorrhagic septicaemia virus), is a single-strand RNA virus that belongs to the genus *Novirhabdovirus* and family Rhabdoviridae. VHS is the disease with the greatest economic impact on farmed salmonids worldwide, as well as on marine fish species like olive flounder (Hong et al. 2010). In order to detect VHSV, a DNA sensor based on quartz crystal microbalance (QCM) has been developed. It uses an oligo-DNA probe specific to a key VHSV gene, the G-protein. The three different immobilisation techniques included immobilising thiol-labelled probe DNA on bare gold, immobilising

amine-labelled probe DNA on gold surface prepared as a carboxyl chip using MPA followed by EDC/NHS activation and immobilising biotin-labelled probe DNA on gold surface following avidin immobilisation on carboxyl chip prior to biotin. The attachment of target DNA tagged with FAM (fluorescein amidites) is visible under a fluorescence microscope. The immobilisation method using biotin-labelled probe DNA is the most successful in identifying target DNA. The probe DNA sequence is 5′-X-TGGTGACTGATAGCGGGT-3′ (X: thiol, amine, biotin) (Hong et al. 2010). During the building of a biosensor to detect bacteria or their genomes, the specificity of the sensor chip is very crucial.

1.4.9.2 Biosensor for *Aphanomyces invadans*

A number of commercially valuable wild and farmed freshwater and estuary finfish species are severely affected by epizootic ulcerative syndrome (EUS) which is known as mycotic granulomatosis, ulcerative mycosis or red spot disease. The invasive oomycete fungus, *A. invadans*, causes EUS. Mycotic granulomas seen in infected fish tissues are diagnosed as EUS using histological analysis, gross examination and culture (Kuan et al. 2013).

Molecular methods like PCR (polymerase chain reaction) and fluorescent in situ hybridisation (FISH) have been applied to assist in the diagnosis of EUS. Gold nanoparticles coated on multiple layers of latex were used in a novel electrochemical genosensor application to detect the epizootic ulcerative syndrome pathogen, *A. invadans*. The 208 bp PCR product of 18S rRNA internal transcribed spacer regions of a real fungal sample is employed. Using a premix sandwich hybridisation assay and capture probes set on a screen-printed carbon paste electrode surface, the PCR product was detected. The PCR product is hybridized with reporter probes conjugated to AuNP-latex spheres. After hybridisation, differential pulse anodic stripping voltammetry is used to identify the gold nanoparticles (Kuan et al. 2013).

1.4.9.3 Aptamer-Based Biosensors for Pathogen Detection

Controlling aquatic illnesses requires a combination of prevention and therapy. It is absolutely necessary to develop speedy diagnostic tools and effective treatments for diseases in aquaculture. Aptamers are functional single-stranded oligonucleotides (ssDNA or RNA) with lengths between 50 and 100 bases that are produced using the exponential enrichment (SELEX) method from a library of random synthesised oligonucleotides. Both simple substances (such as ions, peptides and proteins) and complex molecules are targets for SELEX (viruses, bacteria, cells and even tissues). SELEX uses a cycle-repeating technique to create highly specific aptamers for selected targets. To properly develop the precise aptamers that recognise the targets, several rounds of selection are required.

1.4.9.4 *Vibrio parahemolyticus*

Using whole-bacterium SELEX, DNA aptamer sequences specific to *V. parahemolyticus* have been discovered. These sequences help in rapid isolation/identification of *V. parahemolyticus* in food and environmental samples (Duan et al. 2012). A number of different bacteria, such as *L. monocytogenes*, *E. coli*,

S. typhimurium, *S. aureus* and *S. pneumoniae*, are tested using the specific fluorescently labelled aptamer sequences A1, A1P, A3, A3P and A18P. All of these aptamer sequences showed that *V. parahemolyticus* is their preferred target for binding. It is also found that the examined aptamer sequences are unique to *V. parahemolyticus* (Duan et al. 2012).

1.4.9.5 *Vibrio vulnificus*

With the aid of SELEX, several aptamers have been created targeting *V. vulnificus*. These are evaluated for *S. aureus*, *V. anguillarum*, *E. coli* and *B. subtilis*. Among these, Vapt2 had a significantly lower binding affinity for the other bacterial species and a strong specificity for *V. vulnificus*. Vapt2 bound to *V. vulnificus* at a higher rate than it did to the other bacteria, with a significantly smaller fluorescent signal. Fluorescence microscopy analysis revealed that Vapt2 bound more *V. vulnificus* sequences in the same amount of time as other bacterial sequences. The Vapt2 aptamer can be used to detect *V. vulnificus* as it is highly specific to it (Yan et al. 2018).

By combining 50 nM Vapt2 with various *V. vulnificus* dilutions and watching the fluorescence signal, it is possible to determine how sensitive Vapt2 is to the pathogen. The rate of fluorescence intensity is found to be closely linked with *V. vulnificus* concentrations between 20 and 2.0×10^8 cfu/ml; the LOD is 8 cfu/ml (Yan et al. 2018).

1.4.9.6 *Vibrio alginolyticus*

An opportunistic pathogen called *V. alginolyticus* can infect both humans and fish raised in aquariums. One of the hazardous bacteria in mariculture, *V. alginolyticus*, is precisely targeted by aptamers made using the SELEX technique. The specificity of aptamer candidates (VA2, VA8) is assessed using a flow cytometer. The results indicate that in contrast to the control group, which exhibited no visible fluorescence, the aptamers VA2 and VA8 could specifically attach to the target bacterial strain *V. alginolyticus*. The aptamers VA2 and VA8 have extremely high affinities for the target *V. alginolyticus*, according to additional estimations of the aptamer's binding affinities (Yu et al. 2019).

1.4.9.7 *Vibrio harveyi*

The two aptamers, C14 and C22, are used from two representative groups, one of which is made up of 14 aptamers with a homology of more than 87% (C1, C17, C16, C39, C22, C34, C5, C8, C9, C42, C32, C23, C35 and C24) and the other of which is made up of 13 aptamers with a homology of more than 92% (C14, C25, C48, C7, C37, C28, C29, C15, C27, C30, C6, C31, C38). The software DNAMAN created the homologous tree based on the homology of the aptamers, and the percentage at each node represented the homologous rate of the involved aptamers (Hui-Min et al. 2020). Two aptamers' affinities for binding to the target bacteria, *V. harveyi* and *V. alginolyticus*, are much higher than their affinities for binding to nontarget bacteria, *A. hydrophila* and *E. tarda*. Affinities of two aptamers to bind to *V. harveyi* are also much higher than those to bind to *V. alginolyticus*. Result showed

good affinity and specificity of the two aptamers in separating *V. harveyi* from *V. alginolyticus* as well as the target bacterium from the nontarget bacteria (Hui-Min et al. 2020).

1.4.9.8 *Listeria monocytogenes*

The SELEX process has been developed to produce aptamers that precisely and robustly bound to *L. monocytogenes*. A15, which is picked from eight families of pre-screened aptamers, exhibited a very high level of selectivity and a great affinity for the intended *L. monocytogenes* cells. Using fluorescently labelled aptamer A 15, several different bacterial species, including, *S. typhimurium*, *L. innocua*, *E. coli* and *S. aureus* were examined. The high fluorescence intensity of *L. monocytogenes* in comparison to the other control bacteria, especially the other *Listeria* species, demonstrates exceptional specificity of aptamer A 15 to *L. monocytogenes* (Duan et al. 2013).

1.5 Conclusion

Aquaculture is the fastest growing sector of agriculture globally, and the demand for seafood is increasing day by day due to burgeoning growth of population. To enhance the fish production, the mitigation of abiotic and biotic stress in the aquaculture system is required. Fish disease caused by various pathogenic microbes is the main cause of the crop loss, which retards the aquaculture production enhancement. At the same time, lots of EDCs are contaminating the aquatic environment causing abiotic stress to fish which leads to sex change, loss of immunity, retarded growth, susceptibility to various diseases, etc. Recent developments in different types of biosensors and their use in detection of abiotic and biotic stressors in fisheries and aquaculture have been highlighted. As biotic and abiotic stress is a big problem in aquaculture, sustained effort should be made to develop various biosensor-based technologies in an affordable price. Emphasis should be given in research to miniaturize the biosensors for point of use in aquaculture in the future.

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Application of CRISPR-Cas9 Technology in Fish

2

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Abstract

CRISPR/Cas9-based genome editing allows efficient and targeted enhancement of important aquaculture traits and will probably be the solution to the many present problems in the aquaculture industry. CRISPR/Cas9 has its own advantage over previously used genome editing platform like zinc finger nuclease (ZNF), meganuclease and transcription activator-like effector nuclease (TALEN) in terms of cost effectiveness, designing of construct, ease of delivery of construct and minimising the off-target modification. CRISPR/Cas9-based modification of growth-associated genes in important aquaculture species like red sea bream, channel catfish, common carp, etc. has shown promising result. Successful modifications were done also in grass carp, channel cat fish and zebra fish for acquiring disease resistance and stress tolerance. Apart from the growth- and disease-related traits, several studies were done to improve flesh quality, improve pigmentation, etc. in fish as well as in crustaceans. Besides that, knock-out study with CRISPR/Cas9 system revealed function of many genes which were previously unknown. Highly efficient techniques for disease diagnosis are developed for detection of viral DNA/RNA in host cell based on CRISPR/Cas9-based system. In spite of wide-scale application of CRISPR/Cas9 system, there are a few challenges it may face in the genome modifications in fish which will be required to be resolved in order to achieve the full potential benefits of this wonderful tool of nature.

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Keywords

Genome editing · CRISPR/Cas9 system · Aquaculture · Nutri-fish and high growth

2.1 Introduction

Genetic improvement of farmed animals as well as fishes with organised breeding technique and genetic engineering tools is in place for decades. Though the results are very encouraging as far as both the technologies are concern, genetic improvement through selective breeding has its own limitation in terms of dependency on the heritability of the target trait and long generation interval. Genetic engineering tools offer rapid and effective solution to these problems. With the advent of CRISPR/Cas system, a flood gate of such opportunities has opened in the field of genetic improvement in aquaculture. Specially designed nucleases such as TALEN and ZFN were previously used for genome editing. But due to complexity in designing, involvement of high cost and lack of efficiency, more and more researchers are turning towards the CRISPR/Cas9 system. CRISPR/Cas system allows specific, targeted minor changes in the genome of species of interest and does not involve transfer of foreign gene like in transgenesis, thus addressing the major public safety concern. Because of its flexibility and highly efficient nature of gene targeting in terms of knocking out, modification and introduction of desired sequence in the genome of interest, CRISPR/Cas9 technology has been considered as an important tool for sustainable genetic improvement programme in aquaculture industry.

2.2 Genome Editing Platforms

The basic principle of genome editing tools depends on the cellular DNA repair mechanisms. DNA repair mechanisms are triggered by double-stranded breaks in two main ways: homology-dependent repair, which involves copying a donor sequence that matches the target DNA molecule, and non-homologous end joining (NHEJ), which involves joining ends of the broken DNA and results in mutation because of indels (Wang et al. 2016). All the genome editing tools used this simple basic technique to induce a site-specific double-stranded break in targeted DNA. The second pathway is used for knocking out particular gene, whereas the former is used for the introduction of desired sequence in the genome by supplying a homologous donor sequence.

2.2.1 Zinc Finger Nuclease

Before the discovery of CRISPR system, the genome editing was used to mostly carry out ZFN and TALEN. ZFNs are custom-designed molecular dimer containing

zinc finger protein domain conjoined with FokI endonuclease domain. Each zinc finger contain 3–4 domains; each domain is made up of 30 amino acids. These domains of ZNFs bind with nucleotide triplets, and cutting of DNA is mediated by FokI endonuclease with 5–7 bp spacer sequence (Yang et al. 2022; Smith et al. 2000). ZFNs have some technical difficulties and are costlier to use; also they have disadvantages of being off-target cleavage and undesired mutation (Miller et al. 2007; Pattanayak et al. 2011; Yang et al. 2022).

2.2.2 Transcription Activator-Like Effector Nuclease

TALE was discovered from bacteria of *Xanthomonas* genus. This protein has three domains: a DNA binding domain, a nuclear localisation signal and an activator domain that activates transcription of target gene. Bacteria of *Xanthomonas* genus infect the plant cell and release TALE protein in the cytoplasm. TALE mimics the eukaryotic transcription factors and activates certain genes in plant cell, making them susceptible to the pathogen (Canonne and Rivas 2012).

The DNA binding domain contains monomers that have specific nucleotide. Each monomer contains tandem repeats of 34 amino acids. The first nucleotide at the 5′ end where TALE monomers bind is always required to be thymidine (T). The 12th and 13th number of amino acid are variable and specific to particular nucleotide (Nemudryi et al. 2014). However, this recognition is not absolute and there is some degree of degeneracy. This DNA binding domain of TALE protein may be designed for target DNA segment and fused with FokI endonuclease in similar fashion like in the case of ZFN. The FokI endonuclease cuts the DNA in 12–13 bp spacer sequence. The major limitations of TALEN include requirement of thymidine nucleotide at 5′ end and technical hurdle for cloning the repeat TALE arrays is designing the large-scale identical repeat sequences (Roy et al. 2022). Though both the technologies have improved themselves for genome editing, they still have limitations.

2.2.3 Clustered Regularly Interspersed Short Palindromic Sequence

CRISPR is a short repeated sequence separated by a spacer sequence, and Cas (CRISPR-associated protein) system is a standalone mechanism of bacterial adaptive immune system against any foreign DNA. This mechanism acts in three phases: adaptation, expression and target interference (Lone et al. 2018). During the first phase, the invading DNA is recognised often by recognising a small specific 3–5 bp DNA motif which located 2–6 bp downstream of target DNA. This small sequence is called protospacer adjacent motif (PAM). After recognising the target DNA, cas protein complex cuts the DNA, which is called protospacer. The protospacer is incorporated into the upstream location of CRISPR arrays by cas protein complex; this segment is now called spacer, which remains in the genome of the host as memory of infection. The CRISPR array is transcribed into precursor CRISPR RNA

(pre-crRNA) during the expression stage, which is then processed into mature CRISPR RNA (crRNA). The flanking repeats of the crRNA contain spacer sequence, which remains in an RNA-protein complex (Ione and Marakova). Maturation of crRNA is mediated by either a complex of cas proteins or by a single multi-domain cas protein or by non-cas host RNases in different classes of CRISPR/Cas system (Marakova). In the last phase of interference, the crRNA recognised protospacer sequence in the invading DNA in subsequent infection with the help of PAM sequence which helps in differentiating the spacer sequence available in the bacterial genome (in CRISPR locus) and invading DNA, thus preventing the bacteria from destroying its own genome. Upon recognition, the cas nucleases cleaved the invading DNA and thus inactivated it.

2.2.3.1 Classification of CRISPR/Cas System

The classification of CRISPR/Cas system mechanism becomes highly challenging owing to the diversity of Cas proteins, presence of multiple CRISPR loci, and horizontal transfer of the CRISPR/Cas system. However, in a most recent study based on the architecture of effector module, the CRISPR/Cas system is classified into 2 major classes, 6 types and 44 subtypes (Makarova et al. 2020). In class 1 system, the effector module composed of multiple Cas proteins forms complex structure with crRNA that involved in binding and processing of target DNA. In class 2 system, a single large multi-domain crRNA binding cas protein is involved which performs as functional analogue of multi-protein complex in class 1. Class 1 contains 3 types, that is, types I, III and IV, and 18 subtypes. Class 2 contains types II, V and VI and 26 subtypes.

Due to the involvement of single multi-domain protein for recognition and interference, the class 2 type II system is a widely accepted platform for genome editing. The type II system is called minimal CRISPR/Cas system that includes CRISPR repeat arrays and only four (most often three) genes. Cas1 and Cas2 are involved in acquisition of spacer sequence. The pre-crRNA forms an RNA duplex with transactivating crRNA (tracr-RNA) which is processed by host RNase III into mature crRNA in presence of Cas9 (lone). Cas9 is the signature protein and in the type II system is the large multi-domain protein alone responsible for recognition and cleavage of target DNA.

2.3 Genome Editing Using CRISPR/Cas9 System

2.3.1 Selection of Target Gene

The preliminary step in genome editing is the selection of the target gene. After searching the genomic databases of prospective species, the target gene has to be selected. Whole genome sequencing of about 90 species is completed and available in the database (Bian et al. 2019; You et al. 2020). Such a large genome database for fishes aids the molecular researcher in the selection of number species of aquaculture importance. However, most of the genomes are not well assembled and annotated

(Yang et al. 2022). Function of many genes is still unknown. The successful genome editing depends on the ability to target proper gene. In general, any DNA fragment of 20–25 bp sequence having a PAM motif in either of the strands in downstream can be selected for CRISPR-based editing. Characterisation and development of Cas9 from different organisms with different PAM sites are giving the flexibility in the selection of target gene (Lino et al. 2018). Besides that, Cas9 is genetically engineered for altered PAM sequence providing greater scope in selection of target site. However, requirement of PAM site in the downstream of target DNA limits the full-scale application of CRISPR/Cas9 system (Hartenian and Doench 2015). Availability of PAM site in nontargeted location in the DNA may cause off-target cleavage by Cas9. In aquaculture, the genes related to the traits like growth and muscle development, pigmentation, sterility, disease resistance, fecundity, breeding, immunity, tolerance of toxicity, fatty acid metabolism, etc. so far drew the attention of the researcher (Blix et al. 2021).

2.3.2 Designing of gRNA

The crRNA that guides the Cas9 protein to a specified DNA segment in the native CRISPR/Cas9 system remains in an RNA-RNA hybrid with tracr-RNA, which acts as a scaffold linking crRNA to Cas9 (Roy et al. 2022). This crRNA-tracrRNA complex is programmed in genome editing systems to one-guide RNA (gRNA) or single-guide RNA (sgRNA), which is sufficient to control Cas9-mediated recognition and cleavage of target DNA.

Designing of the optimal guide RNA is the major challenge faced by the researcher in practical field application. An off-target match with 5 bp mismatch could still anneal the gRNA and is recognised by Cas9 (Elaswad et al. 2018; Blix et al. 2021). Specificity of the technique depends on the PAM motif and sequence of gRNA (Elaswad et al. 2018; Blix et al. 2021). The optimal design of sgRNA ensures the increase on target activity and reduces off-target cleavage. In principle, it is easier to design sgRNA simply by finding PAM site (5′-NGG-3′) and identifying 20 bp upstream (Luo et al. 2022). But in practical, the presence of PAM motif in non-target region results in off-target cleavage. Thus only few sgRNA actually find its course towards cleavage at target site. Very high or low GC content in the sgRNA affects the effectiveness (Luo et al. 2022). Targeting non-transcribed strands were found more effective than targeting transcribed strands. sgRNAs targeting the last coding exons are found to be less effective than exons located earlier in the genome (Luo et al. 2022). Single bp mismatch up to 11 bp upstream of PAM completely abolishes the cleavage by Cas9. All of these features are taken into consideration while designing sgRNAs. There are online tools available for designing the sgRNA, and these tools are also able to analyse the possible outcome. Some of these tools are ZiFiT, CRISPRScan, CRISPOR and CHOPCHOP. These tools accept input data in terms of FASTA sequence, gene name, gene ID or genomic coordinates. Using these tools for designing sgRNA reduces large-scale off-target cleavage and increases

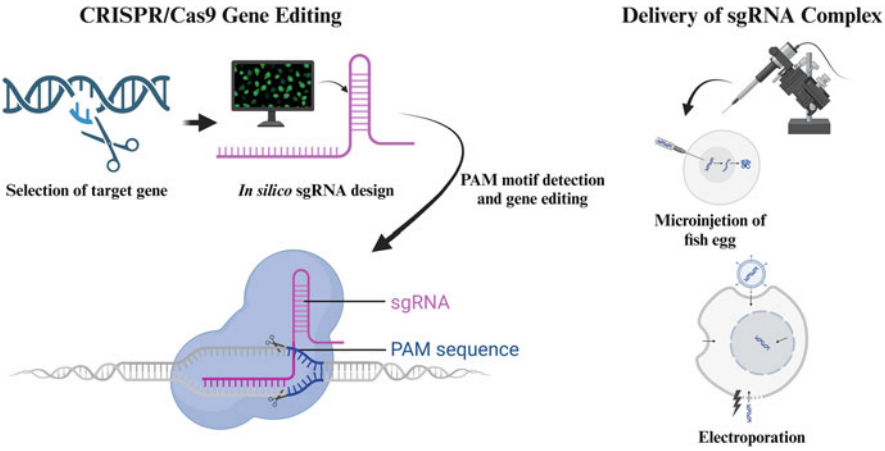

Fig. 2.1 The CRISPR/Cas9 gene editing mechanism in fish and gene delivery system through microinjection and electroporation

specificity. Such platform also analyses the off-target possibility of the sg-RNA in vivo. After designing, the sgRNA is oligo-synthesised.

2.3.3 Delivery of sgRNA Complex

There are two types of CRISPR/Cas9 component delivery, namely, cargo and vehicle deliveries (Lino et al. 2018). Three approaches are commonly used regarding CRISPR/Cas9 cargoes: (1) plasmid DNA encoding both gRNA and Cas9, (2) mRNA for translation of Cas9 alongside guide RNA and (3) guide RNA with Cas9 in ribonucleoprotein complex. These three cargos will be packed and delivered depending on the delivery system and its usability in the in vivo and/or in vitro system.

The gene editing system is delivered using three alternative methods: physical delivery, delivery using viral vectors and delivery using non-viral vectors. Microinjection and electroporation are the most often employed physical delivery systems. In aquaculture, microinjection in the one-cell stage of egg is the preferred method used for delivery of CRISPR/Cas9 complex in vivo. Other physical delivery methods include hydrodynamic delivery.

The delivery using viral vectors is suitable for genome editing study in vitro (Roy et al. 2022). Adeno-associated virus (AAV), full-sized adenovirus and lentivirus vehicles are among the viral vectors. Non-viral delivery methods are less well known than viral vectors or physical delivery methods. But they possess advantages being the efficient virus-free delivery method. The delivery methods and their common features are briefed in Table 2.1. The CRISPR/Cas9 gene editing mechanism along with the delivery of sgRNA complex in fish has been illustrated in Fig. 2.1.

Table 2.1 Different delivery vehicles and their composition, capacity, form of cargo and advantages and limitations (Lino et al. 2018)

Delivery vehicles	Composition	Capacity	Common form of cargo	Advantage/limitation	Reference
Microinjection	Needle	nM levels of Cas9 and sgRNA	DNA plasmid, mRNA (Cas9+sgRNA) and protein (RNP)	Guaranteed delivery into cell of interest, time-consuming, difficult and generally in vitro only	Yang et al. (2022), Horii et al. (2014), Chuang et al. (2017), Nagasawa et al. (2019), Crispo et al. (2015), Raveux et al. (2017), Sato et al. (2015), Niu et al. (2017)
Electroporation	Electric current	nM levels of Cas9 and sgRNA	DNA plasmid, mRNA (Cas9+sgRNA)	Delivery to cell population, well-known technique, generally in vitro only and some cells not amenable	Hashimoto et al. (2016), Chen et al. (2018), Qin et al. (2015), Matano et al. (2015), Paquet et al. (2016), Ousterout et al. (2015)
Hydrodynamic delivery	High-pressure injection	nM levels of Cas9 and sgRNA	DNA plasmid, mRNA (Cas9+sgRNA) and protein (RNP)	Virus-free, low cost, ease of use, nonspecific and traumatic to tissues	Guan et al. (2016), Xue et al. (2014), Zhen et al. (2015)
Adenovirus-associated virus	Non-enveloped ssDNA	<5 kb nucleic acid	DNA plasmid	Minimal immunogenicity And low capacity	Long et al. (2016), Carroll et al. (2016), Platt et al. (2014), Hung et al. (2016), Swiech et al. (2015)
Adenovirus	Non-enveloped ds DNA	8 kb nucleic acid	DNA plasmid	High-efficiency delivery, inflammatory response and difficult scaled production	Voets et al. (2017), Maddalo et al. (2014)
Lentivirus	Enveloped RNA	10 kb and up to 18 kb nucleic acid	DNA plasmid	Persistent gene transfer, prone to gene rearrangement and transgene silencing	Kabadi et al. (2014), Roehm et al. (2016), Zhang et al. (2016) and Platt et al. (2014)

(continued)

Table 2.1 (continued)

Delivery vehicles	Composition	Capacity	Common form of cargo	Advantage/limitation	Reference
Lipid nanoparticles/ liposomes/ lipoplexes	Natural or synthetic lipid or polymers	nM levels of Cas9 and sgRNA	mRNA (Cas9+sgRNA) and protein (RNP)	Virus-free, simple manipulation, low cost, endosomal degradation of cargo and specific cell tropism	Yin et al. (2016), Wang et al. (2016), Miller et al. (2017), Ebina et al. (2013) and Li et al. (2015)
Gold nanoparticles	Cationic arginine-coated AuNP	nM levels of Cas9 and sgRNA	Protein (RNP)	Inert, membrane-fusion-like delivery and nonspecific inflammatory response	Mout et al. (2017) and Lee et al. (2017)

For induced desirable heritable modification in the organism, the delivery of CRISPR/Cas9 complex must be done in the very early stage ideally at one-cell stage so that it can be incorporated in the germ line.

2.3.4 Production and Maintenance of Homozygous Mutant

Sequencing analysis is used to screen mutants. Individuals who are not on the target list should be assessed and separated. Mutants in the F0 generation have varied mosaic patterns of mutation, which may affect the further differentiation of related traits. The F0 individuals are crossed with wild population to generate heterozygous F1 population. The F1 population is crossed to produce homozygous mutant F2 progeny. The third stage is selection and application, which includes selecting phenotypes with CRISPR-induced mutations and establishing new kinds with increased aquaculture values.

2.4 Application of CRISPR/Cas9 in Fisheries and Aquaculture

Most aquaculture species have a shorter generation time, external fertilisation of eggs, embryonic and larval development outside the mother and a well-established method of artificial spawning and larval rearing for many cultured species, which has advantages for genome editing research and applications over farmed terrestrial animals. Most of the aquaculture applications is now limited to improve economically important traits like traits for growth, disease resistance, fatty acid metabolism, sterility, pigmentation, etc. However, the application of CRISPR/Cas system is not limited to improvement of traits. In aquaculture, it has a great potential in functional characterisation of genes, understanding signalling pathway, diagnosis of diseases, etc.; in vivo and in vitro studies have been conducted in over 20 species of aquaculture, importantly starting from zebra fish, Japanese medaka, Nile tilapia, Atlantic salmon, red sea bream, puffer fish, channel cat fish, etc. in teleost; giant freshwater prawn, ridge tail white prawn, *Neocaridina heteropoda* and *Eriocheir sinensis* in crustaceans; and *Crepidula fornicate* in Mollusca.

2.4.1 Improvement of Aquaculture Important Traits

2.4.1.1 Somatic Growth and Production

Most important traits of farmed fishes which are widely studied are growth-related genes. Most of the previous attempts were dependent on transfer of growth hormone genes from different species with inducible promoters. AquAdvantage growth hormone gene of Atlantic salmon was replaced with growth hormone gene from Pacific Chinook salmon. In an alternative approach, targeted silencing of genes that negatively regulates somatic growth has shown encouraging result. The protein suppressor of cytokine signalling 1a is a member of suppressor family that

negatively regulates Jak-Stat pathway. Targeted knockout of *SOCS1a* in zebra fish showed improved growth performance in heterozygous *SOCS1a* mutant (Dai et al. 2015). Another gene which negatively regulates the growth is myostatin (*mstnb*) gene. Myocytes release myostatin that inhibits muscle growth. Knockdown of myostatin gene shows either of the two of the distinct phenotypes, that is, either accelerated muscle growth or hyper-muscular phenotypes.

Multiple studies have been made using CRISPR/Cas9 genome editing for growth enhancement by targeting mstn gene in many fishes like common carp, red sea bream, olive flounder, mud loach, channel cat fish, etc. (Zhong et al. 2016; Khalil et al. 2017; Yang et al. 2022). In 2018, Japanese researcher reported CRISPR technique for the development of a line of red sea bream by knocking out mstn gene which resulted in 16% increase in the skeletal muscle (Kishimoto et al. 2018). Two years later, Chinese scientists reported a strain of mtsn^{-} yellow catfish (*Pelteobagrus fulvidraco*), which shows 37% more muscle mass compared to its counterpart post 210 days of fertilisation. PI3K/AKT/mTOR signalling pathway has a very important role in vertebrates' metabolism and homeostasis. Enhancing PI3K/AKT/mTOR pathway by targeting the regulatory subunit (*P85α*) of PI3K resulted in improved feed conversion efficiency and somatic growth in Gibel carp.

Apart from the studies related to targeted knockout or knockin genes that directly regulate the growth, efforts are being made to target genes that indirectly regulate the growth. Laptene receptor gene controls appetite in fish. Targeted silencing of laptene receptor gene leads fish to eat more, thus gaining more weight. Japan recently legalised the sale of two CRISPR-edited fishes, including the tiger puffer fish and red sea bream, which grow quicker than their counterparts (Hallerman 2021).

Several fishes show sexual dimorphism in which one sex grows faster and larger in size. Generally, in carps, female grows larger than male which is reverse in case of tilapia and channel catfish. Strategies were developed for mono-sex fish production by using either hormonal therapy or strategic breeding techniques. Genome editing using CRISPR/Cas9 system can become a useful tool in controlling the sex of the farmed species. Though the fishes exhibit different types of sex determination system, they share almost similar molecular pathway in terms of primordial germ cell formation, proliferation and steroid synthesis. Knocking out of genes responsible for steroidogenesis in tilapia and zebra fish led to sex reversal from female to male. For example, ovarian aromatase gene *cyp19a1a* when silenced using genome editing resulted in all male offspring due to failed ovarian differentiation (Lau et al. 2016). Sex-determining region in Y chromosome is necessary for male sex determination in mammals. In Japanese medaka, DMY (DM region in Y chromosome) was identified having characteristics of SRY region in mammals. Disruption of DMY using TALEN led to male-to-female conversion of XY male (Luo et al. 2015). A recent study has discovered a sex-determining role of a duplicated copy of anti-mullerian hormone (*amhy*) in Y chromosome of Nile tilapia. Knocking out of amhy gene in XY fish using CRISPR-based technology led to all male-to-female conversions (Li et al. 2015). In the future, with more understanding of growth genes and their associated factors and understanding of cross talk between different

genes contributing to somatic growth, researcher can design farmed fishes in a more efficient way using genome editing approaches.

2.4.2 Disease Resistance and Stress Tolerance

Aquaculture industry is continuously facing challenges due to outbreak of diseases. Inadequate response could result in huge mortality that leads to economic losses. The capacity to survive and resist against diseases is tightly linked with fish's immune system and immunoendocrine interactions. Genome editing approaches may be used for remodelling of such interaction to initiate more efficient immune response against disease and external stress.

Some researchers have generated disease-resistant models by targeting potential viral receptor on the cell membrane. BH-3-interacting domain death agonist (Bid) is responsible for virus-induced apoptosis in teleost fish. Bid-deficient grass carp and rare minnows created using CRISPR/Cas9 technology delayed the viral replication and increased survival time against grass carp reovirus (GCRV) (He et al. 2017). Knockout of junctional adhesion molecule – a gene in grass carp kidney cells – reveals that JAM-A is necessary for GCRV infection and JAM-A-deficient cell line shows resistance against the virus (Ma et al. 2018). In an alternate approach, foreign genes with antimicrobial properties also can be integrated in the fish genome to increase disease resistance. CRISPR/Cas9 HDR system was used to integrate exogenous alligator cathelicidin gene into targeted non-coding region of channel cat fish genome to acquire resistance against certain diseases (Simora et al. 2020).

Apart from targeting the traits to get disease resistance in fishes, several works also have been done to increase the stress tolerance in fishes. Knockdown of fih gene which is an inhibitor of hypoxia-inducible factor increases hypoxia tolerance (Cai et al. 2018). Traits like disease resistance and stress tolerance involve control by many genes; hence, to acquire a desired phenotype, multiple genes may be required to edit. Function of many of these genes is not yet known in case of fishes. CRISPR/Cas9 system being the cheap, efficient platform for genome editing is being used by the researcher to discover function of many of such genes in fishes by targeted knockout followed by studying the altered phenotypes. Du et al. (2015) had demonstrated the role of pVHL (Von Hippel-Lindau) gene in innate immunity in fishes which was previously unknown. Significant research will be required to determine the target genes and their version for genome editing in order to exploit the benefits of CRISPR/Cas9 system in acquiring disease resistance in fishes.

2.4.3 Sterility and Reproductive Confinement

Escape of fishes from aquaculture facilities to the wild is a threat to the native environment. Fishes are of nonnative and invasive nature, alter the ecological balance and impact the genomic diversity of the environment. Thus, it is better to breed sterile animals for aquaculture to prevent introgression with wild stock and to

minimise the poor productivity effects of early maturation (Roy et al. 2022). Effective and practical fish sterilisation programme is crucial to resolve such threats posed by escapee fishes. Manipulation of chromosome set by triplodisation or creating intra-specific hybrid is the most common method of creating sterile fishes in aquaculture system. However, it has been observed that few triploid fishes have also shown some degree of fertility. In another approach, transgenically expressed antisense RNA used for blocking the Gnrh expression failed to completely induce sterility.

Presently, CRISPR/Cas9 technology offers new sterile models in aquaculture by targeting genes responsible for ovulation and oocyte maturation and also by preventing formation of primordial germ cells. Dead end gene (dnd) is a gene responsible for the formation and migration of germ cell in zebra fish (Wargelius et al. 2016). Targeted knockout of dnd gene using CRISPR/Cas9 in Atlantic salmon resulted the formation of germ cell-free gonads in both sexes (Wargelius et al. 2016). Two hormones, FSH and LH, secreted by the pituitary gland are most studied reproductive hormones in teleost. These hormones are responsible for reproductive development and ovulation. Targeted mutation in two subunits of FHS and LH resulted in the delay in puberty and infertility in zebra fishes due to anovulation (Chu et al. 2015; Zhang et al. 2015b).

The problem with the knocking out of genes having reproductive importance is that such modification may not be transferred to F1 generation due to sterile nature of F0. Thus, alternate strategy is required in which a knockin system may be developed for introduction of certain genes in the genome of the organism with inducible "on-off" mechanism to induce sterility (Roy et al. 2022). Nevertheless, some prior knockin work with model fish species medaka and zebrafish suggests that restored fertility for breeding stocks might be possible (Nagasawa et al. 2019; Zhang et al. 2015a, b). This strategy has been developed by the scientist to recover germ cell in germ cell-free dnd mutants of Atlantic salmon. In this technique, the full-length dnd mRNA of wild-type variant of Atlantic salmon is co-injected with CRISPR construct containing gRNA for dnd in the one-cell stage embryo which resulted in the formation of germ cells. Sterlet (*Acipenser ruthenus*) has the shortest reproductive cycle and can be utilised as a surrogate for highly endangered and late-maturing sturgeon fish. The CRISPR/Cas9 system is used to knock out the dnd1 gene and prepare a sterile sterlet host for surrogate production of late-maturing and big sturgeon species (Chen et al. 2018).

2.4.4 Flesh Quality and Omega-3 Metabolism

Typically, the term "flesh quality" refers to a mix of factors including texture, firmness, juiciness, flavour, smell and nutritional content. Omega-3 polyunsaturated fatty acid (n-3 PUFA) content and the absence of intermuscular bones are two generally recognised indications of good quality flesh in all farmed fishes. The nutritional benefit of long-chain polyunsaturated omega-3 fatty acid (n-3 LC-PUFAs) particularly EPA (eicosapentaenoic acid, 20:5n-3) and DHA

(docosahexaenoic acid, 22:6n-3) for neural growth and well-being of general health is well documented. Human cannot synthesise LC-PUFAs; hence dietary supplement is the only solution to meet the requirement. Fishes have the ability to synthesise LC PUFAs with the help of certain enzymes such as elongase and desaturase produced in their body. Transgenic studies were conducted by introducing foreign desaturases into the genome of common carp and zebra fishes which greatly improved n-3 PUFA content in flesh. CRISPR/Cas9 based tool was used for editing fatty acid elongase 2 (*elovl2*) in Atlantic salmon which resulted in inhibition of fatty acid elongation which suggests a key role of this enzyme. This study also reveals the major role of sterol regulatory binding protein-1 (*strep-1*) in fatty acid biosynthesis (Roy et al. 2022). Such study will help in understanding the process of endogenous biosynthesis of fatty acid in fishes, and it will be interesting to see how genome editing tools like CRISPR/Cas9 system will be useful to enhance endogenous biosynthesis of unsaturated fatty acid using fishes' own elongases and desaturases.

Process of forming intermuscular bone and factor associated with it is still unclear in teleost. Signal transducer and activator of transcription (STAT) is responsible for the growth and development of vertebrates. TALEN used for knocking out *stat3* gene in zebra fish leads to malformation of bone and immune system disorder (Xiong et al. 2017). A similar study conducted by Niu et al. (2017) described the role of zinc finger transcription factor Sp7/osterix on bone formation in zebra fish. Thus, systematic study will be required to the molecular process of formation of intermuscular bone in fishes using genome editing tools, and CRISPR/Cas9 system may emerge as a key player in these studies.

2.4.5 Pigmentation

Pigmentation and colour patters are important traits under consideration for ornamental fishes. Size types of pigment cell are found in fishes, and different sets of pigment cells may be available in different species. Cells responsible for pigmentation are also responsible for colour pattern in some cases. With the recent introduction of genome editing tools like CRISPR/Cas9 approaches for examining these pigmentation genes, our understanding has grown, and additional colour mutant fishes have been created (Kimura et al. 2014). Several studies have been done using CRISPR/Cas9 tool to understand the function of genes responsible for pigmentation by targeted gene silencing and analysing the resulted phenotypes. The golden gene (*slc24a5*) is essential for pigmentation, and it is highly conserved across vertebrates (Lamason et al. 2005). Disruption of golden gene in zebra fish resulted in light-coloured eyes in mosaic larvae in founders (Jao et al. 2013). In Atlantic salmon, mutation in tyrosinase, a key enzyme in melanin synthesis and golden gene using CRISPR, leads to various degrees of pigment loss in F0 population (Edvardsen et al. 2014). CRISPR/Cas9 editing of 25 pigmentation genes involved in melanogenesis and pteridine metabolism and the carotenoid absorption and cleavage pathways in Nile tilapia (*Oreochromis niloticus*) produced phenotypes in both F0 and F2

generations. Four kinds of pigment cells have been found in wild-type tilapia, which, along with several naturally and artificially produced colour gene mutations, may be used to examine colour patterns in teleost fishes (Wang et al. 2021). Mutation in tyrosinase (*tyr*) gene in two important fish species loach (*Paramisgurnus dabryanus*) (Xu et al. 2019) and white crucian carp (*Carassius auratus cuvieri*) (Liu et al. 2019) shows varying degrees of pigmentation to albinism. HDL receptor/scavenger receptor B1 *Scarb1* gene responsible for metabolism of carotenoid and *Gch1* (GTP cyclohydrolase 1) gene have certain role in pteridine pathway. Disruption of these genes using CRISPR/Cas leads to whitening of red colour in ornamental common carp (Du et al. 2015). In another study, two Agouti signalling protein genes (*ASIP 1* and *ASIP 2*) were disrupted via CRISPR/Cas9 system and by which black patches disappeared in Oujiang colour common carp (Chen et al. 2018). Red tilapia is a hybrid of red inbred line of Nile tilapia, and other variant/species of tilapia have increasing demand in the market, but inconsistency and non-uniform coloration have become a problem for the aquaculturist. Some fishes show dark red patches in the body which fetch lower market price. Disruption of *slc45a2* (solute carrier family 45 member 2) gene which is responsible for melanin formation leads to production of albino variant with red eyes and coloured skin of Nile tilapia (Segev-Hadar et al. 2021). Studies conducted on pigmentation made researchers able to visually confirm the successful genome editing. Slc mutant fishes show some degree of loss of pigmentation in a wide variety of fishes; thus visual conformation of successful genome editing may be possible by targeting the slc family genes and observation of albino phenotypes in fishes.

2.4.6 Application in Disease Diagnosis

CRISPR/Cas-based system is successfully implemented in detecting several diseases including most recent human nCOV. Cas proteins involved in nucleic acid-based virus detection are lesser known siblings of Cas9 named as Cas12a and Cas13a. CRISPR-based effector enzymes like Cas9, Cas12b, Cas13a or Cas13b have high analytical sensitivity. Cas 12a and Cas13a have provided important programmable tool for double-stranded DNA and RNA targeting and detection, respectively. Both enzymes have characteristic of having a collateral cleave activity that allows these enzymes to indiscriminately cut nontargeted single-stranded DNA/RNA after binding with the targeted nucleic acid. Based on their activities on targeted nucleic acid, important diagnostic tools are developed named DETECTR and SHERLOCK involving Cas12a and Cas13a, respectively.

DETECTR (DNA endonuclease-targeted CRISPR Trans Reporter) has three players, a Cas12a enzyme, a gRNA for targeted binding and a single-stranded reporter molecule having fluorophore reporter and quencher molecule at both the ends of ssDNA. To increase the sensitivity, isothermal recombinase polymerase amplification (RPA) of target is combined with the detection. When the mixture of these compounds comes in contact of target DNA, the gRNA directs the Cas12a to the targeted DNA and led to collateral cleavage of reporter ssDNA molecule

resulting in separation of quencher and fluorophore to generate signal. This technique can detect DNA molecule at very low concentration as low as one DNA molecule per microliter.

On the other hand, SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) involves Cas13a-mediated indiscriminate cleavage of RNA molecule (Kellner et al. 2019). Application of SHERLOCK is not only limited to detection of RNA if coupled with a transcription step in RPA process for converting viral DNA to RNA, thus enabling detection of DNA molecule too. This technique also follows the same principle as DETECTR, where crRNA-activated Cas13a binding with the target RNA cleaved nonspecific labelled RNA to generate signal. Xiao et al. (2021) have demonstrated CRISPR-based detection of *Vibrio vulnificus* using recombinase-assisted amplification and CRISPR/Cas12a system. Detection of *Vibrio parahaemolyticus* was successfully done using PCR amplification followed by CRISPR/Cas12a-based detection (Jiang et al. 2022).

2.4.7 Application in Functional Characterisation of Genes

Genome editing tools can be used to functionally characterise genes and elucidate different regulatory pathways related to them by inducing mutation in DNA sequence. Kiss gene and receptor signalling pathway play an important role in mammalian vertebrates. It has been described that spermatogenesis, folliculogenesis and reproductive capability not impaired in both sexes when *kiss1* and *kiss2* along with its receptor were knocked out in zebra fish indicated that kiss gene and its receptor are not absolutely required for reproductive development in zebra fish (Tang et al. 2015). Such types of studies will help in understanding several physiological pathways in fishes and better understanding of function of genes and their regulatory pathways. Apart from that, CRISPR has the capacity to find out the basis of genetic regulation beyond the control of the DNA sequence of the coding gene, that is, at epigenetic level. The Cas9 involved in epigenome modification is nuclease-inactivated dCas9. The dCas9 lacks nuclease activity and fused with transcription activator or repressor domain known to have epigenetic effect. Specially designed gRNA and dCas9 fused with epi-effector molecules have the ability to achieve desired epigenetic modification such as methylation or de-methylation in the target region to achieve desired phenotypic changes. Several authors have used CRISPR tools to identify several virulent genes in bacteria and their strategy to evade immune system of the host. These studies will speed up the discovery of drugs and disease resistance crops to minimise the agricultural loss.

2.4.8 Other Aquaculture Important Applications

Internal fertilisation in crustaceans is a constraint for genome editing study. However, two new techniques, receptor-mediated ovary transduction of cargo (ReMOT control) and electroporation, have been demonstrated to introduce CRISPR

construct in embryo of crustacean species and demonstrated in cultured crustacean species like model crustaceans *Daphnia pulex* and decapod *Exopalaemon carinicauda* (Xu et al. 2020). Recently, microinjection system has been described by Li et al. (2022) in decapoda crustaceans *Neocaridina heteropoda* and *Eriocheir sinensis* for silencing Nh-scarlet gene responsible for eye development and pigmentation. Genome editing using CRISPR/Cas performed in widely cultured giant freshwater prawn *Macrobrachium rosenbergii* using microinjection showed 100% editing efficiency (Molcho et al. 2022). CRISPR-based gene knockin is performed in molluscan *Crepidula fornicate* by fusing mCherry to endogenous β-catenin to study β-catenin expression during embryonic development. Such fusion of reporter gene is also possible in fishes with the gene that gets expressed in the presence of particular pollutants to determine the presence of pollutants in the aquatic environment.

2.5 Challenges in Application of GE-Based Tools in Fisheries and Aquaculture

In spite of the usefulness of CRISPR-based tool of genome editing for assessing the gene function and improving important traits of aquaculture species, there are several technical challenges which needed to be addressed. Limited knowledge about genes and their associated function in fishes, off-target mutation, efficiency in delivery of cargo, lack of species-wise standardised technique and risk assessment of other ethical issues required to be addressed in order to get public acceptance and regulatory approval.

2.5.1 Lack of Robust Knowledge of Teleost Genomes

CRISPR or any other genome editing technologies required clear and robust knowledge about the genetic background and genomic sequence which is lacking in case of teleost fishes. With introduction of advanced sequencing genome technologies, now more than 70 fish species were decrypted, but this is still less considering huge number of aquaculture species (about 600). Sequencing has already been completed in considerable numbers of species, genes and genomic fragments are not well assembled, genes are poorly annotated and functions of many of the genes are not well known. There is lack of clear understanding of trait-related genes in fishes like in human and plants. For example, zebra fish *Gnrh3/gnrh3* gene which is believed to be hypophysiotropic when knocked out did not affect sexual maturation and gametogenesis in both the sexes (Spicer et al. 2016). But in a recent study, TALEN-induced disruption of *gnrh1* (hypophysiotropic form in medaka) gene resulted in female infertility in Japanese medaka due to anovulation (Takahashi et al. 2016). However, with the help of CRISPR-based genome editing, scientist is able to decipher function of more and more genes which are having economic importance. Improved knowledge of the genome sequences and trait-related genes will help in the designing of gRNAs specific to a single targeted region.

2.5.2 Non-availability of Suitable Cell Lines

For rapid selection of gRNA and optimum CRISPR construct, it is required to be tested in vivo cell culture. But there is a lack of well-characterised species-specific cell line for many fishes, creating major problem for standardisation of CRISPR-based technique. In addition to that, many crustacean and molluscan species do not have well-established cell line, thus restricting the use of CRISPR-based tool to finfishes only.

2.5.3 Development of Standardised Species-Specific Method

The CRISPR construct needs to be delivered into the fertilised eggs at one-cell stage. One-cell stage varies from species to species and time to time. Therefore, it is essential to know the exact timing of delivery of construct for a particular species which is a critical point in genome editing. Any delay in delivery of CRISPR construct may lead to mosaicism.

The most widely used method for delivery of CRISPR construct is microinjection. Though the microinjection is believed to be an efficient method in terms of successful delivery, the number of eggs to be covered in a particular duration of time depends on the expertise of research personnel. Even the most skilled person may deliver CRISPR construct approximately 100 pieces of eggs before one-cell stage passed which is not sufficient for breeding purposes. Egg membrane makes low microinjection success rate for oviparous fish, and in ovoviviparous fishes, there is no established gene editing platform at present. Besides that, egg shell of many species is hard; therefore, separate species-specific microinjection platform is required. Microinjection method is limited to the fish's exhibited external fertilization; it is difficult to access fertilised eggs at one-cell stage for aquaculture species like shrimp. Therefore, different delivery methods like electroporation and non-viral delivery system need to be tested for different types of fishes.

2.5.4 Effect of Whole Genome Duplication

Teleost-specific whole genome duplication (WGD) hinders the efficiency of CRISPR-based genome editing. Many fish species have undergone WGD; essentially salmon in which WGD has expanded salmon-specific fourth round (SS4R) resulted in an extra set of all genes. WGD changes the fate of duplicated genes in three broad categories as follows:

Neo-functionalisation: Duplicated genes acquire new function compared to the ancestral copy.

Sub-functionalisation: Duplicated genes retain some of the functions of original gene.

Non-functionalisation: Duplicated genes completely lost the function.

Due to several rounds of duplication events, teleost contains different sets of chromosomes. WGD is a governing aspect when editing teleost fishes. In case of paralogue genes, the function and sequence of paralogues may be determined, and which one to be targeted needs to be assessed. For fishes with different ploidy levels, the one with the lowest level should be used as model species.

2.5.5 Public Acceptance and Regulations

In spite of the potentiality of the CRISPR system in revolutionising the food sector, the public acceptance of genome edited fish remains a biggest challenge for the aquaculturist. The acceptance of GE products is key to full use of GE technology in food industries. The GE products differ from the transgenic GMOs in a way that the former does not introduce foreign gene into the genome of organism but rather make small changes in the DNA to improve the function of particular trait by removing inferior alleles (Yang et al. 2022). But there is a general debate on whether GE approaches should be considered differently from GMOs, even though there is a possible way to introduce foreign sequence in GE organism using HDR. Besides that, the development of CRISPR-based single-nucleotide modification is a very usual natural process in living organism and epigenome modification where targeted knockout/knockin is crucial in getting regulatory approval. GMO regulations across the countries are different and broadly divided in product- and process-based concept. The product- and process-based regulation is developed depending on how the GMOs are defined in respective guidelines. In product-based regulation, the definition of GMOs is based on final product such as novel combination of genetic material obtained through the use of modern biotechnology. In process-based regulation, GMOs are defined as the process by which genetic material has been altered in a way that is not possible in natural mating or recombination. Countries like the USA, Japan, Canada, Argentina and Republic of Korea have implemented product-based regulation contrary to the countries like India, China, Australia and EU which have implemented process-based regulation. If GMO regulations apply to GE organism, the progress of research and innovation in GE will be largely hampered. It is generally advocated that GE crops generating through NHEJ approach which does not involve foreign gene should not be regulated. The regulatory guidelines for the GE fishes have not been placed yet so far in many countries. Argentina is the first country to enact guidelines for new breeding techniques (NBT) that include genome editing. Other countries that have guidelines for GE crops include Ecuador, Nigeria, etc. It is quite evident that a longstanding debate will exist regarding formulation of GE regulatory guidelines and the GE regulation will differ across various countries. Some countries may not recognise the GE organism as GMOs, thus avoiding stringent biosafety screening. Various regulatory agencies throughout the world, including the EU, Argentina, Brazil, Australia, New Zealand, Canada, the USA and Norway, have begun conversations about how to govern goods derived from the new CRISPR editing technology (Eckerstorfer et al. 2019). The decision should be based on the scientific knowledge about the

benefits and risk associated with the product in sustainable aquaculture. It is the duty of aquaculture scientists to educate the consumer with scientific knowledge about the potential risk, profit, safety and sustainability of GE breeding technique in ensuring global food security and ensuring active participation of general public in the dialogues and decision-making process.

2.6 Commercialisation

Japan is the first country to have commercialised CRISPR genome edited fish in its list of cultivable fish which has already reached to the market for consumption. The red sea bream and tiger puffer fish were developed by the Kyoto based start-up Regional Fish Institute with Kyoto University and Kindai University. Red sea bream is produced using cage culture in Kyushu, and Seto Inland Sea fish is a very prized fish in Japan due to its taste. The myostatin knockout using CRISPR resulted in 1.2–1.6 times higher muscle yield and improved feed conversion efficiency by 14%. Tiger puffer fish which is popularly known as torafugu in Japan is edited by silencing four leptin receptor genes responsible for controlling appetite. This leads fishes to develop more appetite for feed there by gaining weight faster and become 1.9 times heavier than their counterpart. Genome edited red sea bream and tiger puffer fish given are named as *madai* and 22-seiki *fugu*, respectively, in Japan. Japan, where GMOs have to undergo biosafety screening, has exempted CRISPR-based genome edited organism as there was not any involvement of foreign gene, considering that such knockout/silencing of particular gene may occur naturally through mutation.

Another product developed by the AquaBounty Company is genetically edited Nile tilapia named FLT-01 which has been approved by regulatory authority of Argentina since it does not contain any foreign DNA-alike AquAdvantage transgenic salmon developed by same company.

2.7 Future Aspects and Conclusion

Genome editing has great potential in accelerating the process of aquaculture breeding. It is strongly believed that commercialisation of aquaculture product through genome editing will largely depend on CRISPR/Cas9-based genome editing platform. Genetic improvement is still in its infancy in aquaculture sector. With the rapid evolution of sequencing platforms and whole genome sequencing study of numerous fishes, the aquaculture breeding is entering into post genomic era. In the future, it will be possible to target multiple genes across the chromosome to improve complex traits. The continuous improvement of the CRISPR/Cas9 technique is attributed to the biological and technical advantage of fish species. Its application in aquaculture as a new breeding technology will revolutionise the sector by increasing the production and quality. Application of CRISPR is not only limited to targeting genes and getting desirable phenotypes; its application has extended to

identification of trait-related genes, functional annotation of the trait-related genes and signal pathways.

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Nanotechnological Applications in Aquatic Health Management

3

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Abstract

Aquatic resources around the world are in strenuous position due to ever-increasing anthropogenic activity and extreme climate change. Deterioration of water quality parameters by the presence of several abiotic and biotic stressors has threatened the aquatic health in terms of environmental degradation, loss of natural habitat and aquatic biodiversity, human health and livelihood. Even though the uses of numerous traditional remedies are in practice, the extreme nature of the present situation demands innovative approaches to improve the aquatic health conditions. In this context, nanotechnological interventions are showing tremendous potential to improve the situation in a sustainable way. Distinct groups of nanomaterials are being used to improve the basic water quality parameters and also to remediate some extremely persistent toxic elements like hazardous metal ions and organic pollutants. Similarly, the nanomaterials are also being applied as therapeutic agents against biotic stressors like pathogenic bacteria and parasites and also to improve the immunogenic parameters of the aquatic animals. This chapter focuses on some of the key interventions involving nanostructured materials to tame some important abiotic and biotic stressors to aqua life, thus improving the overall health of aquatic resources.

Keywords

Aquatic health · Nanostructured materials · Abiotic stressors · Biotic stressors

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3.1 Introduction

The ever-increasing demand for protein and the positive health effects by aquatic protein sources made aquaculture and fisheries as important food-producing sectors globally. In 2020, the total world's fisheries and aquaculture production touched around 214 million tons including 178 million tons of aquatic animals and 36 million tons of algae with an estimated total value of $460.2 billion (FAO 2022). Fisheries and aquaculture representing the leading sector with the fastest growth are one of key livelihood options generating jobs for approximately 58.5 million people around the world (FAO 2022) contributing immensely to attain projected sustainable development goals. With regard to economic significance and food security, aquaculture is regarded to be among the most significant food production systems, and the continued growth of this sector is a crucial component to safeguard global nutrient stability (Béné et al. 2016; Jennings et al. 2016). However, environmental deterioration, pollution by toxic elements, improper management practices, repeated disease occurrence, more use of drugs and improper nourishment of fish, are among the key problems that are hampering the sustainability of the sector (Bondad-Reantaso et al. 2005; Mishra et al. 2018; Subramaniam and Masron 2021). The maintenance of viable aquaculture requires a combined approach of analysing, assimilating and using new scientific and technological solutions. Nowadays, aquatic ecosystems are largely being affected by the pollutants such as heavy metals, pesticides and other harmful nutrients such as ammonia and phosphorus discharged from both point and non-point sources (Fan et al. 2021a, b; Łuczyńska et al. 2018; Mallin and Cahoon 2020). These pollutants both in higher and lower quantities kill or accumulate inside the fish and render toxic effect on the immune system's neurological activity and fish behaviour and physiology and also damage the vital organs like gills, kidney and liver (Heath 2018; Obasohan et al. 2010). Poor water quality in the ecosystem leads to disease condition such as fin rot/tail rot, papilloma, hyperplasia, liver damage, neoplasia and ulceration (Zeitoun and Mehana 2014). Few surveillance studies also noticed the occurrence of more diseased fishes in the polluted site when compared to non-polluted sites in aquatic ecosystem (Austin 1998).

Hence there is an immediate need for sustainable technologies that could efficiently reduce the toxic effect of pollutants to aqua life, efficiently deliver the drugs to diseased fishes, reduce antibiotic usage, manage the water quality and also act as a nutrient supplement. In this context, nanotechnological interventions provide solutions to deal with these ever-growing problems to boost production and also attain sustainability in this sector. Nanotechnology is defined as the branch of science that understands, creates and controls the properties of materials within nanoscale, a dimension less than 100 nm (Fajardo et al. 2022). Nanotechnology integrates various fields such as chemistry, physics, engineering and biotechnology and functions in multiple dimensions of structural elements (Chandra 2016). Nanoparticles are employed in a variety of sizes and shapes, including dendrimers (Chan et al. 2017), nanocapsules (Torchilin 2006), nanospheres (Donbrow 1991) and nanotubes (Reilly 2007), among others. Functionalised nanomaterials can be created through bottom-up approach (arranging atom by atom) or by top-down

approach (fragmenting large materials to nanoscale) utilising physical, chemical, optical, magnetic and electrical processes (Roy et al. 2012). The most common types of nanomaterials are nano-metal, metal oxide, nanotubes and nanospheres, quantum dots, nanoceramics and nanoshells, etc. (Stone et al. 2010). Potentially an infinite number of elements can be used to synthesise a vast series of nanomaterials with unique size characteristics laden with varieties of chemical, physical, optical and other properties. Not only size, nanomaterials having unique three-dimensional shape incorporating many unique chemical properties are also currently being synthesised (Dasgupta et al. 2014; Zheng et al. 2012). Nanotechnology has immense potential to bring revolutionary enhancements to fisheries and aquaculture systems to lower costs, boost efficiency and lessen our impact on the environment (Luis et al. 2019; Shah and Mraz 2020). According to a forecast, the global market for food-related applications of nanotechnology would rise at an average pace of more than 24% from 2019 to 2023, reaching $112.48 billion (Fajardo et al. 2022). Sustainable and targeted development of nanotechnology with environmentally friendly, non-hazardous, natural approach will help in achieving comprehensive development of aquaculture and fishery industry resulting in common and inclusive benefits. This chapter discusses some key nanotechnological interventions to remediate or reduce important abiotic and biotic stressors hampering aquaculture and fishery production systems.

3.2 Nanomaterials Against Abiotic Stressors

Recently, nanotechnological interventions are being reported extensively for the remediation of pollution from aquatic bodies. Various kinds of nanostructured materials containing carbon, alumina, zeolite, iron, etc. are being reported to improve the water quality parameters by removing ammonia, nitrate, nitrites, heavy metals and other pollutants.

3.2.1 Phosphate and Ammonia

For example, iron oxide nanoparticles distributed on zeolite (EL-MNP@zeolite) were reported to simultaneously eliminate toxic ammonia (NH_4^+) and phosphate (PO_4^{3-}) concentration from aqueous solutions (Xu et al. 2020). An initial concentration of 10 mg L^{-1} EL-MNP@zeolite could be able to eliminate 43.3% of NH_4^+ and 99.8% of PO_4^{3-} (Fig. 3.1). The maximum adsorption capacities for NH_4^+ and PO_4^{3-} under optimal circumstances achieved were 3.47 and 38.91 mg g^{-1}, respectively. Similarly, in another study, a complex (MHHPC) of magnesium hydroxide (MgOH) and hydrogen peroxide (H_2O_2) (Mg to H_2O_2 ratio of 2:1) was created with oxygen-releasing properties (Li et al. 2020). MHHPC's oxygen-releasing, nutrient-removal abilities and pH-adjusting properties were assessed in pure and eutrophic water and show that they were able to continuously release oxygen during one-week period and simultaneously remove PO_4^{3-} and NH_4^+ (below 0.5 mM) from the

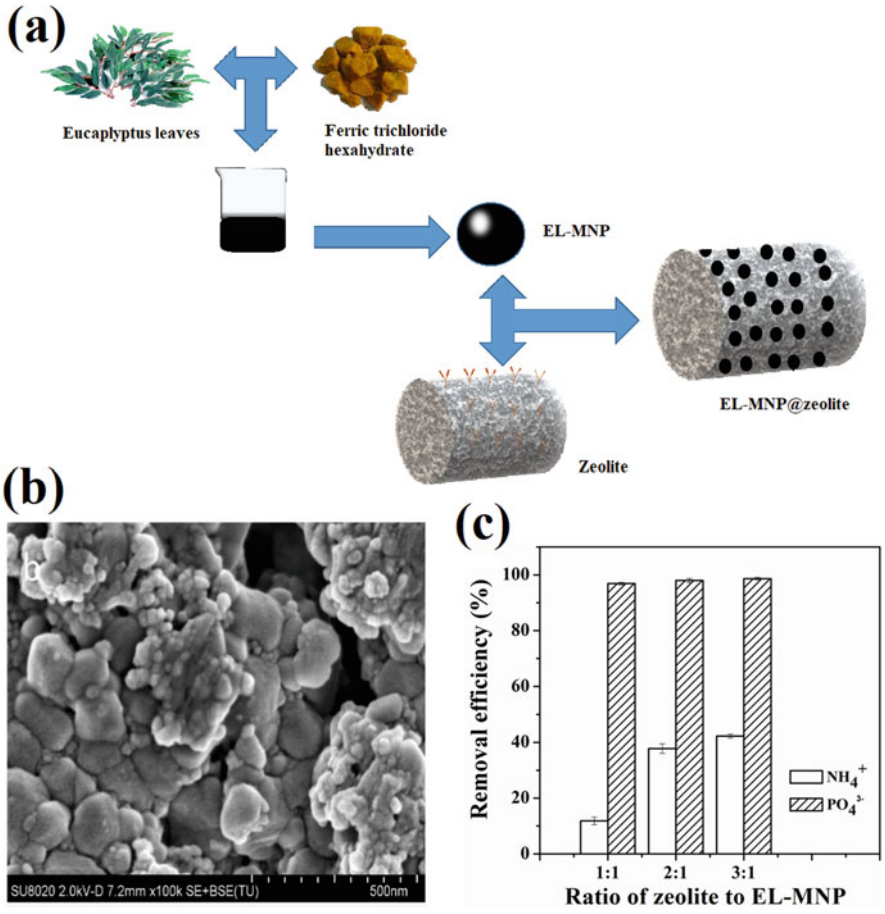

Fig. 3.1 Synthesised of EL-MNP@zeolite (**a**), SE images of EL-MNP@zeolite (**b**) and NH_4^+ and PO_4^{3-} removal efficiency by EL-MNP@zeolite (Xu et al. 2020)

aqueous phase. In another work, nitrate (NO_3^-) was removed electrochemically by nZVI (nano-zerovalent iron) anode magnetically immobilised on RuO_2–IrO_2/Ti plate with NH_4^+-oxidising properties (Hong et al. 2022). Generally, the limiting factor for removal of NO_3^- using nZVI through cathodic reduction involves generation of ammonia (NH_3) as the principal reduction intermediary. However, in this study, NO_3^- reduction with concurrent electrochemical oxidation of intermediate NH_4^+ improved the NO_3^- removal as N_2. The process with high NO_3^- removal efficiency (90.2%) and N_2 selectivity (70.6%) involves few major steps, first interaction of nZVI on the anode with NO_3^- due to the applied electric field followed by reduction of NO_3^- by surface-adsorbed H* produced during water electrolysis process and then RuO_2–IrO_2/Ti plate-mediated NH_3 oxidation under high voltage (Fig. 3.2). Similarly, to remove phytate-PO_4^{3-} from eutrophic water bodies, a

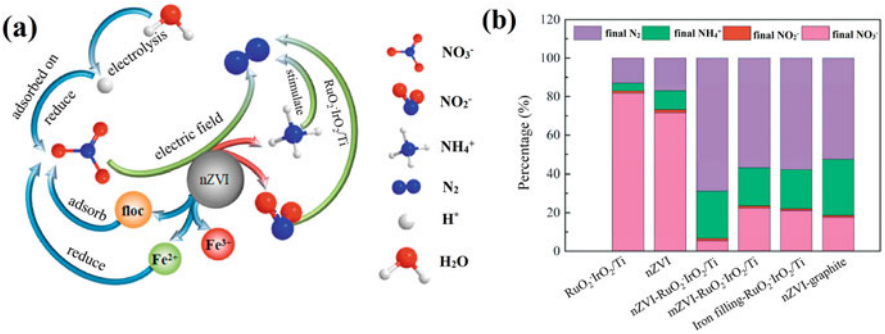

Fig. 3.2 Schematic illustration of electrochemical nitrate removal (**a**) and effect of anode materials on the percent concentrations of NO_3^-, NO_2^- and NH_4^+ ions in solution (**b**) (Hong et al. 2022)

metal-organic framework fabricated with phytase in highly ordered silica and nano-Fe_3O_4 was employed (Dave and Modi 2019). The phytate-PO_4^{3-} was catalysed by phytase and the released phosphate is adsorbed by the Fe_3O_4 nanoparticles. The Fe_3O_4 and phytase-loaded silica microcosm could be able to remove 60–80% of phytate-PO_4^{3-} and reduced the growth of photoautotrophs by 50%. Zirconium oxide (ZrO_2) nanoparticles (nZrO_2, 10.91 nm) immobilised in aluminium alginate beads (nZrO_2-Al-alig) were used as adsorbents for the removal of PO_4^{3-} from water (Biftu and Ravindhranath 2020). Under optimum condition, the phosphate adsorption capacities of nZrO_2 and nZrO_2-Al-alig were 126.2 mg/g and 173.0 mg/g, respectively.

3.2.2 Heavy Metals

Not only nutrients, heavy metals and organic pollutants were also reported to be removed by the nanomaterials. For example, humic acid modified nano-hydroxyapatite was able to remove 97.68% Cu and 100% methylene blue from water under optimum conditions (Wei et al. 2020). Even remediation of uranium (VI) from aqueous environment was achieved using cellulose-based adsorbents impregnated with nano-Fe_2O_3 (Rule et al. 2014). The nano-iron-impregnated cellulosic adsorbent had 100% adsorption efficiency at pH 7 when impregnated with 6 wt % Fe_2O_3 within 150 min. Nano-zerovalent iron was also reported to have the capacity to remediate toxic hexavalent Cr(VI) from industrial waste water to Cr (III) (Fig. 3.3) (Du et al. 2022). It was reported that the release of Fe^0 from the nZVI causes reduction of Cr(VI) to $Cr(OH)_3$ which was also found to be the dominant species on the surface of nZVI prior to transforming to Cr_2O_3. The synthesised nZVI had high Cr(VI) removal capacity (100 mg g^{-1}) and within 180 min 51.9 % Cr (VI) was transformed to Cr(III) and the rest as particulate Cr species. However, its removal mechanism was found to be influenced positively and negatively by the

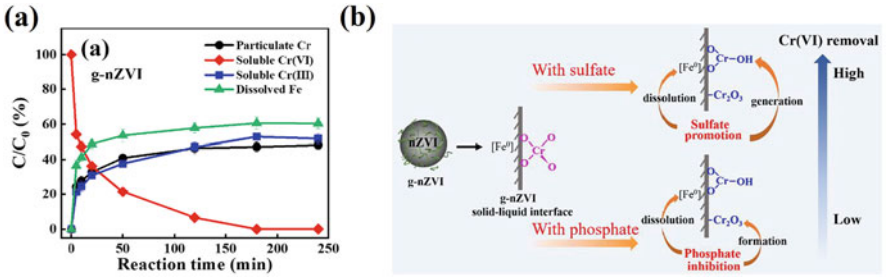

Fig. 3.3 Variations in Cr and dissolved Fe(III) of g-nZVI (0.5 g L^{-1}) as a function of reaction time at initial Cr(VI) concentration of 50 mg L^{-1} (**a**) and schematic illustration of Cr(VI) adsorption and reduction on the interface of g-nZVI (**b**) (Du et al. 2022)

presence of sulphate and phosphate ions. The sulphate ion was reported to trigger the release of Fe^0 from nZVI core facilitating reduction of Cr(VI) to Cr(III), and phosphate ion inhibits the same due to complexation. SiO_2-coated nano-zerovalent iron (SiO_2-nZVI) was employed to degrade p-arsanilic acid, a typical organoarsenic compound, and simultaneously remove the released arsenic (Lv et al. 2020). In the presence of SiO_2-nZVI, p-arsanilic acid (10 ppm) completely oxidised to As(V), NH_4^+ and phenolic compounds. The formation of lepidocrocite and magnetite on the surface of SiO_2-nZVI played a vital role in adsorbing the released As(VI) and thus helped in reducing the As level below 0.04 mg L^{-1}. In another study, nitrogen-doped carbon foam loaded with nZVI (nZVI@NCF) was used for simultaneous removal of Cd(II) and naphthalene from water (Li et al. 2022). Under optimum conditions, the adsorption capacity of nZVI@NCF for Cd(II) in water was 13.9 mg g^{-1}, and the degradation rate for naphthalene (10 ppm) reached almost 100%.

Not only n-ZVI, zerovalent nickel nanoparticles (nZVN) were also reported for removal of Cr(VI) (Camps et al. 2020). The nZVN (100–200 nm) was prepared by simple grinding reduction method and has 96% removal efficiency at 10 ppm concentration. Nano-zerovalent copper (n-ZVC) was also reported to remove Cr (VI) in combination with pistachio shell powder (Kumar et al. 2021). The combination system at the dose of 0.1 g/L exhibited Cr(VI) adsorption capacity of 110.9 mg/g. A composite colloid consisting of nano-ferrous sulphide (n-FeS) and polyacrylic acid salt was used to remove Cr(VI) from contaminated water (Yao et al. 2020). The composite colloid had Cr(VI) maximum removal amount of 432.79 mg/g as compared to 218.29 mg/g in case of only n-FeS. Nanoribbons prepared from aramid amphiphiles with chelating head groups, namely, dodecane tetraacetic acid or tetraxetan and diethylenetriaminepentaacetic acid or pentetic acid, were employed for removal of lead (Pb) from contaminated water (Christoff-Tempesta and Ortony 2021). Under optimum condition, the synthesised nanoribbon had adsorption capacity of 72 mg/g.

3.2.3 Other Pollutants

A biofilm membrane bioreactor (BF-MBR) using composite of nano-attapulgite clay and hydrophilic urethane foams (AT/HUFs) was employed as a biofilm support to treat petroleum refinery wastewater (PRW) (Jiang et al. 2019). For a hydraulic retention duration of 5 h, the COD (500 mg/L), NH_4^+ (15 mg/L) and turbidity (180 NTU) removal efficiency using BF-MBR were 99.73%, 97.48% and 99.99%, respectively, which were 23%, 20% and 6% higher than in the control bioreactor. Hydrophobic nanosponge prepared by modifying melamine formaldehyde sponge modified by silylation of amino silicon oil (ASO) and aminopropyltriethoxysilane (APTES) was used for removal of diesel fuel from water (Li et al. 2019). The diesel fuel adsorption capacity of hydrophobic nanosponge was 88 g/g, and it helped in better survival of fishes by removing diesel fuel-contaminated water. Nano-porous sponge material, derived from thermal cross-linking among 2,2,6,6-tetramethylpiperidin-1-yl)oxyl-oxidised cellulose nanofibers, branched polyethylenimine and citric acid, was used for removal of organic dyes from water (Riva et al. 2020). The dye sorption capacities of this nanosponge were 22.58, 23.85, 88.96 and 121.53 mg/g for Naphthol Blue Black, Orange II Sodium Salt, Brilliant Blue R and Cibacron Brilliant Yellow, respectively.

To remove harmful algal growth from eutrophic water bodies, nano-Cu_2O/SiO_2 was suggested which can kill harmful cyanobacteria like *Microcystis aeruginosa* through reduction of chlorophyll a, protein and polysaccharide content (Fan et al. 2021a, b). At the same time, the application of nano-Cu_2O/SiO_2 at the rate of 10 mg/L did not hamper probiotic algae growth, namely, *Cyclotella* sp. (Fig. 3.4). Mesoporous nano-MgO coupled with microfiltration membrane separation was used to remove excess natural organic matter from water with an adsorption capacity of 446 mgC/g-MgO (Zhou et al. 2020). The Mg^{2+} ion release from MgO contributes to

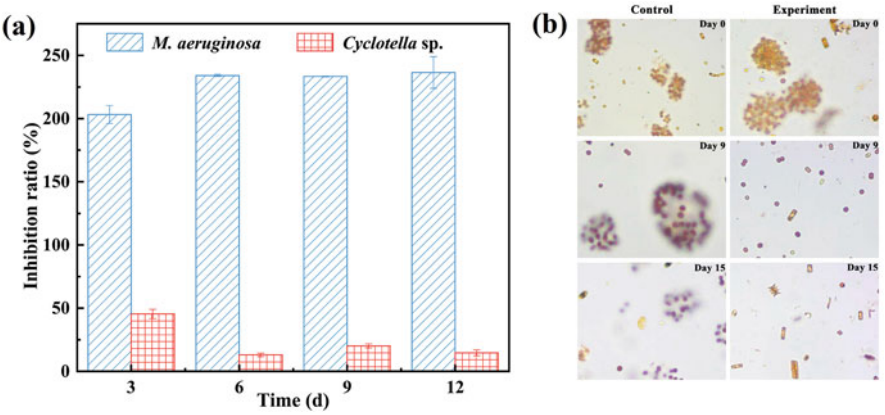

Fig. 3.4 Inhibition ratios between *M. aeruginosa* and *Cyclotella* sp. by nano-Cu_2O/SiO_2 (**a**); algal community structure due to nano-Cu_2O/SiO_2 application (**b**) (Fan et al. 2021a)

92% removal of organic carbon via coagulation while the rest 8% was removed by adsorption.

Amine-functionalised graphene oxide nanocarrier was covalently attached to PersiManXyn1 for effective removal of dyes from water (Ariaeenejad et al. 2021). The immobilisation of enzyme in the nanocarrier has maximised its activity and improves its stability during storage. The immobilised PersiManXyn1 enzyme could catalytically reduce the methylene blue solutions within 150 s with superior reusability (94% dye removal after 15th cycle). Nano-olive stones were used for removal of methylene blue from water, and at pH 10, highest (71 %) methylene blue removal efficiency was observed (Al-Ghouti and Dib 2020). Biochar-loaded nano-iron/nickel was reported to remove 2,4,6-trichlorophenol from water with degradation rate of 39.7–71.6% (Liu et al. 2019). Very recently, it was discussed that microplastics from the wastewater can be removed efficiently with augmentation of nanomaterial-enabled strategies in the wastewater treatment plants (Goh et al. 2022).

3.3 Nanomaterials Against Biotic Stressors

The deterioration of water quality led to the emergence of pathogens that are extremely harmful to the aquatic animals. Disease outbreak is considered as the most significant barrier to fisheries and aquaculture sustainability and its development (Mishra et al. 2018). Nanotechnology has enormous potential in this context by providing new perspectives on drug delivery and disease diagnosis. In the case of drug delivery, some approaches involving nanotechnology include coating of labile or environmentally sensitive drug molecules with synthetic or biopolymers to protect the drug (Fam et al. 2020). These kinds of approaches have the benefit to enhance the therapeutic efficiency by controlling the drug release and targeted tissue release and enhancing the bioavailability through high absorption rate (Kadam et al. 2012).

3.3.1 Nanovaccines

There have been extensive studies on the development of nano-delivery of vaccines for aquaculture applications. For example, mucoadhesive chitosan-complexed nanovaccine was developed for the control of highly contagious bacterial disease *Columnaris*, caused by *Flavobacterium columnare*, in the farmed tilapia (Kitiyodom et al. 2021). The study showed that immersion of fish in chitosan-complexed nanovaccine followed by experimental infection on the 30th day resulted to 78% relative percentage survival as compared to 89% mortality in control fish (Fig. 3.5). Similarly, a mucoadhesive nanoencapsulated vaccine (EncapFlavoNP^{++}) was developed using cationic lipid-based nanoparticles (polysorbate 80 and cetyltrimethylammonium bromide) combined with antigen obtained from *Flavobacterium oreochromis* (Bunnoy et al. 2022). The use of EncapFlavoNP^{++} at dilution of 1:100 and 1:200 improved the immunity by enhancing the antibody level specific to *Flavobacterium Oreochromis* in *Lates calcarifer* (Asian sea bass).

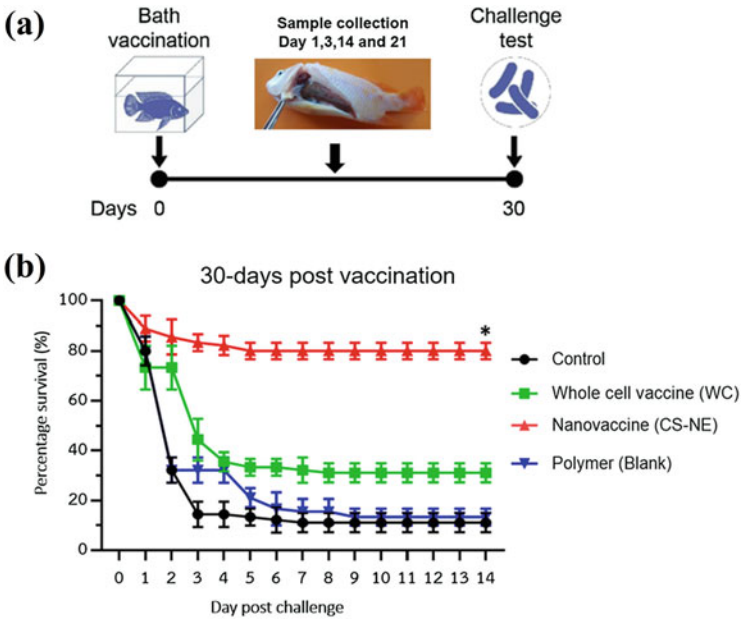

Fig. 3.5 Vaccination schedule of mucoadhesive chitosan-complexed nanovaccine in red tilapia (*Oreochromis* sp.) (**a**) and percentage survival after immersion challenge with 1×10^6 CFU/mL *Flavobacterium columnare* F–K17/1 strain (**b**) (Kitiyodom et al. 2021)

Upregulation of immune-related genes such as MHC-IIα, IgM and DCs in the gills, skin, liver, peripheral blood lymphocytes (PBLs), head kidneys and spleen was observed as compared to the control. Moreover, nanoDNA vaccine encoding the glyceraldehyde-3-phosphate dehydrogenase gene of *Edwardsiella tarda* was prepared by conjugation with poly(lactic-co-glycolic acid)-chitosan polymer (Leya et al. 2021). This nanovaccinated *Labeo rohita* after challenging with *Edwardsiella tarda* exhibited an increase in total serum protein, globulin concentration and less mortality as compared to control. In addition, immunoglobulin (IgM), which is essential for defence mechanisms, was found at a higher level in serum and mucosal tissues (skin, gill and gut) in the vaccinated group as compared to the control. Not only polymers, inorganic nanocarriers like modified halloysite nanotube were also reported for developing a vaccine against streptococcosis disease in tilapia (Pumchan et al. 2022). Halloysite nanotubes were modified with chitosan, APTES and their combination and loaded with inactivated *Streptococcus agalactiae* (Fig. 3.6). Among these nanocarriers, halloysite nanotubes modified with chitosan could properly release loaded antigen and could protect the fishes from streptococcosis disease with relative percentage survival of 75.00 ± 10.83%.

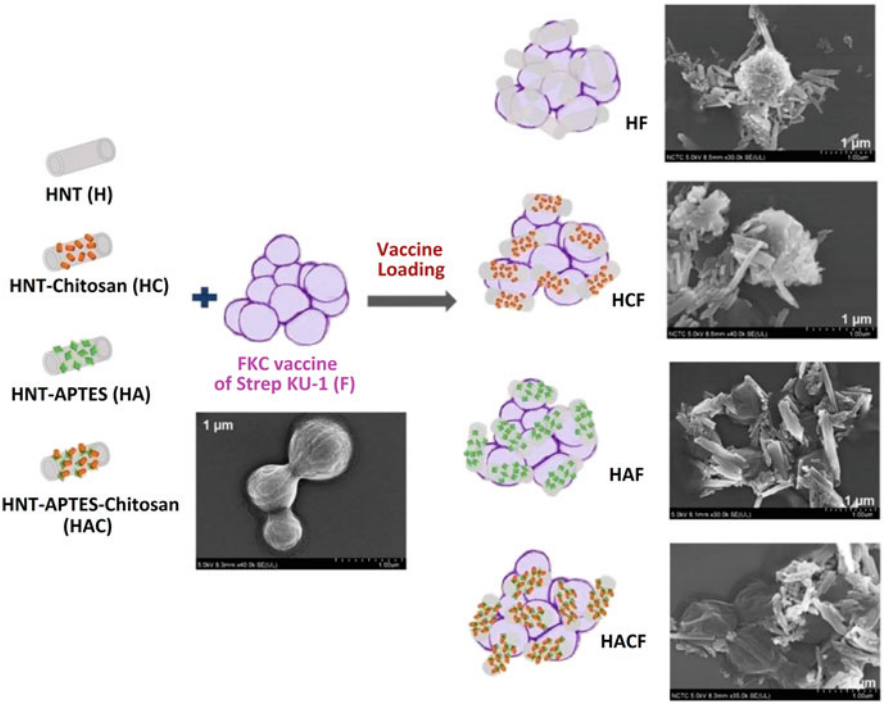

Fig. 3.6 The schematic diagram proposing the surface binding characteristics of HNT moiety on the Strep KU-1 surface (Pumchan et al. 2022)

3.3.2 Nanoantibiotics

Though there are many studies on vaccine development for fishes, very few studies exist on developing nanoantibiotics to break antimicrobial resistance in fish pathogenic bacteria. In one such study, Tetracycline-loaded calcium phosphate nanoparticles (8 ± 5 nm) were prepared which resulted in the breaking of tetracycline resistance by *Escherichia coli*, *Salmonella kentucky* and *Shigella flexneri* with lowering of minimum inhibitory concentration (MIC) to 20–40 µg/ml as compared to 150–180 µg/ml by free tetracycline (Mukherjee et al. 2016). Further, the death due to Shigella infection in zebra fish larvae was also found to be inhibited by tetracycline-loaded calcium phosphate nanoparticle treatment. Multi-morphological silver nanoparticles, synthesised using cashew nut shell liquid, were shown to have significant antibacterial activity (ZOI, 12.5–15.5 mm), MIC (124–312 µg/mL) and minimum bactericidal concentration (MBC) (128–322 µg/mL) against bacteria associated with fish diseases, namely, *Aeromonas bestiarum*, *Aeromonas hydrophila*, *Edwardsiella tarda* and *Pseudomonas fluorescens* (Velmurugan et al. 2014). Nano-silver prepared with marine fungal (*Aspergillus terreus*) chitosan was found to show antibacterial properties against nine fish sepsis-causing bacteria,

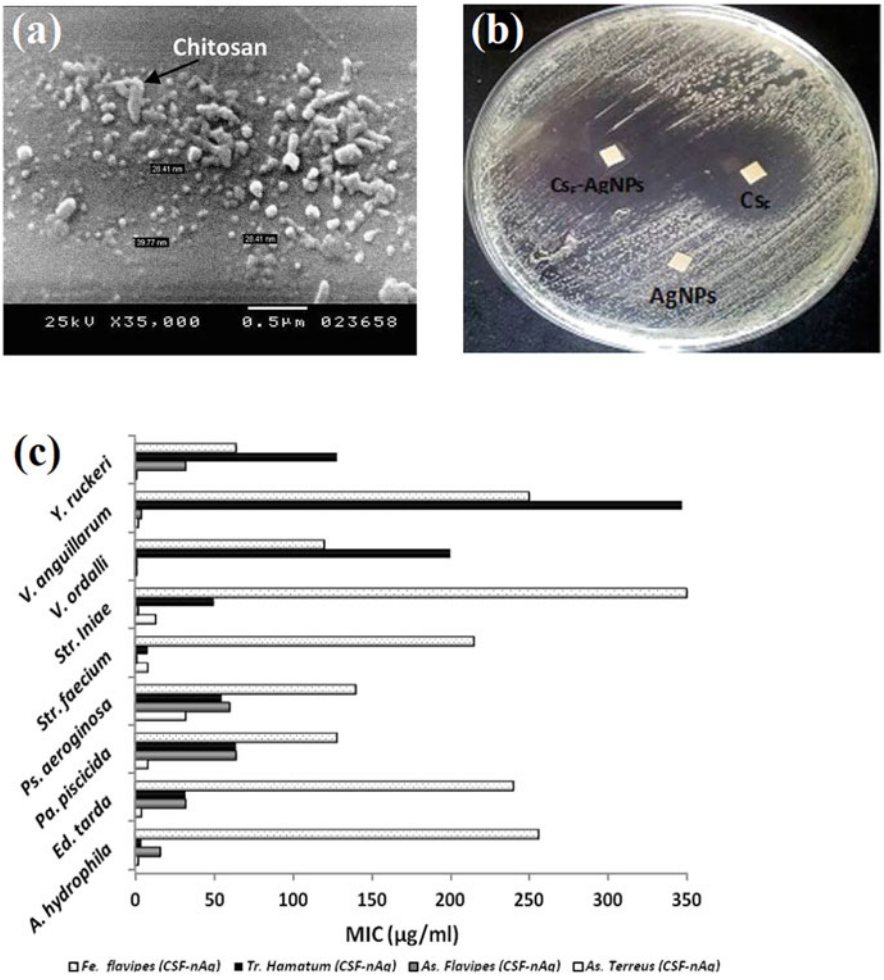

Fig. 3.7 SEM image of chitosan-based silver nanoparticles (**a**), antibacterial activity of *A. terreus* chitosan-based silver against *A. hydrophila* (**b**) and MIC of different fungal chitosan-based nano-silver against sepsis bacteria (**c**) (Barakat and Gohar 2015)

namely, *A. hydrophila*, *E. tarda*, *Pasteurella aeruginosa*, *P. piscicida*, *Streptococcus iniae*, *S. faecium*, *Vibrio ordalli*, *V. anguillarum* and *Yersinia ruckeri* (Barakat and Gohar 2015) (Fig. 3.7). The MIC of chitosan-capped silver nanoparticle was 7.9 µg/ml whereas only chitosan and silver nanoparticles separately showed MIC of 27.2 and 18.2 µg/ml, respectively.

3.3.3 Nano-immunomodulator

Besides nano-delivery of traditional therapeutics, application of nanoparticles to improve immune response and disease resistance in fishes is extensively studied. The effect of dietary nano-chitosan was observed as a feed supplement on growth performance, innate immunity and antioxidant activity of Nile tilapia, *Oreochromis niloticus* (Abdel-Tawwab et al. 2019). Due to dietary nano-chitosan supplementation, antioxidant-stimulated activity was observed along with an increase in catalase, superoxide dismutase, lysozyme and respiratory burst activity. The effect of a nano-selenium-supplemented diet was also tested in monosex Nile tilapia after challenging with *Aeromonas hydrophila* (Rathore et al. 2021). The study showed that nano-selenium supplement (1 mg/kg of feed) resulted better growth and feed utilisation significantly and also improves the haematological, serum biochemical, immune parameters and antioxidant activities. Nano-selenium supplementation (0.7 mg/kg^{-1} feed) in Nile tilapia also showed resistance against *Streptococcus iniae* infection with 26.66% mortality as compared to 93.33% in control (Neamat-Allah et al. 2019). Similarly, in European sea bass (*Dicentrarchus labrax*) dietary supplementation of selenium nanoparticles at the rate of 0.5–1 mg/kg diet resulted in optimal growth performance, antioxidative status, immune-related genes and haemato-biochemical indices (El-Kader et al. 2021). Not only fin fishes, nano-selenium supplement in Chinese mitten crab (*Eriocheir sinensis*) was also reported to reduce hypoxia stress and improve disease resistance against *Aeromonas hydrophila* (Qin et al. 2016). Similarly in another study, the effect of selenium-loaded chitosan nanoparticles on immune-related gene expressions, cytochrome P450, heat shock protein and transcriptomic modulation of caspase 1 of Nile tilapia was observed (Ibrahim et al. 2021). The study showed downregulated expression of myostatin gene and upregulated glutathione peroxidase, superoxide dismutase and catalase expression. It was also found that upon challenge with *Aeromonas hydrophila*, 93% survival rate was observed in tilapia fish when fed with selenium-loaded chitosan nanoparticles at 2 g/kg feed as compared to 45% in control. Upregulated expressions of caspase 1 and downregulations of cytochromes P450 and heat shock protein were also observed. A combination of nano-selenium and vitamin C in the feed supplement (1 mg nano-Se + 500 mg vitamin C/kg of food) was reported to significantly enhance the growth-related parameters with a reduction in feed conversion ratio in Nile tilapia (Dawood et al. 2020). Their combination increased intestinal villus length and width and the number of goblet cells.

Not only selenium but many other nanoparticles were also tested to boost immunity in fish and efficacy against fish pathogenic bacteria. Nano-zinc oxide as dietary supplement in Nile tilapia was reported to improve the total antioxidant capacity and antioxidant enzymes (Awad et al. 2019). A supplementation at 30 mg/kg diet significantly improved the lysozyme activity, bactericidal activity and IgM level in the tilapia fish. Nano-delivery of *Spirulina platensis* as a dietary supplement was also tested in Nile tilapia (Elabd et al. 2020). Nano-spirulina dietary supplementation at 0.5%/kg feed significantly increased growth performance, immunological parameters, biochemical assays, antioxidants and digestive enzymes.

Upon being challenged with pathogenic bacteria *A. veronii* (9×10^8 CFU/ml), fishes fed with nano-spirulina showed 100% protection as compared to 50% mortality in control. Immersion of zebra fish larvae in water-soluble nano-scale β-glucan solution (500 μg/mL) was reported to enhance the immunomodulatory properties in fish with upregulation of immune functional genes including TNF-α, β-defensin, IL-1β, lysozyme, IL 12, IL 10 and C-Rel when challenged with pathogenic bacteria *E. tarda* (Udayangani et al. 2017). Water supplementation of silica nanoparticles, prepared from rice husk using *Trichoderma harzianum*, was shown to reduce lead (Pb) concentration in the muscles of Nile tilapia when subjected to Pb concentration of 0.088 mg/L (El-Gazzar et al. 2021). Silica nanoparticle supplementation also increased the growth, haematological parameters, antioxidant capacity and immune-related gene expression of IL-1β. Magnetite (Fe_3O_4) nanoparticle supplementation (1.0 mg/L aqueous suspension) in Nile tilapia in the presence of mercury (0.025 mg/L) was also reported to significantly reduce the bioaccumulation of mercury in fish muscle (Mahboub et al. 2021).

3.3.4 Nanoantiparasites

Like bacterial pathogens, nanotechnological interventions were also made while managing parasite infection in fishes. In order to design immunoprophylatic nano-formulation against scuticociliatosis caused by endoparasite *Uronema marinum*, poly-D,L-lactide-co-glycolic acid (PLGA)-encapsulated nanovaccine was developed and tested on adaptive and innate immune response in kelp grouper (*Epinephelus bruneus*) (Harikrishnan et al. 2012). PLGA-encapsulated vaccine significantly enhanced respiratory burst (RB) activity and complement activity and α2-macroglobulin. Fish immunised with PLGA-encapsulated vaccine showed enhanced serum lysozyme activity, antiprotease activity and antibody level. The cumulative mortality due to scuticociliatosis was found to be 20% with PLGA-encapsulated vaccine as compared to 30% with only vaccine. Anthelmintic praziquantel-conjugated nanobioparticle consisting of chitosan-N-arginine and alginate was reported for control of intestinal trematodes in highly infected *Corydoras schwartzi* (Madrid et al. 2021). The administration of the developed anthelmintic resulted in no sign of visible alteration or tissue damage in the intestinal mucosa. Not only endoparasitic infection, nano-delivery of active compounds has also been tried against fish ectoparasites. Nanoemulsion formulation of antiparasitic molecule cypermethrin was developed using PEG diblock copolymer through a spontaneous emulsification technique (Sarkar et al. 2022). The developed nanoemulsion was tested against fish ectoparasite *Argulus bengalensis* at embryonic and adult stages (Fig. 3.8). It was shown that the nanoemulsion could disrupt the natural developmental stage of the ectoparasite at 0.001 ppm and calculated LC_{50} against the adult parasite to be 0.005 ppm.

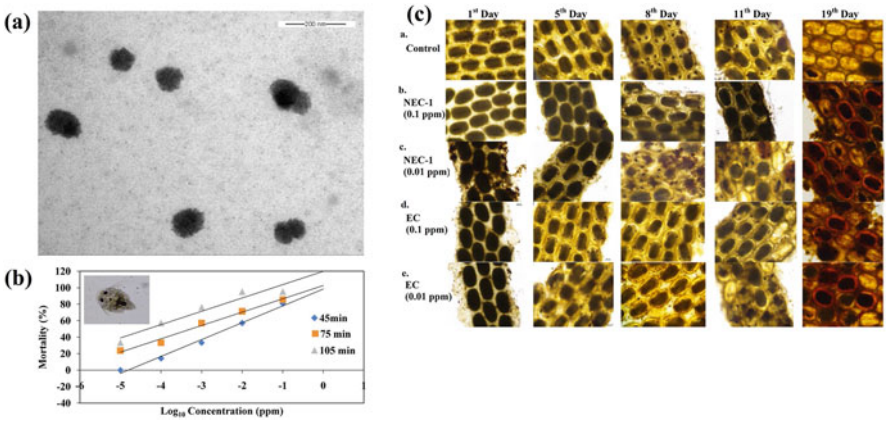

Fig. 3.8 TEM micrograph of cypermethrin nanoemulsion prepared with poly[poly-(oxyethylene-200)-oxyazelaoyl] (**a**); in vitro mortality of adult *Argulus bengalensis* treated with different concentrations of cypermethrin nanoemulsion (**b**); effect of cypermethrin nanoemulsion treatment on egg hatching stages of *Argulus bengalensis* at different days (Sarkar et al. 2022)

3.4 Conclusion and Future Prospect

A wide range of publications on the use of nanostructured materials for the remediation of contaminants and several other abiotic stressors from aquatic resources are available. The most prevalent are uses of multifunctional nanomaterials like zerovalent metal, metal oxides, etc. to maintain highly toxic ammonia and other toxic nutrients at threshold level. Besides these, interventions were also made to remediate hazardous metal ions, petroleum waste, toxic algal growth, etc. from the water using nanostructured materials. Against biotic stressors like pathogenic bacteria, parasites, etc., innovative strategies like development of nanovaccine, nanoantibiotics and dietary and water supplementation with nanomaterials like nano-selenium, nanosilver, nano-chitosan, etc. were reported. DNA vaccine using nanocarrier was reported to perform better with improved immunogenic properties and defence mechanisms as compared to conventional one. Nanomaterials and their conjugates with traditional antibiotics were reported to improve the antibacterial efficacy against antimicrobial-resistant pathogens. Dietary and water supplementation with nano-selenium, nano-chitosan, nanosilver, etc. was reported to enhance the resistance against several pathogenic bacteria and improve the growth performance. Despite several reports on the use of nanostructured materials for the improvement of water quality parameters, the results are still restricted to laboratory observation. There is an immediate requirement to explore these new materials in real field condition where their performance can be better judged in holistic manner along with their probable hazardous characteristics towards nontarget aquatic life. In case of nanotechnological interventions against biotic stressors, no toxicity was found against the target animals in most of the cases. However, extensive studies are

needed on their suitability in farm trial and there is a need to generate toxicity data against nontarget organisms during field applications.

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Transgenerational Phenotypic Plasticity in Fishes

4

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Abstract

Phenotypic plasticity, which is sometimes referred to as an environmental driven phenotypic variation, is the capacity of a genotype to create a variety of phenotypes under different environmental conditions. It is critical to include both the genetic architecture and environmental sources of phenotypic variation when examining the trait since environmental change can alter the genetic makeup and plasticity of traits. As a capacity for quick genetic adaptation, plasticity is vital in helping organisms to cope with rapidly changing environments. Transgenerational plasticity refers to a parent's capacity to modify their offspring's phenotype without introducing genetic modifications into the offspring. The environmental experiences of the parent generation and/or prior generations have an impact on this genotype (TGP). Through this phenotypic transition, offspring can prime their physiology to more closely fit the environmental circumstances. Parents can modify the offspring's phenotype that is cued by the environmental experiences in parental generation and/or previous ancestors without involving a genetic change in offspring termed transgenerational plasticity (TGP). Offspring are primed to adapt their physiology to the environment through this phenotypic shift. Transgenerational plasticity provides a real-time compensatory response to environmental fluctuations which

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is likely to be advantageous by increasing the fitness of species since the climate is changing more rapidly than natural selection that can keep up with adaptation. Recently, TGP is increasingly recognised as a mechanism by which organisms can act much quicker in environmental changes. Several studies have reported that parental exposure to environmental stressors has beneficial effects on offspring. Moreover, transgenerational plasticity and immune priming can be used for the development of novel strategies for producing the robust progenies. Enhanced resistance against various pathogens and abiotic stressors can be developed within and even across generations of offspring which will provide a huge possibility for commercial applications in the fisheries and aquaculture sector. Although there is ample proof that the environment has an impact to phenotypic variation, the underlying mechanisms are still not entirely understood. Mechanistically, parental effects and epigenetic inheritance (DNA methylation, histone modifications and RNA-mediated modifications) have been proposed in facilitating the transgenerational plasticity and programming of the offspring. Understanding the molecular processes by which environmental information is received to switch genes for alternate phenotypes in the context of global climate change and disease outbreaks in the culture system is therefore of highest importance. In this chapter, we have introduced the concept of transgenerational phenotypic plasticity, summarised the evidence of transgenerational plasticity in fishes and highlighted the potential molecular mechanisms.

Keywords

Phenotypic plasticity · Transgenerational plasticity · Parental exposure · Epigenetic inheritance

4.1 Introduction

The genotype of the progeny is based on the parental (maternal and paternal) DNA; however, the phenotype of the progeny can be plastic and is impacted by a wide range of environmental influences. The ability of a given genotype to create various phenotypes in response to various environmental situations is known as phenotypic plasticity (Pigliucci et al. 2006). The originality and diversity of morphological, physiological and behavioural features of an organism's phenotypic and life cycle traits are made possible by its strong interest in ecology and evolution, as well as its significant significance for agriculture and related fields (Stillwell et al. 2007; Crozier and Hutchings 2013). Though phenotypic plasticity is widely observed in the wild; however, for a long time, it was a neglected mechanism, and only recently mechanisms involved in phenotypic plasticity have been analysed. Variation in plasticity among genotypes is classically known as genotype-environment interaction. Plastic characteristics will alter phenotypes without altering the genetic architecture in the scenarios of rapid environmental change and climate change. Additionally, a number of biotic and abiotic stimuli trigger this non-genetic

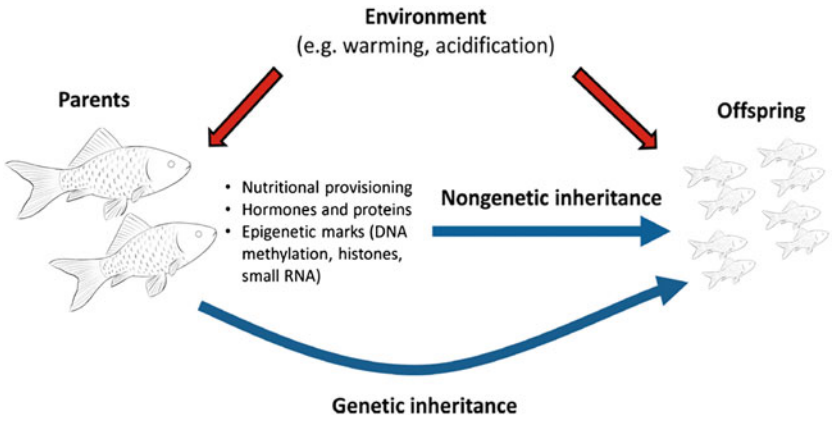

Fig. 4.1 Parents have both genetic and non-genetic (plasticity) effects on their children's phenotypes (Munday 2014). Through a number of non-genetic pathways, the environmental experiences of parents can influence the phenotypes of their offspring. When parents have experienced the same environment and their descendants perform better in it, this phenomenon is known as transgenerational acclimation

inheritance of phenotypic plastic responses (Ellers 2010; Bernhardt et al. 2020; Srikant and Drost 2021). From the applied perspective, the appropriate degree of plasticity depends on the expected environmental conditions. Since they offer a selective advantage in changeable contexts or during adaptation to a rapid environmental transition, adaptive plastic responses are particularly crucial where the altered phenotype is linked to better fitness. Non-genetic phenotypic plasticity is now regarded as one of the key processes by which animals can adapt to the rapidly changing environment and other drivers of global change. Therefore, understanding and forecasting the reactions to scenarios of global change require a thorough understanding of the broad-scale causes of phenotypic plasticity (Jeremias et al. 2018; Fox et al. 2019).

There was a long-standing question whether the plasticity observed within generations can influence the subsequent generations. Phenotypic plasticity is also represented by the parental effects which are known as transgenerational phenotypic plasticity (TGP), where parental/ancestral environmental experience can be inherited/transferred to influence the offspring's phenotype (Fig. 4.1) (Donelson and Munday 2015; Clark et al. 2019). The impacts of TGP have been observed in a wide variety of traits and taxa, and it has recently been abundantly obvious that these effects are pervasive and diversified. When parents from diverse settings give their children different proteins, nutrients, hormones, epigenetic factors or antibodies, transgenerational plasticity may result. TGP can influence the growth, immunity, viability, phenology and reproductive timing in offspring. Mechanistically, transgenerational phenotypic plasticity may be mediated by parental effects or by epigenetic inheritance (DNA methylation, histone protein modifications and tiny RNA molecules are examples of DNA modifications that occur without affecting the

DNA sequence information) (Bell and Stein 2017; Roy et al. 2021). The link between the surroundings that the parents and their children experience determines some of the adaptive significances of transgenerational plasticity. However, little is known about individual fitness and if these changes are adaptive in light of the rapid environmental changes. Recent reports suggest that epigenetic inheritance and TGP are crucial for species' ability to adapt to climatic changes. Hence, understanding the relationship between plasticity and evolution has taken on increased importance in rapid global climate change scenarios (Jeremias et al. 2018).

In this book chapter, we will introduce key concepts of transgenerational plasticity and summarise the current evidence of TGP in aquatic animals (fish/shellfishes) under different environmental changes and potential molecular mechanisms by which parental environmental information passes on to the offspring.

4.2 Evidence of Transgenerational Phenotypic Plasticity in Fishes

Organisms will be exposed to novel, potentially stressful conditions as a result of the fast-changing climate. Thermal changes in the environment are expected to be particularly challenging for aquatic life and ectothermic species because their physiology is directly dependent on ambient temperatures. Whenever a species or population experiences environmental stress, natural selection can stop it from going extinct through evolutionary rescue. However, these quick environmental changes and man-made activity might be too fast for evolution to keep up with. In this situation, phenotypic plasticity can become a potential solution for organisms to buffer against environmental changes and thereby buy time for evolutionary rescue (Fig. 4.2) (Fox et al. 2019). Furthermore, transgenerational plasticity where parents pass the environmental cues/experiences to their progenies to influence their phenotype can increase the population persistence and will successfully buy time for an evolutionary rescue to happen. Recently, a growing number of studies evidenced and reported the transgenerational effects in aquatic/marine organisms upon parental exposure to environmental stressors, and it promises the application of conservation methods (Donelson and Munday 2015; Roth and Landis 2017; Bernal et al. 2018; Eirin-Lopez and Putnam 2021).

4.2.1 Temperature and Ocean Warming

Temperature is the principal regulator of transgenerational phenotypic plasticity. Early-life environmental experiences can have an impact on a female's reproductive tactics and maternal investment. This may be a crucial mechanism for protecting animals from the harmful effects of climate change. Due to the restrictions placed by the higher energetic costs of a warm environment, exposure to high temperatures can alter maternal strategy. This has assisted mothers in conditioning/preparing their offspring to make up for higher energetic costs and damage when progeny

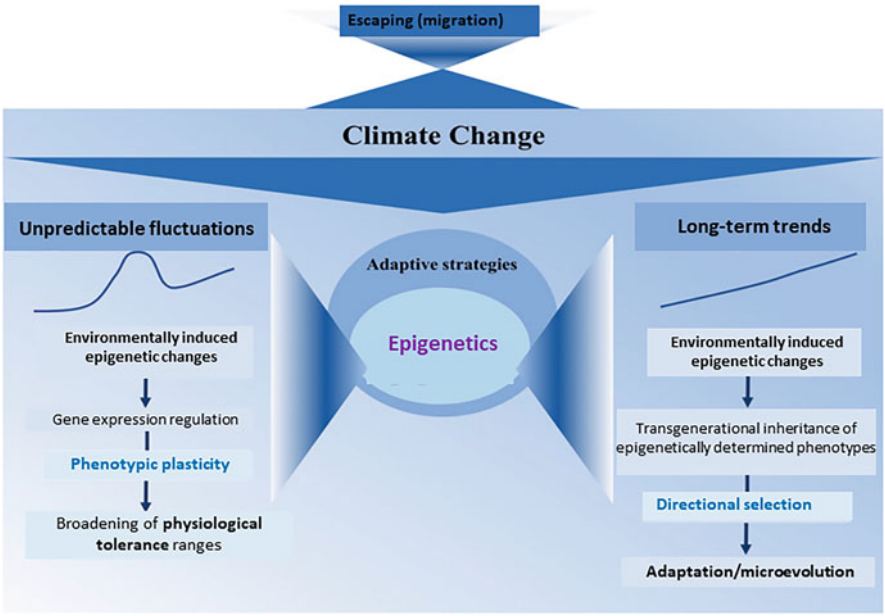

Fig. 4.2 The conceptual graphical representation of epigenetic roles in phenotypic plasticity as a coping mechanism for aquatic animals under climate change to deal with environmental challenges (Jeremias et al. 2018)

experience the same temperature stress as their mothers. For example, thermal transgenerational effects of fastest-growing offspring were observed in sheepshead minnow fish (*Cyprinodon variegatus*) whose parents had experienced the same temperature exposure over 30 days prior to fertilisation (Lee et al. 2020). Thermal conditions experienced by the mother and grandparents throughout development might affect the size of the egg and quantity of yolk produced by the offspring. As transgenerational thermal effects in Atlantic salmon (*Salmo salar*), significantly larger eggs were produced in the F2 offspring as a result of the F1 mother's experiences prior to egg fertilisation (Jonsson and Jonsson 2016; Bernal et al. 2022). In another study, thermal conditioning during early/juvenile life has influenced maternal reproductive strategies in the three-spined stickleback (*Gasterosteus aculeatus*). However, the distribution of antioxidants in eggs rather than egg size may be a mediator of the maternal thermal environment transgenerational influences on offspring's phenotype (Kim et al. 2017). The cumulative impact of higher temperatures on egg hatchability in three-spined marine sticklebacks caused by grandparental thermal effects that were passed down to the following generations suggests that parental and grandparental TGP have significant fitness implications that are very environment dependent (short- or long-time exposure) (Shama and Wegner 2014). It was discovered in a study on largemouth bass

(*Micropterus salmoides*) populations from heated lakes that despite the generationally temporary nature of these changes, largemouth bass can mitigate the sub-lethal effects of warming by changing physiological processes that have an effect on the aerobic scope. However, it does not change in response to warming in terms of its maximum thermal tolerance (White and Wahl 2020). In another study, differential effects of developmental thermal plasticity observed in guppies (*Poecilia reticulata*) when fishes were exposed to warmer temperature showed better performance than the cold-developed fish which might be advantageous for a tropical animal such as guppies (Le Roy et al. 2017).

Though transgenerational plasticity through non-genetic inheritance has the capacity to buffer populations against ocean warming, not a lot is known about the duration of these impacts and whether they accumulate across generations. In a study, coral reef damselfish model (*Acanthochromis polyacanthus*) investigates how TGP can influence/compensate the negative effects of ocean warming projections over two generations (+1.5 °C in the first generation and subsequently +3.0 °C in the second generation). When grandparents or parents were exposed to warm weather, the offspring's aerobic capacity rose along with immune system, inflammatory and cellular stress-related gene expression (Bernal et al. 2022). In light of this, the study demonstrates how future generations' adaptability to global warming may be influenced by the warming conditions experienced by both prior and present generations. Moreover, another study shows that cold-shock exposure promotes the metabolic trade-off in early life stages in tilapia fish. The availability of energy during reproduction may be impacted by parental cold exposure, and the structure of metabolites enables transgenerational responses to temperature change. In order to more accurately forecast the adaptation mechanisms in fish populations, knowledge of such metabolism-related transgenerational impacts in fish is important (Wang et al. 2022). However, transgenerational and developmental plasticity observed in various capacities between the species and different traits, therefore, is plausible that species- and trait-specific plasticity has evolved, changed by the functions those features play in life history (Shama and Wegner 2014). There is evidence from numerous studies that TGP plays some part in setting the stage for the offspring's response to high temperatures, but there is also evidence that TGP had very modest effects of ambient temperatures on thermal performance in lake trout salmonids over generations (Penney et al. 2021).

4.2.2 Sex Determination and Sex Ratios

Fish reproduction and sex determination are genetically determined; however, it is also related to the environment or a combination of the both. Usually during a crucial phase of early development, temperature is one of the most significant environmental elements that might influence reproduction and sex determination (Honeycutt et al. 2019). Due to the high energy cost of reproductive performances, various species have evolved to reproduce only within a limited heat range (Geffroy and Wedekind 2020). So there is a concern that rise in global temperature, and ocean

warming can affect the reproductive performance and operational sex ratios to the fishes. However, interestingly, recent findings suggest the importance of transgenerational plasticity by parents to mitigate the negative impacts of climate change on their offspring (Shama and Wegner 2014; Donelson et al. 2017). Recent research on coral reef damselfish (*Acanthochromis polyacanthus*) revealed that non-genetic and non-behavioural mechanisms allow parents to modify the gender and sex ratios of their offspring during the first generation of life at elevated anticipated future ocean temperatures (+1.5 °C and +3.0 °C above the present-day average temperature). This evidence showed the potential of transgenerational plasticity in early life development to mitigate few impacts of climate change on reproductive traits (Donelson and Munday 2015). Another study on coral reef damselfishes discovered that gradual temperature increases over two generations (parents, +1.5 °C, offspring, +3.0 °C) increased the F2 generation's reproductive output (Donelson et al. 2016). The expression of the male gonadotropin receptor genes (Fshr, follicle-stimulating hormone receptor, and Lhcgr, luteinising hormone/choriogonadotropin receptor) in the gonads was also found to match such pattern of enhanced reproduction, indicating that Fshr and Lhcgr may play a role in regulating reef fish male reproductive potential. Moreover, lower levels of Fshb expression in females imply that the vitellogenesis-related Fshb (follicle-stimulating hormone, beta-polypeptide) is susceptible to high temperatures in females (Veilleux et al. 2018).

4.2.3 Immunity and Defence

Immune priming is an adaptive strategy that improves the defensive capacity upon exposure to stimuli from pathogens, pathogenic components, beneficial microbes or abiotic cues. After the subsequent challenge, it mounts a faster and/or stronger defence response resulting in the increased resistance and/or stress tolerance (Salmela et al. 2015; Sheehan et al. 2020). Priming can be durable and maintained throughout the life span (within generation) and can even be transmitted to subsequent generations, therefore representing a type of immunological memory (Roy et al. 2020). Transgenerational immune priming (TGIP), which protects offspring from repeated interactions with pathogens that may persist through generations, is another way that parental and ancestral immunological experiences can increase progenies' survival (Green et al. 2016; Beemelmanns and Roth 2017; Roy et al. 2022). A growing number of studies described the phenomenon in diverse taxa including invertebrates (Salmela et al. 2015; Tetreau et al. 2019) and vertebrates (Beemelmanns and Roth 2016b; Roth et al. 2018). Nevertheless, the underlying mechanisms are diverse, and priming may cause changes at different levels such as physiological, transcriptional, epigenetic and metabolic levels. Following parental generational priming by exposure to a live or dead *Vibrio* sp., a recent study in brine shrimp (*Artemia franciscana*) model reported the incidence of transgenerational immune memory, which provides a chance for the production of offspring with improved resistance against *Vibrio* infections. It demonstrates how TGIP could be

applied to parental conditioning and the development of transgenerational innate immunological memory in offsprings for the purpose of disease prevention in shrimp farming, ultimately lowering significant economic losses (Roy et al. 2022). Additionally, the Pacific oyster (*Crassostrea gigas*) showed evidence of transgenerational immunity to *ostreid herpesvirus* 1 (OsHV-1) infection (Lafont et al. 2019).

Later life stages (within generation) and oyster larvae of the following generation have shown improved survival after exposure to poly(I:C), particularly when their mothers were exposed to poly(I:C) before spawning. The increased larval survival may be a result of the mother's providing of antiviral substances in the eggs. In another study, biparental and bacteria (*Vibrio*)-specific transgenerational immune priming was observed in pipefish (*Syngnathus typhle*) (Beemelmanns and Roth 2016a). Maternal investment and offspring immune defence was also studied in the mouthbrooding cichlid fish (*Astatotilapia burtoni*) (Keller et al. 2017). The findings indicated that maternal investment and the increase in immunological activity in response to an immune challenge are expensive traits; therefore, if both are present at the same time, not only do mothers appear to be affected, but also offspring appear to be hindered in their capacity to respond to an exposure to a potentially virulent pathogen.

Transgenerational immune priming can also be brought on by abiotic temperature shock. In the brine shrimp Artemia model, exposure to environmental heat stress or nonlethal heat shock causes transgenerational inheritance of robustness towards lethal heat stress and resistance against pathogenic *Vibrio campbellii*, and the acquired phenotypic traits were passed down to three succeeding generations (Norouzitallab et al. 2014). In the following study, the parental generation was exposed to the heat-shock protein-inducing compound phloroglucinol early in life, and this resulted in transgenerational inherited resistance against biotic infections with bacteria (*V. parahaemolyticus* AHPND strain and *V. harveyi*) and abiotic (thermal stress) in the brine shrimp model. This increased resistance was passed down to three additional generations (Roy et al. 2019). Another study examined the effects of the combination of biotic immune challenge and abiotic heat stress in the parental generation on the offspring in pipefish (*S. typhle*) (Roth and Landis 2017). Simultaneous exposure to an abiotic and a biotic factor in the parental generation had interactive effects on the performance of the offspring. The temperature effect outweighed the impact of the immune challenge, indicating that there are only a limited number of resources that can be devoted to phenotypic transgenerational effects.

4.2.4 Ocean Acidification and Salinity

Elevated temperatures are not the only situation where transgenerational acclimatisation occurs. Climate change's effects on marine fishes are a concern, but it is unclear how ocean acidification might be adapted to or acclimated to through transgenerational plasticity. Under circumstances that simulate the impacts of future ocean acidification, parental effects can have a major impact on how well marine

creatures perform. The offspring (juveniles) of anemonefish (*Amphiprion melanopus*) whose parents were exposed to high CO_2 levels and seawater acidification did not suffer from the detrimental effects on growth and survival (Miller et al. 2012). In a study, coral reef fish was used to determine the molecular response of short-term, developmental and transgenerational exposure to high CO_2. The effects of ocean acidification on reef fish are determined by a combination of plasticity and parental phenotype, according to transcriptional research (Schunter et al. 2018). In another study in European sea bass (*Dicentrarchus labrax*), two (F1 and F2) generation populations were exposed to predicted ocean acidification treatment (pH 7.6). Ocean acidification's transgenerational transcriptome impacts on the olfactory epithelium were discovered by gene expression profiling to be linked to increased virus resistance (Cohen-Rengifo et al. 2022). In another study, pre-conditioning of the adult green sea urchin (*Psammechinus miliaris*) with low pH demonstrates that it prepares the larvae for future new conditions by preloading with antioxidants and can significantly impact offspring outcomes (Clark et al. 2019).

Salinity has significant effects on the physiology of fish/aquatic organisms, and changes in salinity have been also predicted in view of the climate change scenario. Not much studies have reported on the transgenerational plasticity to respond in such salinity changes. Recently, transgenerational plasticity effects and adaptive potential to salinity change have been studied in the three-spined stickleback (*Gasterosteus aculeatus*) model, and results suggested that transgenerational plasticity may vary between the life stages and early life stages which are particularly vulnerable to increased salinity (Heckwolf et al. 2018). Another study examined the eastern oyster (*Crassostrea virginica*) transgenerational plasticity and ability to adapt to low salinity (Griffiths et al. 2021). The findings show that transgenerational plasticity is unlikely to significantly contribute to low-salinity tolerance and that parental genotype primarily controls variation in larval size, pointing to evolutionary adaptation as a potential mechanism by which oysters might be able to survive future salinity declines.

4.2.5 Pollutants

Many pollutants and emerging pollutants like microplastics are considered as potential threats to exposed fish/aquatic organisms; additionally it may have an impact on the performance and phenotypes of their progeny. However, little is known about how these contaminants and newer pollutants may affect future generations of offsprings. Recent studies indicate that exposure of pollutants to the parents tends to be costly for both parent and offspring generations. Transgenerational effects of endocrine disruptors were studied in an estuarine fish model, that is, inland silversides (*Menidia beryllina*) (Decourten et al. 2020). The F0 and F1 generations suffered unfavourable consequences from parental larval exposure (F0) to endocrine disruptor chemical, including decreased larval development and survival, malformations, skewed sex ratios, decreased egg production, poor immunological

function and changed gene expression. Additionally, exposed fish showed heritable epigenetic changes (DNA methylation in certain genes) over the course of three generations, indicating the transfer of effects through epigenetic mechanisms. According to the findings, it is crucial to conduct assessments spanning several generations to ascertain the entire scope of consequences resulting from prenatal pollutant exposure.

Recent studies demonstrated that environmentally relevant concentrations of microplastics (polystyrene) had adverse transgenerational effects on the reproduction of marine medaka (*Oryzias melastigma*) (Wang et al. 2019) and adaptation of life history traits and trade-off between growth and reproduction (Wang et al. 2021). Microplastics promoted the maternal transfer of phenanthrene to offspring embryos in marine medaka and concurrence of microplastic aggravated transgenerational toxicity (Li et al. 2022). In the European whitefish (*Coregonus lavaretus*), pre-fertilisation exposure of sperm to nanoplastics showed transgenerational impacts on offspring's phenotype and decreasing offspring's survival, growth and swimming performance (Yaripour et al. 2021). These studies provided new insights on the transgenerational effects of microplastics which indicates that in aquatic habitats, microplastic/nanoplastic pollution may have substantial ecological and evolutionary effects.

4.3 Established and Potential Molecular Mechanisms of TGP

Though poorly investigated, the epigenetic mechanism has been proposed in facilitating the transgenerational plasticity and programming of the offspring and how the phenotypic response to environmental factors is passed down through genetics (Burggren 2016; Jonsson and Jonsson 2019). Epigenetic inheritance may aid in adaptability to new environmental conditions, which is especially advantageous for the survival of the exposed population and subsequent generations as they are anticipated to be able to cope with the fast-changing environment (Fig. 4.2).

It is possible that the key mechanism enabling intra- and intergenerational phenotypic plasticity is epigenetic control, which can influence heritable traits without affecting the genetic code (DNA sequence) (Burggren 2016; Roy et al. 2019, 2022). The primary epigenetic mechanisms include the following:

(a) DNA methylation: it involves chemically altering DNA bases by adding a methyl group.
(b) Histone protein modifications: altering the histone proteins linked to DNA limits the shape of the chromatin and DNA accessibility.
(c) Non-coding RNAs: non-coding RNAs can regulate the expression of genes and create intricate genome-wide RNA regulatory networks (Fig. 4.3) (Roy et al. 2020, 2021).

Environmental factors can have a direct impact on epigenetic markers, which serve as a key modulator of phenotypic responses to environmental cues. It is

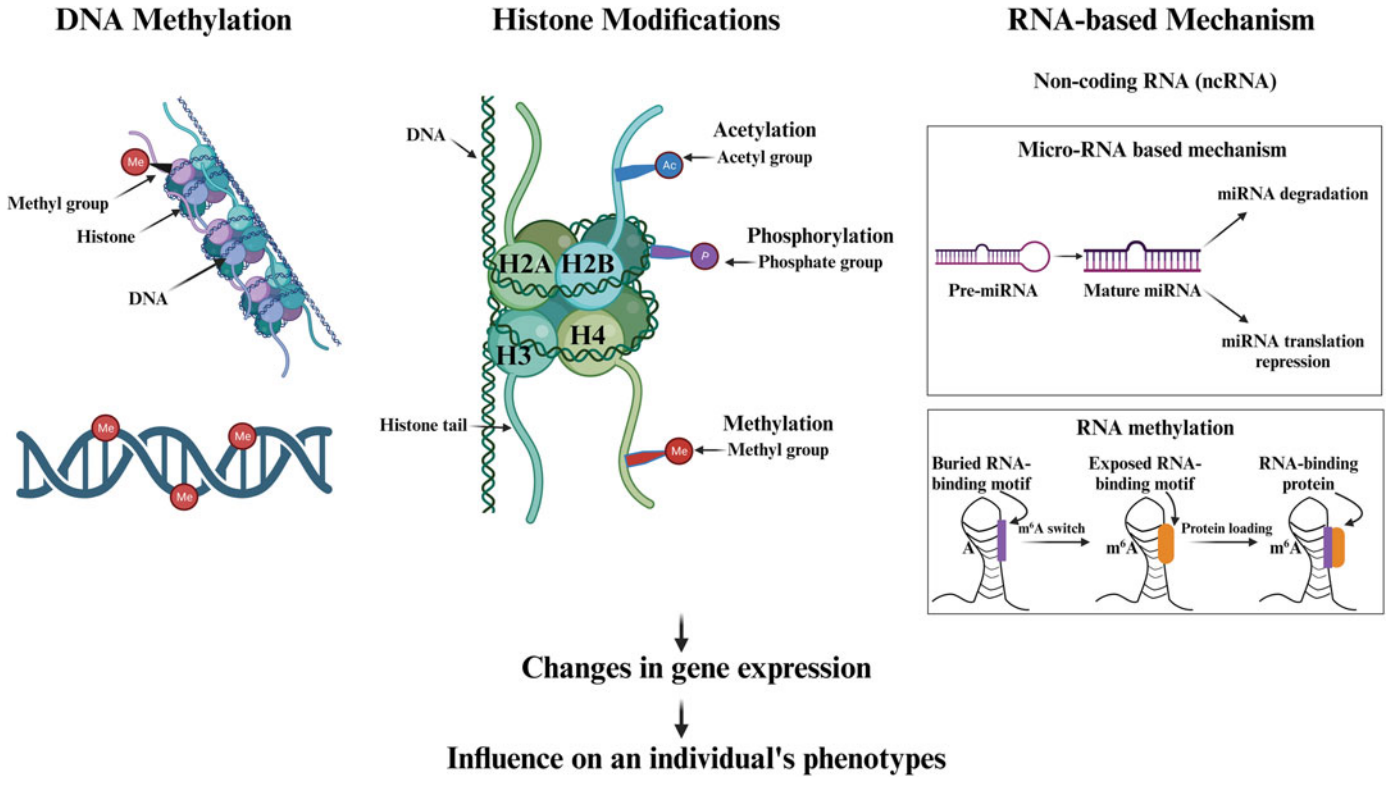

Fig. 4.3 Main epigenetic modifications involved in transgenerational plasticity (Roy et al. 2021). The term "epigenetic modifications" refers to a variety of chemical changes to DNA, histones or non-coding RNA that affect phenotypic and gene expression without altering the genomic sequence. (1) Since DNA is methylated, the degree of methylation—hyper- or hypomethylation—has an effect on how genes are expressed. (2) Chemical modifications to histones include

interesting to note that some specific epigenetic patterns may endure in the chromatin throughout generations and serve as the foundation for long-term adaptation.

A relatively new yet exciting area is that of the evolution of epigenetically inherited traits. But during gamete and early embryo development, epigenetic reprogramming (the erasure and re-establishment of epigenetic markers) may prevent the transgenerational epigenetic inheritance. Although DNA methylation is the most stable and well-studied epigenetic process in fish and shellfish, it is still unclear how much DNA methylation is being reprogrammed in fish (Dorts et al. 2016; Herman and Sultan 2016). Typically, fishes' environmental-induced DNA methylation patterns do not undergo significant reprogramming (Wellband et al. 2021).

Through transgenerational epigenetic inheritance, parents' capacity to prepare their offspring for heat stress is crucial in situations of global warming and climate change (Bernal et al. 2018). Global DNA methylation/hydroxymethylation and gene expression implicated in the epigenetic alterations were seen during gametogenesis and embryogenesis in the marine stickleback (Gasterosteus aculeatus) under ocean warming scenarios (Fellous et al. 2022). An interaction between the DNA methylation enzyme (DNMT) and histone modifications (histone acetylation) was found in zebrafish (*Danio rerio*) which regulates the developmental thermal plasticity and alters fitness in thermal responses to environmental change (Loughland et al. 2021). Environmental variation experienced during captivity and maturation has been shown to influence the gamete DNA methylation in fish Atlantic salmon (*Salmo salar*) (Wellband et al. 2021). In Chinook salmon (*Oncorhynchus tshawytscha*), the genetic architecture of DNA methylation also varied depending on the environment in which the fishes were raised; hatchery-reared offspring displayed greater additive and nonadditive genetic variance, whereas seminatural stream channel-reared offspring displayed greater maternal effects (Venney et al. 2021).

Through the process of transgenerational immune priming, parental immunological knowledge is passed on to offspring, protecting them from repeated pathogen exposure. As a strategy to prevent disease outbreaks, parental generation brine shrimp were exposed to the plant-derived phloroglucinol compound to induce the transgenerational inheritance which significantly a core set of innate immune genes' expressions was increased in following generations.

Results demonstrated that the transgenerational inheritance of the resistant brine shrimp progenies is a result of epigenetic mechanisms such as DNA methylation, m6A RNA methylation, histone methylation and acetylation playing an underlying role in the regulation of gene expression (Roy et al. 2019). In a second investigation, parental generation was primed by exposure to the live or dead *Vibrio* sp. for the production of transgenerational innate immunological memory in brine shrimp.

Fig. 4.3 (continued) methylation, acetylation, phosphorylation and sumoylation, which can be applied to either the histone tails or the histone core itself. (3) MicroRNAs, small RNAs like siRNAs or long non-coding RNAs are responsible for mediating posttranslational changes of RNA. Additionally, mRNA methylation has been documented

Significantly increased innate immune-related gene transcription and epigenetic changes in histone modifications (H3 and H4 acetylation) and RNA m6A methylation were linked to disease resistance in offspring (Roy et al. 2022). In the pipefish (*Syngnathus typhle*), grandparental immune priming induced immune gene expressions and epigenetic regulation processes including DNA-methylation and histone modifications while transferring immune-related information across generations in grand offspring (Beemelmanns and Roth 2016b).

4.4 Future Outlook and Conclusions

Transgenerational phenotypic plasticity's evolutionary significance is currently a hot topic in climate change scenarios. If parental environmental experience may predict their offspring's environment, populations that demonstrate adaptive transgenerational phenotypic plasticity should be better prepared to deal with environmental change situations. Therefore, it is likely that plastic rescue is commonly used to achieve evolutionary rescue. The fastest-growing sector of the food production business, aquaculture and fishing are soon becoming essential elements of global food security. Producers have had to rely more and more on rigorous culture-based systems as a result of increasing demand in recent years, which strains the animals and eventually causes illness outbreaks.

The sustainability of production is currently constrained by high mortality rates; losses from diseases, mainly bacterial and viral ones; and subsequently low yields. Disease outbreaks have been a continually serious problem. Immune priming and transgenerational plasticity, as we currently understand them, may eventually have implications for the development of cutting-edge drugs to treat diseases brought on by microorganisms. Through immune priming, disease resistance can be increased within a generation or even between generations. Brood stock populations may be stimulated with biotic (exposure to microbial challenge/microbial components) or abiotic stressors (particular environmental stimuli as heat-shock/HSP-inducing chemical) stressors in order to transmit enhanced immunity to the following generations of offspring. This technique offers several possibilities for commercial applications in the fishery and aquaculture sectors since it may result in offspring that are stronger and more resistant to certain infections.

However, research also demonstrates that while parental participation promotes child survival, it is time- and energy-intensive. When the following criteria are satisfied, transgenerational plasticity is predicted to emerge: (1) environmental variation is predictable beyond one generation; (2) parents' and offspring's environments are accurate predictors of each other's environments; (3) the cost of plasticity is less than the benefits it offers; and (4) parents and offspring are better able to bear the costs of plasticity. The investigation into transgenerational plasticity in fisheries is still in its early phases; hence there are numerous unresolved issues. The lack of progress in understanding transgenerational phenotypic plasticity continues to be the biggest obstacle to usage in the fishery sector. Understanding how phenotypic plasticity and genetic changes interact to promote population

adaption to climate change can help us better predict the effects of a changing environment.

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Heat Shock Proteins in Fish Health Management

5

Vikash Kumar, Suvra Roy, Tanushree Banerjee, Bijay Kumar Behera, and Basanta Kumar Das

Abstract

All types of cells, including those of bacteria and mammals, include a group of highly conserved proteins called heat shock proteins. They play a significant role in protein maturation, re-folding, degradation and maintaining homeostasis and good health in fish species. In the presence of varied internal and external environmental challenges, some Hsps are constitutively expressed in specific locations, whereas others are quickly increased as a basis for self-defence and stress response for organisms. There are reports which suggest that in vivo production of Hsps, in response to both abiotic-like hyperthermia and hypoxia and biotic stressors such as microbial infections, leads to efficiently increase protein stability and enhance host immunity and resistance to microbial infection. Therefore, developing an alternative agent or strategy to enhance Hsps levels inside animals resulted in enhanced immunity and disease resistance in the host.

Keywords

Heat shock proteins · Aquaculture · Immunity · Disease resistance

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5.1 Introduction

The fastest-growing food-producing industry in the world, aquaculture, includes all methods of cultivating aquatic animals and plants in freshwater, brackish water or marine environments. Its average annual growth rate is 7.1%. However, disease outbreak is thought to be a key barrier to the growth of this industry, resulting in enormous economic losses each year globally (Defoirdt et al. 2004; Kumar et al. 2023a). The aquatic environment independent of their host is more favourable to pathogenic bacteria than the terrestrial environment, and consequently, pathogenic bacteria like *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., etc. can reach very high densities around the aquatic animals and can be problematic to several aquatic species (especially the larval stages), as in many cases it can cause total mortality up to 100% (Defoirdt et al. 2004, 2011). Traditional approaches, such as using antibiotics and disinfectants, had only inconsistent success in preventing or treating aquatic infections. However, because of the emergence of (multiple) resistance, their use in the aquaculture industry is under intense scientific and public criticism. Due to these worries, FAO (Food and Agriculture Organization) has determined that the development of alternative control measures in aquaculture is urgently needed, with a focus on prevention, which is likely to be more affordable than cure (Defoirdt et al. 2011).

Improved management techniques can reduce the risk of disease by preventing stress (due to high stocking density, handling temperature, dissolved oxygen, etc., improving water quality (e.g. by bioaugmentation), avoiding the spread of pathogens (e.g. by quarantine), etc. Moreover, in recent years, alternate strategies like developing natural ingredients that give immunity to pathogens and/or boost immune response in a way conceptually similar to probiotics, vaccinations or immunostimulants have been successfully tested in aquatic animals, and results showed that they can be used in aquaculture system to prevent the aquatic disease (Niu et al. 2014). These molecules are also frequently referred to as heat shock protein inducers (Hspi), as it enhances the production of Hsps in the host and provides protection counter to biotic and abiotic stressors. The Hsps, comprising 5–10% of total cellular proteins, function as molecular chaperones, increased 2–3 folds when cells are exposed to stressful conditions and perform a multitude of housekeeping functions including eliciting immune responses against pathogenic microbial infection, protein homeostasis, etc. that are vital for cellular survival (Kumar et al. 2022a, b). Hence, utilisation of natural substances/molecules could be an emerging therapeutic target to stimulate HSP production in the host.

5.2 Heat Shock Proteins (Hsps)

The heat shock proteins (Hsps) are early and highly conserved proteins that are present in the majority of living things, including archaebacteria, prokaryotes and eukaryotes. In times of stress, Hsps are crucial for controlling cellular metabolism (Dong et al. 2020, 2021). These proteins can be found throughout the intracellular

space of eukaryotes, including the cytosol, mitochondria, endoplasmic reticulum (ER), chloroplast and nucleus. Hsp60, Hsp70, Hsp90 and sHsps are only a few of the families that the Hsps are typically divided into based on their function, molecular mass and sequence homology (Table 5.1). They can also be classed according to their closest size family (e.g. Hsp84, Hsp85 and Hsp86 in Hsp90 kDa family).

In the early 1930s, the effect of heat shock was performed on the *Drosophila* (fruit fly). However, Hsps was first discovered in 1962 following the experiment performed on the *Drosophila* model; when the salivary gland of *Drosophila* was exposed for 30 min at 37 °C nonlethal heat shock and after recovery at 25 °C, the gene termed as 'puffing' was observed in the chromosome of the recovering cell (Ritossa 1962). A decade later, Tissiéres et al. (1974) observed that in response to heat shock, the salivary gland of *Drosophila melanogaster* has increased expression of proteins (chromosome puffs) with molecular masses of 26 and 70 kDa and coined the term heat shock proteins (Hsps). Similar observations were also reported from prokaryotes and eukaryotes in the following years, suggesting that the upregulation

Table 5.1 Major heat shock protein (Hsp) families and their function in crustaceans

Protein	Members	Monomer mass (kDa)	Location of Hsps	Function
Hsp90	Hsp82, Grp94 and HtpG	82–96	Cytoplasm, nucleolus and nucleus	Is essential for growth in high temperature, facilitates polypeptides folding and promotes stability and regulates regulatory proteins such as transcription factors and kinases
Hsp70	DnaK, Grp78, Hsc70, Bip, Kar2, ssa, ssb, ssc, ssd	67–76	Cytoplasm, nucleus, mitochondria, endoplasmic reticulum	Modulate protein secretion and folding, import of proteins into organelles, tolerance to environmental, physiological and pathological stresses, cross-protection and degradation of immature proteins
Hsp60	GroEL, Hsp65, cpn60 and Rubisco-binding protein	58–65	Mitochondria	Chaperonin, assembly of oligomeric protein and folding of the monomeric protein and maintain growth in stress conditions
sHsps	Hsp20, Hsp27, HspB2, HspB3, HspB7, HspB9 and HspB10	18–40	Cytoplasm and nucleus	Protection of protein from irreversible denaturation during stress, sequestration of unfolded proteins into aggregates, storage depot for unfolded protein, apoptosis inhibition and stabilisation of the cytoskeleton

of Hsps in response to heat shock is an ancient and universal mechanism. Apart from sub-lethal or nonlethal heat shock that induces the synthesis of Hsps, exposure of cells to any kind of stresses such as heavy metals, salinity, chemicals, glucose analogs, amino acid analogs, microbial infection and plant-derived compounds that can also induce the production of Hsps.

The stressful condition arising from either biotic or abiotic factors can have harmful effects on the internal organisation of the cells leading to protein unfolding and increased susceptibility to microbial infection. The cells recognise the adverse accumulation of unfolding proteins that can be the result of a variety of stressors including heat shock, oxidative stress, toxic substances or heavy metals. They activate an ancient, highly conserved signalling pathway leading to the significantly increased expression of Hsps. Ananthan et al. (1986) showed that living cells injected with denatured proteins are sufficient to induce Hsp response in eukaryotes (Ananthan et al. 1986). Hsps through its molecular chaperone activity binds non-covalently with a hydrophobic exposed segment of unfolded proteins and prevents misfolding or aggregation of inappropriate polypeptides. Additionally, it also mediates the transport of immature proteins to choose an organelle to target for final packaging and repair or to denature misfolded proteins via the proteasome or lysosomes (proteolysis), which cannot be repaired.

In crustacean aquaculture, the bacterial, viral and parasitic pathogens co-exist with aquatic animals without causing the disease in an ideal or optimum environmental condition. However, the environmental condition might deteriorate very rapidly in either intensive or semi-intensive systems, causing stress to the cultured animals (De La Vega et al. 2004). Moreover, the Hsps have been shown to play a crucial role in the maintenance of structural integrity under osmotic stress (in case of change in salinity) by stabilising protein homeostasis under both abiotic and biotic stressful conditions. It has been also demonstrated that Hsps provide thermal tolerance and cross-protection against microbial pathogens by enhanced immune response and resistance against bacterial, viral and parasitic infection.

5.3 Type of Heat Shock Protein

The predominant class of Hsps, which are widely researched protein in crustaceans, comprises four major and broadly conserved families, that is, Hsp90, Hsp70, Hsp60 and small heat shock proteins (sHsps). These molecular chaperones boost the immune response to microbial infection by engaging in promiscuous interactions with a wide spectrum of immature and unfolded proteins (Kumar et al. 2023b, c) (Fig. 5.1).

5.3.1 Heat Shock Protein 70 (Hsp70)

One of the most well-studied and highly conserved chaperones in crustaceans is Hsp70. During normal physiological condition, Hsp70 are involved in de novo

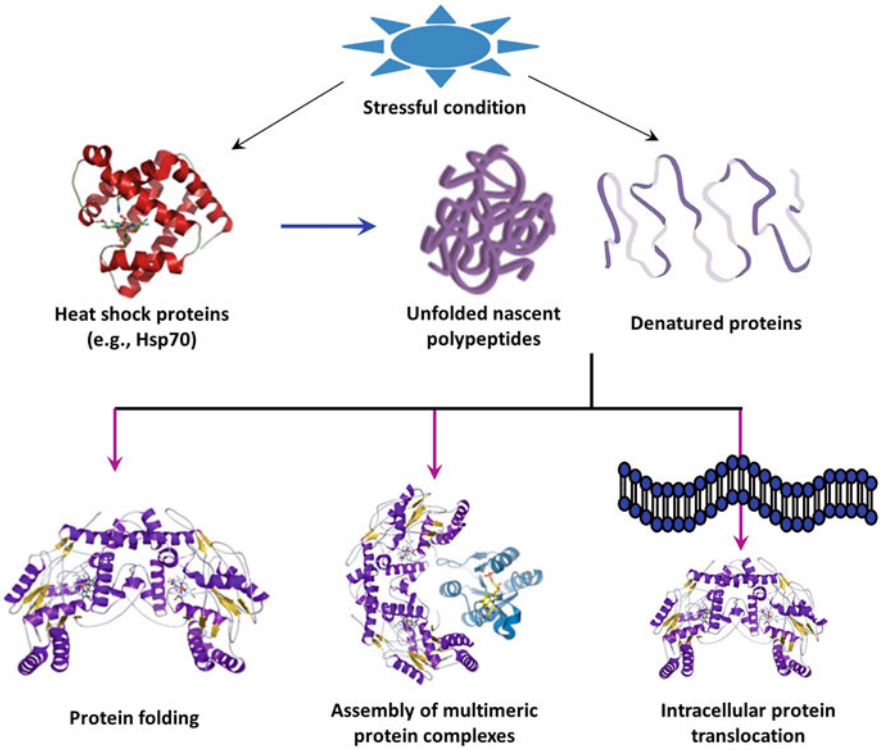

Fig. 5.1 Functional role of heat shock proteins as molecular chaperone

folding of polypeptides. However, during stressful conditions, it avoids the aggregation of denatured and unfolded proteins and mediates the refolding of aggregated proteins (Mayer and Bukau 2005). Three domains make up the bulk of the Hsp70 family: nucleotide binding domain (NBD), also known as the 44-kDa adenosine triphosphatase (ATPase) binding domain (ABD), bind to and hydrolyse adenosine triphosphate (ATP) (Flaherty et al. 1991). The variable 18-kDa peptide binding domain (PBD) or substrate binding domain (SBD) interacts with unfolded proteins and binds with extended polypeptides (Lo et al. 2004). Lastly, a C-terminal domain, which is 10-kDa and contains highly conserved sequences of EEVD terminal, is present in all Hsp70 families of eukaryotes. Cytosolic Hsp70, which includes the stress-inducible Hsp70 and the constitutively expressed heat shock cognate 70 (Hsc70) protein, makes up the majority of the Hsp70 multigenic family (which may be upregulated under stress condition), glucose-regulated protein 78 (Grp78), mitochondrial Hsp75 and heavy chain endoplasmic reticulum binding proteins (Bip). Interestingly, the introns are present in constitutive expressed Hsc70 protein genes, not in inducible Hsp70, suggesting that lack of introns in inducible form of

Hsp70 family allows rapid expression and accumulation of heat shock proteins during stressful conditions.

The cytosolic forms of the Hsp70 family's inducible Hsp70 and cognate Hsc70 are reportedly closely related (Yang et al. 2014). However, there are also few reports that suggest membrane-bound forms of inducible Hsp70 and cognate Hsc70. The Hsc70 proteins, expressed constitutively, were reported to play an important role in protein homeostasis under normal physiological conditions. In fact, the Hsc70 serve as housekeeping chaperones or professional protein that mediates important physiological functions such as membrane translocation, folding of newly generated polypeptides, protein complex creation and disassembly and the breakdown of poorly folded or immature proteins (Hartl and Hayer-Hartl 2002). The Hsc70 protein is always associated with immature or newly synthesised proteins and stabilises the unfolded polypeptides and forms a stable protein complex, for example, reduced carboxymethylated α-lactalbumin (CMLA), apocytochrome *c* and *Staphylococcus* nuclease thermally unstable mutant.

Hsp70 proteins are considered very important in maintenance of cellular homeostasis against environmental stressors. As compared to constitutively expressed Hsc70, the inducible Hsp70 proteins are involved polypeptide folding, cytoprotection, thermal and osmotic stress tolerance and enhanced immune response against microbial infection. Once Hsp70 is released from cells, in response to stressful condition, it acts as a messenger and mediates communication between the cells' inferior protein composition with immune system resulting in initiation of immune response against inappropriate polypeptides. Although the transcription patterns of Hsp70 and Hsc70 genes are quite different, they share common structural features, containing 44-kDa N-terminal ATPase binding domain (ABD), 18-kDa peptide binding domain (PBD) or substrate binding domain (SBD) and a 10-kDa C-terminal domain (Yang et al. 2013). Exposure of crustaceans to stress conditions resulted in increased production of Hsp70 which leads to increased thermal and osmotic tolerance and cross-protection against microbial infection (Bedulina et al. 2013), for example, induced Hsp70 increases thermotolerance in *Artemia franciscana* (brine shrimp) (Roy et al. 2019), *Macrobrachium rosenbergii* (scampi), *Homarus americanus* (American lobster), *Mytilus edulis* (blue mussel), *Exosphaeroma gigas* (isopod), *Scylla paramamosain* (mud crab) and *Gammarus pulex* (freshwater crustacean) which was also reported to provide cross-protection against microbial infection in *Artemia franciscana* (brine shrimp) (Roy et al. 2022), *Scylla paramamosain* (mud crab) (Huang et al. 2013), *Macrobrachium rosenbergii* (scampi) (Kumar et al. 2018) and *Litopenaeus vannamei* (whiteleg shrimp) (Junprung et al. 2017). Hsp70 also plays a crucial role in osmotic stress tolerance of aquatic species including *Homarus americanus* (lobster), *Apostichopus japonicus* (sea cucumber), *Argopecten irradians* (Bay scallops), *Tigriopus japonicus* (copepod), *Portunus trituberculatus* (Japanese blue crab), *Scylla paramamosain* (mud crab) and *Artemia franciscana* (brine shrimp). These studies demonstrate that Hsp70 proteins play a significant role in the regulation of cellular immune response resulting in enhanced protection in host cells from microbial infection.

5.3.2 Heat Shock Protein 90 (Hsp90)

Heat shock protein 90 are highly conserved molecular chaperone, reported from all living organisms starting from bacteria to humans, and play an important role in signal transduction, immune response and resistance to microbial infection. Under normal physiological conditions, Hsp90 were reported to be present in very high concentrations in the bacteria and eukaryotic cell cytosol, which gets further upregulated in response to stressful conditions (Welch and Feramisco 1982). Hsp90 also interacts with nuclear or cytoplasmic proteins, including tyrosine kinases, serine/threonine kinases and β-subunit of G protein. This molecular chaperone is distinct in several ways. Unlike Hsp70, Hsp90 binds with native-like proteins and does not bind with unfolded proteins (Jakob et al. 1995). In addition, Hsp90 performs multiple functions, for example, protein refolding, cell signalling and degradation of abnormal of immature protein under both normal and stressful conditions, and protects the animals from adverse conditions. There are several reports that suggest that Hsp90 can induce protection in host animals against abiotic stress, for example, increased Hsp90 secretion has been reported to induce immune response and provide protection against salinity stress in *Pennisetum glacum* and *Crassostrea hongkongensis*. In addition, Hsp90 induction has also been reported to be associated with repair and folding of polypeptides and provide protection against thermal stress in *Sterechinus neumayeri* (Antarctic sea urchin) and *Apostichopus japonicus* (sea cucumber). There are also few reports which demonstrate that increased expression of Hsp90 can induce protection against microbial infection, including parasitic in *Pieris rapae* against *Pteromalus puparum* infection, bacterial in *Acanthamoeba castellanii* and *Scylla paramamosain* (mud crab) against *Vibrio harveyi* and *Staphylococcus aureus* infection and viral in *Scylla paramamosain* (mud crab) against white spot syndrome virus infection.

5.3.3 Heat Shock Protein 60 (Hsp60)

Hsp60, a chaperonin family of proteins which has very unique distinct ring-shaped or toroid quaternary structure, was reported primarily as a mitochondrial protein (Xu and Qin 2012). The key protein after it is imported to the mitochondria, in an ATP-dependent manner, Hsp60, mediates folding of native polypeptides. Apart from playing a central role in defence ability against stress response, Hsp60 helps in the maintenance of cellular homeostasis. There is very limited information available about Hsp60 of crustaceans including *Anemonia viridis* (sea anemone), *Dreissena polymorpha* (zebra mussel) and *Litopenaeus vannamei* (whiteleg shrimp), and most of the studies have been focused on mammalian Hsp60. Moreover, most of the studies have indicated that Hsp60 have possible role in cellular processes and induce protection against abiotic including osmotic and thermal stress and microbial infection.

5.3.4 Small Heat Shock Proteins (sHsps)

Small Hsps, an ATP-independent molecular chaperone family, are apparently most diverse in structure and consist of monomers with molecular size ranging from 12 to 42 kDa. They are reported to contain three domains, namely, an amino-terminus, the carboxyl-terminal extension and conserved α-crystalline domain. The family of sHsps shows variation in their pattern during reversible interactions with proteins and protection with irreversible aggregation (Kriehuber et al. 2010). As indicated above, the sHsps in terms of sequence and size displays high heterogeneity and generates large dynamic oligomers, which are often composed of 24 subunits. They have α-crystallin conserved domain, which is commonly referred as prominent family member of eye lens α-crystallin protein. sHsps are functionally ATP-independent molecular chaperones that interact with large number of partially folded target proteins to prevent the aggregation during stress-induced unfolding (Mchaourab et al. 2010). Interestingly, the unfolded or immature proteins, which are often stored by sHsps, are refolded to their native structure in the presence of other molecular chaperones, for example, Hsp70 proteins. sHsps not only form soluble complex with unfolded polypeptides but also sequestered unfolded proteins into the aggregates, especially when unfolded proteins are massive in cells (a special trait of sHsps related to their passive holdase function) and their remodelling by ATP-dependent molecular chaperone. In invertebrates, sHsps are shown to provide cross-protection against biotic and abiotic stresses, for example, Zhu et al. (2013) reported that mRNA transcription of sHsp (Hsp20) from *Pieris rapae* was regulated by *Pteromalus puparum* parasitisation, which indicates that sHsps are potentially involved in host response to parasitisation. The brine shrimp (*Artemia franciscana*) have been demonstrated to possess ATP-independent sHsps, that is, p26, artemin, ArHsp21 and ArHsp22. These sHsps were synthesised in the embryos and reported to be induced by stressful conditions. Another study in *A. franciscana* reported that sHsps promote increased survival of *Artemia* cysts in quiescence and diapause state when they are exposed to stresses such as temperature extremes, desiccation, anoxia and radiation.

5.4 Functional Importance of HSPs

5.4.1 Protein Homeostasis

Exposure of animal to stressful condition, for example, extreme heat or cold, might have deleterious effects on growth performance and overall health of aquatic animals resulting in decreased tolerance to pathogenic microbes. However, organisms can compensate variations of environmental stress by modification of their cellular and molecular functions and structures (Peck et al. 2014). One of the major molecular responses that is activated in cells during biotic or abiotic stress is the enhanced production of heat shock proteins (Hsps). As molecular chaperone, Hsps stabilise unfolded nascent polypeptides or denaturing proteins and either refold those

polypeptides, assembled to multimeric protein complexes or translocate folded proteins intracellularly (Fig. 5.1) (Tomanek 2010). Hence, Hsps response might be considered an evolutionary and ecologically important factor for stress adaptation in aquatic animals. In addition, Hsps also plays an important role in setting the aquatic animals and enhanced tolerance limits to different stressful conditions. Nevertheless, the physiological responses to stressful conditions are not uniform among different taxonomical class of crustaceans. For instance, it has been demonstrated that few aquatic marine species are capable of inducing heat shock protein response against thermal stress. However, there are also some reports that suggest that these marine organisms simply do not have the necessary physiological mechanism or lost such capacity to produce heat shock protein response. Hence, more work is needed to unravel the Hsps mechanistic nature in aquatic invertebrate protein homeostasis.

5.4.2 Cross-Protection

Cross-protection also referred as cross-tolerance is described as enhanced tolerance to physiological perturbation following an initial transient and albeit different stress (Sung et al. 2011). An important cross-tolerance or cross-protective role of Hsps has made base for development of new therapeutic strategies that possibly targets overexpression of Hsps induced by a mild stress resulting in generation of protective response in aquatic invertebrate against lethal exposure to other stressful stimuli and diseases (Piano et al. 2004). There are reports that showed that microbial, osmotic or thermal stress itself induces the production of Hsps that leads to enhanced overall heath and tolerance to osmotic, thermal stress and microbial infection. For instance, injection of bacterial or virus particles to mud crab, *Scylla paramamosain*, induces the production of Hsps (Hsp70 and Hsp90), which induces the production of Hsps that are involved in enhanced immune response and cross-protection against microbial infection. Nonlethal heat shock has been demonstrated to induce production of Hsp70 in *Artemia franciscana* resulting in induced thermotolerance and enhanced resistance to pathogenic *Vibrio campbellii*. The heat shock proteins, for example, Hsp70, have also been reported to induce thermotolerance and induce protection from microbial infection in *Exosphaeroma gigas*, *Hyale hirtipalma* and *Gammarus pulex*; Hsp90, Grp78 and Hyou1 in *Sterechinus neumayeri*; and Hsc70 in *Penaeus monodon*. The salinity or osmotic stress influences the heat shock protein response in crustaceans, for example, salinity stress induces Hsp70 expression in *Potamocorbula amurensis* and *Scylla paramamosain* and Hsp60 in *Portunus trituberculatus* and provides cross-protection to aquatic animals against salinity and microbial stress.

There are few studies which showed that natural- and/or plant-based compounds are potential heat shock protein inducing (Hspi) molecules, involved in generating protective immunity in crustaceans against abiotic and biotic stresses (Kumar et al. 2020b; Kumar and Bossier 2018, 2019; Kumar and Roy 2017). Study demonstrated that pretreatment of *A. franciscana* with Tex-OE® (isolated from *Opuntia ficus indica*, prickly pear cactus) modulate the transcription of Hsp70 and enhances the

immune system tolerance in *A. franciscana* against salinity and thermal and microbial stress. In another study, the *A. franciscana* pretreated with phenolic compound, pyrogallol or β-hydroxybutyrate short chain fatty acid and poly-β-hydroxybutyrate (PHB) stimulates the production of Hsp70 and protects *Artemia* from pathogenic bacterial infection (*V. harveyi* and *V. campbellii*). There are also reports that suggest that feeding heterologous (prokaryotic Dnak) or homologous Hsp70 *Artemia franciscana* (brine shrimp) enhances the production of Hsp70 and enhances the survival of *A. franciscana* larvae upon challenge with pathogenic *V. campbellii*. Sinnasamy et al. (2016) demonstrated that feeding bacterial (*E. coli*) overexpressing heat shock proteins, that is, DnaK, GrpE and DnaJ (equivalent to Hsp70, Hsp40 and Hsp20), provides protection to Pacific white shrimp, *L. vannamei*, from *V. harveyi* infection.

5.5 Heat Shock Protein-Inducing (Hspi) Compounds: A Promising Approach to Combat Stressors in Aquaculture

An industry that produces food quickly is aquaculture. Since 1970, the industry has expanded at an average annual rate of 8.9%, compared to only 1.2% for catch fisheries and 2.8% for terrestrial farmed meat production systems during the same time period. However, the aquaculture industry's rapid growth has also resulted in more negative environmental effects. The industrial method produces large amounts of dirty effluent that is filled with uneaten feed and excrement. Aquaculture releases nutrients and different organic and inorganic substances, including ammonium, phosphorus, dissolved organic carbon and organic matter, into the aquatic environment. The receiving water bodies' environments deteriorate as a result of the excessive nutrient levels. Additionally, the drained water might introduce new disease species and boost the prevalence of pathogenic microbes. Additionally, the animal is more vulnerable to the spread of disease due to the damaged aquatic environment. For example, the crab aquaculture business has become socioeconomically and environmentally unsustainable as a result of disease outbreaks over the past ten years, particularly those caused by bacterial illnesses (Kumar et al. 2018, 2020a). According to estimates by the FAO, the cost of economic losses caused by disease outbreaks in the aquaculture sector is over US $9 billion year or almost 15% of the value of global production of farmed fish and shellfish (Kumar et al. 2019a, b; Tran et al. 2020). Antibiotics and other conventional techniques that have been used in the past to mitigate stressful situations have had very little success. Antibiotics are the most well-known and often used medications for treating bacterial diseases in the aquaculture of crustaceans. However, extensive use of antibiotics, particularly when used at subtherapeutic levels, can eradicate both harmful and helpful bacteria and inevitably leads to the creation of antibiotic-resistant strains that can cause environmental issues in ecosystems (Defoirdt et al. 2007). Additionally, the presence of antibiotic residues in commercial aquaculture products poses a risk to human health because they may cause allergic reactions and toxicity issues as well as change the normal microbiome in the human stomach.

Therefore, the creation of natural goods or substances derived from plants is required to reduce stressful conditions in aquatic environments and improve crustacean immune reactivity.

Natural remedies made from marine seaweeds and medicinal plants are being examined as potential substitutes for preventing stressful situations in the aquaculture of crustaceans. In addition to having antiviral, antibacterial and antiparasitic capabilities, plant-based chemicals are abundant in secondary metabolites and phytochemical compounds that improve crustacean health and growth performance by increasing feed intake and digestibility (Kumar and Bossier 2018, 2019). Additionally, it has been discovered that plant-based substances have the ability to naturally increase the heat shock protein in animals (Niu et al. 2014). These substances are also frequently referred to as heat shock protein inducers (Hspi) (Baruah et al. 2017). Cross-protection is said to be a function of the heat shock proteins, particularly Hsp70, in crustacean species. For instance, the general stress response in shrimps that upregulates the synthesis of Hsp70 shields the animals from subsequently occurring secondary heterologous environmental and physiological stressors (Roberts et al. 2010). Hsp70 is known to play a protective role in the molecular chaperone activity that prevents nascent polypeptides from misfolding, aids in the formation and dissolution of macromolecular complexes, facilitates co- and post-translational folding and controls translocation. Hsp70 is also known to promote resistance to microbial infection, induce thermotolerance, guard against osmotic stress, prevent oxidative toxicity and damage and protect against osmotic stress (Roberts et al. 2010). These findings amply demonstrated the important function HSPs play in host immunity and health. Therefore, using plant-based or naturally occurring heat shock-inducing chemicals would be a comprehensive strategy to develop resistance in the crustacean species against further harmful environmental shocks. Additionally, it might be a good candidate for usage as an anti-stress drug in the aquaculture of crustaceans.

5.6 Conclusion

The fastest-growing food-producing industry in the world, aquaculture, includes all methods of cultivating aquatic animals and plants in freshwater, brackish water or marine environments. Its average annual growth rate is 7.1%. Disease outbreak, which results in considerable economic losses each year globally, is seen to be a major barrier to the growth of this industry. The aquatic environment independent of their host is more favourable to pathogenic bacteria than the terrestrial environment, and consequently, pathogenic bacteria like *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., etc. can reach very high densities around the aquatic animals and can be problematic to several aquatic species (especially the larval stages), as in many cases it can cause mortality up to 100%. Conventional approach, such as antibiotics and disinfectants, had limited success in the mitigation or cure of aquatic diseases. Additionally, their usage in the aquaculture sector is under severe scientific and public scrutiny due to development of (multiple) resistance. Because of such

concerns, there is an urgent need for the development of alternative control technique in aquaculture, with the emphasis on prevention, which is likely to be more cost-effective than cure.

In keeping with such approaches, interest is developing in compounds/molecules that confer protection and/or enhance immune reactivity to pathogens in a manner conceptually equivalent to vaccines or immunostimulants. Hsps are the most abundant and ubiquitous intracellular proteins, present in all organisms. They perform a multitude of housekeeping functions vital for cellular survival. They also play key roles in eliciting immune responses against many diseases, leading to the formulation of strategies to fight infections. However, there are many obstacles for developing a strategy to induce (or deliver) multiple Hsps in the host (in open systems). Recently, several plant-derived compounds and/or natural products have been demonstrated to induce Hsps within the host and to confer protection against a variety of abiotic and pathogenic biotic agents. For instance, in the gnotobiotic brine shrimp *Artemia* host, it was observed that exposure of the shrimp to a plant-derived compound caused a significant increase in the resistance of the host towards pathogenic vibrios. Interestingly, the *Vibrio*-protective action of the compound was mediated by the induction of Hsps. Similar results have also been observed in other aquaculture species of commercial importance (i.e. freshwater prawn, carp). Hence, potential applications of Hsps and compounds inducing Hsps could become new protective modality for disease management in aquaculture production systems.

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Immunostimulants: Boon for Disease Management in Aquaculture

6

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Abstract

Aquaculture served as protein-rich food for rising world's population and a source of income for the population relying on fishery and fishery-related activities. However, aquaculture industry adopts intensive practices to combat the demand of rising population. And fish disease outbreak is a threat associated with the intensive practices in aquaculture industry, thereby causing production loss and economic inefficiency. Fishes and shellfish primarily depend on innate immune system to maintain its health condition. Immunostimulants are one of the prophylactic measures which are gaining wide interest for its effectiveness in fish disease management. Immunostimulants evolved as superior prophylactic measures as sources are many and inexpensive, primarily target innate immunity, are biodegradable and are effective against a wide range of fish pathogens. In this backdrop, a detailed understanding on types and use of immunostimulants in aquaculture is necessary, and this chapter discusses its different types, mechanisms, the efficiency of immunostimulant in enhancing fish and shellfish immune parameters, how it differs from vaccines, doses and time of application, efficacy and limitations.

Keywords

Immunostimulant · Mechanism · β-Glucan · Herbs · Vitamins · Limitations · Efficacy

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6.1 Introduction

Disease outbreak is one of the threats concerning world aquaculture. The rising demand for fish production due to the increase in world's population has pressurised the aquaculture sector to produce more. This has led the aquaculture sector to adopt high stocking density, feeding at higher rate, use of antibiotics and crowding of aquatic animal which ultimately resulted in stressful environment for culture organisms and disease outbreak. Shrimp industry across the globe is suffering economic loss due to disease outbreak. There is lack of effective remedial control against viral diseases, and specific vaccines for many diseases are ascertained. Fishes largely depend on non-specific defence mechanisms for their health, and thus, prophylactic measures like immunostimulants can be a solution. Immunostimulants are chemicals, drugs, stressors or action that enhances the non-specific or innate immune response by interacting directly with cells of the system activating them. According to Sakai (1999), immunostimulants are categorised based on their sources: algae-derived, bacterial, animal-derived, nutritional factors and hormones/cytokines. Studies on the use of immunostimulants in aquaculture are under progress, and many are tested and confirmed for its efficiency in aquaculture industry.

6.2 Differences Between Vaccine and Immunostimulants

In the history, immunostimulants were considered as substances that can enhance innate/non-specific immunity (Tafalla et al. 2013), but they can induce both specific/adaptive and non-specific/innate immunities (Ganguly et al. 2010). Immunostimulants basically increase innate immune response, but their administration along with vaccine enhances adaptive immune response which acts against the antigen. Immunostimulants are referred to as adjuvants if used along with vaccine. Immunostimulants such as β-glucans and TLR ligands can help to induce trained immunity in broodstock fish and their offspring; thus, they can serve as alternative to conventional vaccination (Zhang et al. 2019). Vaccines are nonpathogenic preparations of the pathogen that induces specific immune response in the host, enabling it to recognise and destroy the pathogen when it encounters it later (Thompson 2017). Vaccine acts on specific immune response and provides long-lasting protection in humans and animal. Vaccination imparts protection against one or two specific pathogens while immunostimulants can act against a wide range of pathogens. Generally, immunostimulants work on complement activation, phagocytosis efficiency and cytokine secretion. Immunostimulants like zymosan, glucans and lipopolysaccharides are considered as true immunostimulants as they act on non-specific immune system and do not require a specific immune mechanism (Barman et al. 2013). Though vaccines and immunostimulants act differently, their use in combination provides a better protection to the immune system of fish by facilitating the use of lower dose of vaccine. Immunostimulants increase the potential of vaccine (Wang et al. 2017). A brief account on difference between vaccine and immunostimulants is given in Table 6.1.

Table 6.1 Difference between vaccine and immunostimulants

Vaccine	Immunostimulant
Acts largely on specific immune response	Acts largely on non-specific immune response
One or two doses is required	Requires frequent doses
Provides long-term protection	Provides short-term protection in general
It can act specifically to one or two pathogens	It can act on a wide range of pathogens
Not suitable for smaller-size fish and shrimps	Suitable for smaller fishes and shrimps, for example, larvae
Costly and trained persons are required	Cost-effective process

6.3 Fish Immune System and Mechanism of Action of Immunostimulant in Fish Immune System

Fish immune system comprised of both innate and acquired immune responses. Both are associated\interdependent with humoral and cellular response mechanisms. Innate immune response is the first line of defence facing the pathogens entering a fish body. Fish possess physical barriers like scales, skin, gill's epithelial cell and mucus while exposed to aquatic environment. Pathogens may get prevented from entering these barriers; if crossed these barriers, the pathogen must encounter cellular and humoral component of innate immune response. Cellular component comprised of mononuclear (monocytes-macrophages and dendritic cells) and polymorphonuclear phagocytes (neutrophilic granulocytes). These cells facilitate phagocytosis of the pathogen by recognising the pathogen through PAMPs (pathogen-associated molecular patterns) with the help of PRRs (pathogen recognition receptors). PRRs in fishes are TLRs (Toll-like receptors), RIG-1-like receptors, C-type lectin receptors and NOD-like receptors. The binding of PRRs to PAMPs activates many cell signalling pathways in fish and induces production of nuclear factor-κβ, inflammatory cytokines, IFNs (interferons) and chemokines. After phagocytosis, the killed and digested pathogen is presented by macrophages and dendritic cells to lymphocytes. Non-specific cell-mediated cytotoxicity to kill xenogenic and allogeneic cells in fish is performed by non-specific cytotoxic cells (NCCs) and natural killer (NK)-like cells. Humoral responses in innate response of fish immune system are exhibited by different types of proteins and glycoproteins, namely, antibacterial peptides, lysozymes, cathepsins, chitinase, complement, chemokines, serum protease inhibitors (α2 macroglobulin, α1 antitrypsin) and acute phase proteins. Cytokines are secreted proteins, namely, chemokines, IFNs, ILs (interleukins), lymphokines and TNF, which are involved in innate and adaptive immune response of fish (Thompson 2017).

Adaptive immunity works based on specific response towards a specific pathogen. It memorised the pathogens and could eliminate them quickly upon reencountering. Adaptive immune response comprised of cellular response by

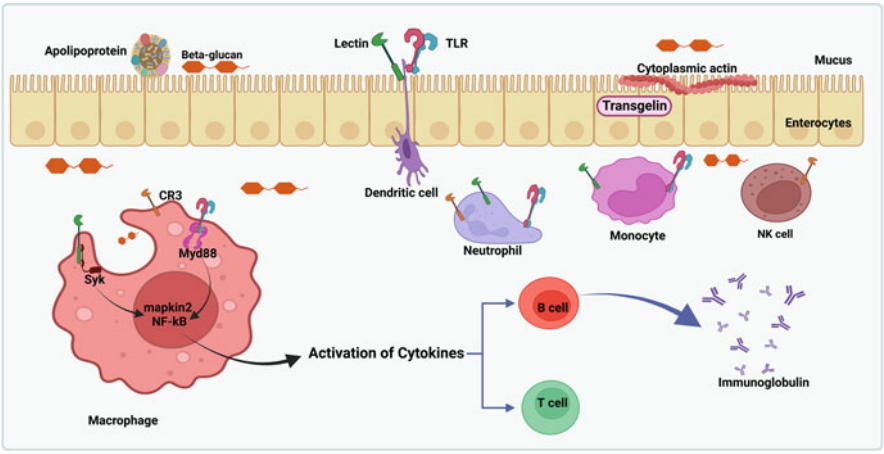

Fig. 6.1 Mechanism of action by β-glucan in fish immune system. (Adopted and modified from Machuca et al. 2022)

T- and B-lymphocytes and humoral response by immunoglobulins. The function of components in adaptive immune response is facilitated by major histocompatibility complex (MHC-I and MHC-II), B-cell receptors, T-cell receptors and recombination activator genes (*RAG1* and *RAG2*). MHC is a set of cell surface molecules to identify a foreign molecule. It facilitates the binding of appropriate T-cells on the peptide fragments of pathogen. The major function of B-lymphocytes is production of antibodies. Signals from T-helper cell induce B-cells to produce antibodies. Previously, IgM was the only reported antibody from fish; later IgD and IgT were also reported to be produced by teleosts. The function of antibody is opsonisation of pathogen and activates complement pathway and agglutination of pathogen (Thompson 2017).

Immunostimulants stimulate the activities of NK cells, T-cells, B-cells, phagocytic cells, macrophage, lysozyme, complement and inflammatory agents in fishes. They can elevate the activity of phenol oxidase, phagocyte, SOD (superoxide dismutase), respiratory burst and increase in total haemocyte count in shrimps. Some immunostimulants were found to activate Toll-like TLRs and their various functions including identification of several endogenous microorganisms and ligands and activation of ideal immune response for each antigen (Takeda et al. 2003; Heine and Lien 2003). β-Glucans can enhance the phagocytic activity of antigen-presenting cells (APCs) which is associated with the recognition of PAMP in β-glucans by PRRs in APCs. This may activate the downstream signalling cascade such as production of RNI (reactive nitrogen species), ROS (reactive oxygen species) and antibody (Hodgkinson et al. 2015). Figure 6.1 depicts how dietary β-glucan acts on fish body. Through diet, β-glucan reaches the intestine of fish. Apolipoprotein, transgelin and cytoplasmic actin 1 are some of the proteins present in enterocyte of the intestinal wall. TLR (Toll-like receptor) and lectins are present on immune cells. These proteins and receptors help β-glucan assimilation into the

systemic circulation. Lectin recognises β-glucan as a foreign particle and together with spleen tyrosine kinase (Syk) activates mapkin2 (mitogen-activated protein kinase) and NF-kβ. NF-kβ is also activated by TLR and Myd88 (myeloid differentiation primary response adapter protein 88). The activation of NF-kβ led to activation of cytokines. Cytokines help in proliferation of T- and B-cells and secretion of immunoglobulin by B-cell. Thus, the fish is in a state of enhanced immune condition (Machuca et al. 2022).

Shellfish lack specific immune system, and the immune defence mechanism is rendered by non-specific cellular and humoral components. Cellular component comprised of haemocytes (hyaline, granular and semi-granular cells). Activities of haemocytes comprised of recognition, melanisation, phagocytosis, cytotoxicity and communication between cells. Humoral component comprised of lectins (agglutinins: proteins or glycoproteins), prophenoloxidase (ProPo) system, antimicrobial compounds, serine proteinase inhibitors and clotting-by-clotting proteins. The presence of β-1,3-glucans, lipopolysaccharides and peptidoglycans can activate the ProPO system in shellfish. Immunostimulation can enhance immune parameters like haemocyte count, respiratory burst, PO (phenoloxidase) activity, phagocytic activity, agglutination titre, lysozyme and SOD (superoxide dismutase) activities.

Immunostimulants are recognised as foreign body while they enter shrimp haemocoel. Following this, haemocyte has pattern recognition protein (PRP) which recognises PAMP in the immunostimulant and activates cascade of cellular signals including ProPO system, phagocytosis, respiratory burst and melanisation (Fig. 6.2). This altogether leads to a state of enhanced immune condition in shrimps. In this enhanced immune state, if a pathogen tries to enter the haemocoel, there will be recognition of PAMP (of pathogen) and PRP (of haemocyte) leading to phagocytosis, encapsulation, melanisation, nodule formation and finally death of pathogen.

6.4 Fish Mucosa as First Entry Point of Immunostimulants in Fish

Mucosa is the first line of defence in fish body towards the entry of a pathogen. It is also the first layer of fish body which interacts while subjecting to immunostimulant diet or immersion treatment. External stimuli will be recognised by the mucosal tissue; thus, initiating alterations leading to production of cytokines, peptides and hormones altogether starts an immune response. Immune response in mucosa is controlled by mucosa-associated lymphoid tissue (MALT). There are four types of MALT in teleost, that is, skin-associated lymphoid tissue (SALT), situated at the skin; gill-associated lymphoid tissue (GIALT), situated at the gill; gut-associated lymphoid tissue (GALT), situated at the gut; and nose-associated lymphoid tissue (NALT), situated at the nose. GALT possessed cells (granulocytes, macrophages, lymphocytes, plasma, T-cell, B-cell, intraperitoneal lymphocytes, goblet cells, epithelial cells and neuroendocrine cells) to regulate immune response (Vallejos-Vidal et al. 2016).

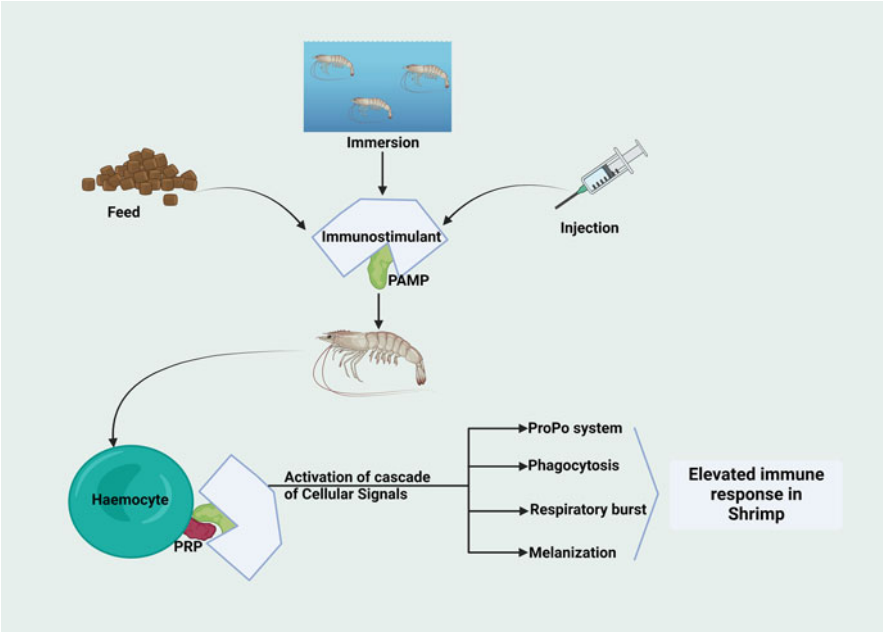

Fig. 6.2 Mechanism of action by immunostimulant in shellfish immune system. (Adopted and modified from Kumar et al. 2022)

6.5 Types of Immunostimulants Used in Aquaculture

6.5.1 Synthetic Chemicals

6.5.1.1 Levamisole

Levamisole possessed antihelminthic property. Levamisole acts to enhance phagocytic and NBT (nitroblue tetrazolium) reaction and increase antibody-producing cells. It also induces immune response in rainbow trout (Kajita et al. 1992) and *Pseudoplatystoma reticulatum* (Zanon et al. 2014). It stimulates non-specific defence mechanism in *Clarias fuscus* and provides protection against *Acinetobacter lwoffii* (Li et al. 2006). Bath treatment of rainbow trout in levamisole helps the fish in development resistant to *Yersinia ruckeri* (Ispir 2009). It enhances macrophage activity in barbel chub (*Squaliobarbus curriculus*) (Li et al. 2011). Dietary levamisole enhances immunity against infection of *Aeromonas hydrophila* and growth and survival in *Cirrhinus mrigala* (Bhatnagar and Lamba 2016).

6.5.1.2 FK-656

FK-656 is heptanoyl-y-glutamyl-(L) meso-diaminopimely-(D)-alanine. It is a peptide which is effective against bacterial infection, like *Aeromonas salmonicida*. It enhances the resistance of rainbow trout towards *A. salmonicida* and elevates

humoral antibody titres and splenic-producing antibody in yellow tail (Kitao et al. 1987).

6.5.1.3 MDP

MDP (muramyl dipeptide) is a *Mycobacterium* derivative and its chemical name is *N*-acetylmuramyl-L-alanyl-D-isoglutamine, derived from *Mycobacterium*. It increases resistance in rainbow trout against *A. salmonicida*. MDP-Lys increases the activities like phagocytic, respiratory burst and migration of kidney leucocytes (Kodama et al. 1993).

6.5.2 Bacteria and Derivatives of Bacteria

6.5.2.1 Bacteria

Vibrio anguillarum bacterin is an efficient vaccine for the fishes in salmonid group. It is an inactivated whole cell vaccine which can be administered orally, bathing or injection (Sakai 1999). *V. anguillarum* bacterin can stimulate immune responses in fish and shellfish. Haemocyte activity was found to enhance in black tiger shrimp (*Penaeus monodon*) while treated with *Vibrio* bacterin (Horne et al. 1995). It stimulates disease resistance in rainbow trout against *A. salmonicida* (Norqvist et al. 1989). *Clostridium butyricum* bacterin activates leucocyte, phagocytosis and superoxide anion production in rainbow trout, thus enhancing its resistance towards vibriosis (Sakai et al. 1995). *Achromobacter stenohalis* cells are reported to use as immunostimulants in fishes. It stimulates immune responses of kidney cells and complement activity. Inactivated form of *A. stenohalis* was reported to activate immune responses while imparting resistance against *A. salmonicida*. Dietary *V. anguillarum* cells (formalin killed) can enhance immune response, growth and protection against *Vibrio harveyi* in banana shrimp (Patil et al. 2014). Inactivated cells of *V. harveyi* biofilm cells can enhance growth, survival, immune response and health of *Penaeus vannamei* (Nagaraju et al. 2019).

6.5.2.2 Lipopolysaccharides

LPS (lipopolysaccharide) is present in cell wall of Gram-negative bacteria. They are effective at very low doses, often present in bacterin preparations as contaminants and used in immunising programmes. It can induce immune responses like complement alternative pathway, phagocytic activity and superoxide anion production of macrophage in fishes. It also enhances production of macrophage-activating factor and B- and T-cell proliferation in fishes. LPS can stimulate immune responses like haemocyte activity and phagocytic activity. Immunostimulatory effects of LPS in Atlantic salmon, red sea bream (*Pagrus major*) and goldfish were discussed in the review by Barman et al. (2013). Dietary LPS (derived from cell wall of *Pantoea agglomerans*) was proven to enhance growth and non-specific immune response in fry of *Oncorhynchus mykiss* (Skalli et al. 2013). It enhances innate immune response in rainbow trout and is effective in preventing *A. hydrophila* disease (Nya and Austin 2010).

6.5.2.3 Freund's Complete Adjuvant

FCA (Freund's complete adjuvant) contains killed *Mycobacterium butyricum*. It increases activities of fish immune system. In rainbow trout, it increases activities of phagocytosis, NK cell and respiratory burst and thus enhances defence against *V. anguillarum* infection (Kajita et al. 1992). FCA was found to provide adjuvant effect on *Pasteurella piscicida* vaccine (Kawakami et al. 1998).

6.5.3 Polysaccharides

6.5.3.1 Glucans

Glucans are long chain polysaccharides extracted from yeast. They are most promising and most studied immunostimulants in aquatic species. Immunostimulatory effects of β-glucans comprised enhancement of lysozyme activity, complement activity, phagocytic and bactericidal activity of macrophages and antibody responses (Thompson 2017). Glucans used as immunostimulants in fish comprised of yeast glucan (β-1-3- and β1-6-linked glucan) (derived from *Saccharomyces cerevisiae*) and β-1,3glucan (VST) (derived from *Schizophyllum commune*) (Pais et al. 2008). β-Glucans are most commonly used immunostimulants, and sources investigated include plants, yeast, seaweed, mushrooms shiitake, maitake, reishi, fungus and bacteria (Sirimanapong et al. 2015). β-Glucan (derived from cell wall of *S. cerevisiae*) can increase resistance in Atlantic salmon against *Vibrio salmonicida*, *V. anguillarum* and *Y. ruckeri* (Robertsen et al. 1990). Dietary administration of β-glucan increases resistance to infections in fishes. Injection of 100–1000 μg glucans/fish increases resistance against *A. hydrophila* (Selvaraj et al. 2005). Glucan-induced resistance was reported to transmit maternally (Misra et al. 2004). Dietary β-1,3-glucan was found to modulate immune response (haemocyte count, superoxide anion production and phenoloxidase activity) in *P. monodon* (Chang et al. 2000). Some of the recent studies revealed the immunostimulatory impacts of β-glucans, for example, improved growth, intestinal morphology, stress resistance and immunity on fish-crowding stress were observed in β-glucan-fed *Oreochromis niloticus* (Dawood et al. 2020) and improved thermal tolerance, immune response and disease resistance against *A. salmonicida* in golden mahseer (*Tor putitora*) (Akhtar et al. 2021), etc.

6.5.3.2 Peptidoglycan

Peptidoglycan is a polymer of β-(1-4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid, derived from cell wall of bacteria. The activity of peptidoglycans increases after hydrolysis. Peptidoglycans promote growth and enhance resistance towards pathogens; thus overall immunity of aquatic animals can be improved (Wang et al. 2017). Peptidoglycan-enriched diet was able to upregulate the antimicrobial peptides (AMPs) in the skin, gills, gut and liver of rainbow trout (*O. mykiss*) (Casadei et al. 2013). In another study, it was proven that its oral administration can enhance innate immune response in rainbow trout (Casadei et al. 2015).

6.5.3.3 Chitin and Chitosan

Chitin is a polysaccharide present in exoskeletons of crustacean and insect and fungi and yeast cell wall (Sakai 1999). They are mostly administered through dietary supplementation and can be used as feed additives according to the studies conducted. Injection or dietary administration of chitin may have immunostimulatory effect on several fishes. Dietary chitin and chitosan enhance parameters like lysozyme and superoxide anion production in *Cyprinus carpio* (Gopalakannan and Arul 2006). Chitin-supplemented diet can enhance myeloperoxidase and alkaline phosphatase activity in *Labeo rohita* (Kumar et al. 2019) and ProPo and RBA of haemocytes in *Macrobrachium rosenbergii* (Kumar et al. 2015). Chitin injection has been also proven to enhance immune response, for example, enhanced RBA (respiratory burst activity), phagocytic activity and natural cytotoxic activity in *Sparus aurata* (Esteban et al. 2000) and enhanced haemocyte count, RBA, PO and phagocytic activity in *Litopenaeus vannamei* (Wang and Chen 2005). Chitosan injection or feeding can enhance immune parameters in fish and shellfish. Dietary chitosan can enhance superoxide anion production, phagocytic activity, lysozyme and alternative complement in *O. niloticus* (Abu-Elala et al. 2015).

6.5.4 Plants and Animal Extracts

6.5.4.1 Ete (Tunicate) and Hde (Abalone)

Ete is an extract from the marine tunicate, *Ecteinascidia turbinata* (Ete), while Hde is a glucoprotein fraction of water extract (Hde) from abalone, *Haliotis discus hannai*. Injection of Ete (tunicate) improves the phagocytosis in eel fish and thus increased its survival while challenged with *A. hydrophila* (Davis and Hayasaka 1984). Also, injection of Hde enhances phagocytic activity in rainbow trout and thereby increases its resistance against *V. anguillarum* infection (Sakai et al. 1991).

6.5.4.2 Firefly Squid

Extract from firefly squid, *Watasenia scintillans*, was reported to enhance immune defence mechanism in rainbow trout. It enhances production of superoxide anion, phagocytic activity of macrophages and the lymphoblastic transformation of lymphocytes in vitro.

6.5.4.3 Herbs and Medicinal Plants

The use of several Chinese herbs as immunostimulant has growing interest in aquaculture recently. Chinese herbs can enhance non-specific immune response such as bacteriolytic and leucocyte activities. The use of Chinese herbs has advantageous over other immunostimulants as they are natural and contain many active compounds that are tested through long screening process in humans and other animals which ensure their safety towards development of drug resistance or residue (Dugenci et al. 2003; Khanna et al. 2007). Chinese herbs (*Rheum officinale* and *Polygonum cuspidatum*) contain chrysophanic acid or chrysophanol (1,8-dihydroxy-

3-methyl-anthraquinone), and they exhibit antibacterial and anti-fungal properties. In catla, dietary chrysophanic acid provides better immunity and enhances the upregulation of immune-related genes against *A. hydrophila* (Harikrishnan et al. 2021). Chinese herbs are effective against various fish pathogens and can be supplemented through fish diet. The use of extracts from *Astragalus membranaceus* and *Lonicera japonica* is also reported to be used as immunostimulant in cultured fish species (Ardo et al. 2008). *Cynodon dactylon*, *Emblica officinalis* and *Adhatoda vasica* were known to improve the immune system in *Carassius auratus* and reduced microbial infection (Minomol 2005). Effectiveness of these herbal stimulants was studied in *Poecilia sphenops* (Ardo et al. 2008). Herbal treatment enhances phagocytosis, RBA (respiratory burst activity), plasma lysozyme activity, etc. Astragalus extract was found to enhance phagocytic activity in Nile tilapia (Yin et al. 2006) and soft-shelled turtles (*Pelodiscus sinensis*) (Yin et al. 2009). Ginger extracts, *Azadirachta indica* (neem), *R. officinale*, *A. paniculata*, *I. indigotica*, *L. japonica*, *Lonicera* extract, *A. membranaceus* (roots and stems), *Isatis tinctoria*, *Polygonum multiflorum*, *Glycyrrhiza glabra*, *Angelica membranaceus*, *A. sinensis*, green tea, cinnamon and American ginseng were some of the herbs examined in many fishes (rainbow trout, crucian carp, Nile tilapia, common carp, rainbow trout, large yellow croaker, *Catla catla*, black rock fish, *Oreochromis mossambicus*) for their immunostimulatory effects. Some of the studies reported that herbal extracts provide resistance in fishes against bacterial infection, namely, *A. hydrophila* and *Citrobacter freundii*.

The use of plant extracts is beneficial as they could lower treatment cost and be environmentally friendly, biodegradable and less likely to induce drug resistance in pathogens and typically possessed multiple mechanisms of actions. Plant extracts can induce stress reduction, appetite, growth, immunostimulation, anti-pathogen and maturation in culture species. They contain several active compounds like tannins, alkaloids, terpenoids, saponins, glycosides, flavonoids, phenolics, essential oils, etc. Herbal extracts or plant extracts used in combination of different sources or single sources were studied for their immunostimulatory effects. The extracts from plants like *Gracilaria* and *Asparagopsis* can be used in place of antibiotics to enhance immunity in shrimps. There is a growing interest over previous decades for the use of plant extracts as shrimp immunostimulants. Herbal or plant extracts should be applied in advance of the onset of diseases, and this will ensure a prior protection and prevent loss (Ghosh et al. 2021). The use of *Syzygium cumini* (leaf) powder; *Psidium guajava* leaf powder; *Forsythia suspensa* methanolic extract; *Solanum nigrum* root ethanolic extract; *Gracilaria tenuistipitata*, *Gracilaria fisheri*, *Gracilaria vermiculophylla* and *Gracilaria verrucosa* extracts; and *Sargassum hemiphyllum*, *Sargassum duplicatum*, *Sargassum fusiforme* and *Moringa oleifera* (ethanolic) extracts as immunostimulants in shrimps was studied (Ghosh et al. 2021). A combination of extracts from nine different plants (*Annona squamosa*, *Aloe vera*, *Citrus aurantifolia*, *Azadirachta indica*, *Andrographis paniculata*, *Ocimum sanctum*, *Allium cepa*, *Coriandrum sativum* and *Psidium guajava*) was found to be effective in stimulating immune response of *P. monodon* against *V. harveyi* (AftabUddin et al. 2017). Mixing of different parts of five herbs (*Hygrophila*

spinosa, *Acalypha indica*, *Picrorhiza kurroa*, *Zingiber officinale* and *Tinospora cordifolia*) was found to enhance immune responses in *Fenneropenaeus indicus* and protects against *V. harveyi* (Rajeswari et al. 2012).

6.5.4.4 Glycyrrhizin

It is a glycosylated saponin with one molecule of glycyrrhetinic acid (animal product). It can enhance immune responses like lysozyme activity, phagocytic activity, RBA and lymphocyte count in fishes. Glycyrrhizin was tested in fishes like yellowtail and rainbow trout (Mehana et al. 2015).

6.5.5 Vitamins, Precursors of Vitamin and Trace Element

6.5.5.1 Vitamins

In fish, Vitamin C (ascorbic acid) is involved in many physiological functions including reproduction, growth and development, wound healing, response to stressors and lipid metabolism. Vitamin C at higher does was found to improve immune responses (macrophage activity, cell proliferation, NK cell activity, complement and lysozyme activity) in fish (Verlhac et al. 1996). It also imparts resistance against bacterial infections (*Edwardsiella ictaluri*, *E. tarda*, *V. anguillarum*, *A. hydrophila*, *A. salmonicida*) and parasitic infection (*Ichthyophthirius multifiliis*) as discussed in the review by Barman et al. (2013). It was observed that higher dietary inclusion of vitamin C in feed of immunocompromised *L. rohita* can neutralise immunosuppression due to aflatoxin B1-contaminated feed (Sahoo and Mukherjee 2003). Insufficient dietary level of vitamin C in juvenile shrimp leads to reduction of growth rate, reduction of feed conversion ratio and reduction of wound healing capacity and more prone to stress. It causes black dead syndrome in juvenile shrimp (Lightner et al. 1979). Vitamin C also imparts disease resistance in *P. monodon* postlarvae and juveniles against baculovirus, *V. harveyi*, and saline shock (Merchie et al. 1998). Dietary vitamin C was able to enhance non-specific immune response in Japanese eel (*Anguilla japonica*) (Shahkar et al. 2015).

Vitamin E can enhance immune response against infection in fishes, for example, Japanese flounder (*Paralichthys olivaceus*) (Villegas et al. 2006). Further, the deficiency of vitamin E was found to reduce immunity in trout against *Y. ruckeri* (Blazer and Wolke 1984). A combination of vitamin C and vitamin E was reported to have beneficial response in fishes (Wahli et al. 1998). Vitamin E supplementation in diet can enhance SOD, weight gain and total haemocyte count in *P. monodon* (Lee and Shiau 2004).

6.5.5.2 Precursors of Vitamin

Precursor of vitamin A like carotenoids was also reported to be used as immunostimulants. They play an important role in both cellular and humoral defence mechanisms in fish immune system. It can enhance phagocytosis, non-specific cytotoxicity, lysozyme activity and complement activity in fishes (Amar et al. 2004; Yanar et al. 2007). The dietary supplementation of carotenoid leads to increase

phagocytic, complement and lysozyme activities in several shrimps and fishes (Wang et al. 2017), namely, β-synthetic carotenoids and astaxanthin which can enhance non-specific immune defence mechanism in rainbow trout (Amar et al. 2004); carotenoid modulates immune defence in common carp and protects against *A. hydrophila* infection (Anbazahan et al. 2014).

6.5.5.3 Trace Element

Trace elements are also reported to enhance immune responses in fish and shrimps. Trace elements such as cobalt, chromium, copper, iodine, iron, molybdenum, manganese, selenium and zinc are important components of hormones and enzymes and play an important role in biochemical processes and physiological functions (Dawood et al. 2018). Supplementation with trace elements in diet was reported to give beneficial effects, namely, dietary Zn improves growth, survival and immune parameters of *L. vannamei* (Lin et al. 2013); dietary inorganic copper resulted in enhancement of growth and immune responses of juvenile beluga (*Huso huso*) (Mohseni et al. 2014); dietary selenium enhances growth, immune response and antioxidative response in *Carassius auratus gibelio* (Zhu et al. 2017).

6.5.6 Hormones

Growth hormone (GH) can stimulate immune system by activating immune competent cells like lymphocytes, macrophages and NK cells. The treatment of exogenous growth hormone (GH) in fish can enhance production of superoxide anions of leucocytes. Prolactin is a growth hormone which can enhance the immune-related activities, for example, production of superoxide anions of leucocytes in rainbow trout (Sakai et al. 1996). Lactoferrin is another growth hormone which can act as immunostimulants (Sakai 1999). It is a glycoprotein and proven to enhance non-specific immune responses of gilthead seabream (*S. aurata)* during dietary administration (Esteban et al. 2005). Cytokines can be used as immunostimulants as they act as modulators in the immune system.

6.5.7 Microalgae

Microalgae like *Arthrospira* (spirulina) *platensis*, *Haematococcus pluvialis* and *Chlorella* spp. are commonly used as nutritional supplements for fish and shellfish. They are rich in bioactive substances, proteins, vitamins, polysaccharides, etc. and emerging as good immunostimulants for fishes. Oral vaccines are developed based on microalgae like *Chlamydomonas reinhardtii* and *Dunaliella salina* and cyanobacteria. There are very few researches which evaluate microalgae as immunostimulant (Ma et al. 2020). Dietary supplementations of microalgae in fish and shellfish were studied in recent past, for example, *A. platensis* in diet leads to improve immune response and resistance against *Pseudomonas fluorescens* infection in *O. niloticus* (Mahmoud et al. 2018); *Chlorella vulgaris* can enhance ProPO

activity, haemocyte count and survival of *Macrobrachium rosenbergii* postlarvae to *A. hydrophila* infection (Maliwat et al. 2017).

6.5.8 Others

6.5.8.1 Nucleotides and Metabolites

Nucleotides and metabolites are potential immunomodulators. Oral administration was shown to give beneficial impact on immune functions, vaccine efficiency or disease resistance in fishes like rainbow trout, coho salmon, Atlantic salmon, common carp, hybrid striped bass and hybrid tilapia (Barman et al. 2013). Dietary nucleotides can enhance immune response in salmonids and protect against *Piscirickettsia salmonis*, *Vibrio anguillarum*, *Piscirickettsia salmonis*, infectious salmon anaemia virus and sea lice (Burrells et al. 2001). Dietary yeast ribonucleic acid can enhance phagocyte respiratory burst, thus providing protection to juveniles of *L. rohita* against *A. hydrophila* (Choudhury et al. 2005). Growth and health performance of red sea bream (*Pagrus major*) was improved while fed with inosine and low concentration of nucleoside by-products (NBPs) (Hossain et al. 2016).

6.5.8.2 Whole Microorganism

Whole microorganisms like yeasts (*Saccharomyces cerevisiae* or *Candida utilis*) and fungus (*Mucor circinelloides*) were reported to be used as immunostimulants. Their use in the form of oral administration or injection was reported to enhance both humoral and cellular immune responses in fishes. Its use imparts protection in fishes (channel catfish, rainbow trout and gilthead sea bream) against pathogenic bacteria (Ortuno et al. 2002; Rodriguez et al. 2004).

6.5.8.3 Lentinan, Schizophyllan and Oligosaccharide

They can enhance immune response mechanisms in fish encompassing phagocyte activity, lysozyme activity and complement activity.

6.5.8.4 EF203

EF203 is product derived from fermentation of chicken eggs. It enhances activity of leucocytes, phagocytosis and chemiluminescence in rainbow trout while administered orally and protects against infection of *Streptococcus* (Yoshida et al. 1993).

6.6 Factors Affecting the Efficiency of Immunostimulants

6.6.1 Immunostimulant Administration: Timing and Dosage

Immunostimulants are administered independently or in combination with a vaccine as adjuvant. It can be administered prior to occurrence of a disease to ensure protection against a predicted seasonal disease. As an adjuvant in vaccination,

immunostimulants can be administered prior to vaccination. Dosage and timing of each immunostimulant need to be determined properly. For example, alginic acid and glucan-treated sea bass showed significant increase in complement activity post 15 days of treatment, significant elevations in serum lysozyme and heat shock protein (HSP) concentration post 30 days and no difference among treated and control in terms of non-specific or specific immune parameters, growth, survival and conversion indexes post 45 days (Bagni et al. 2005; Li et al. 2010). Dosage of immunostimulants needs to be regulated properly in order to ensure its efficacy and potency. Efficacy differs according to dosage, that is, vitamin E when orally administered at different dosages showed different patterns of immunostimulation in gilthead seabream (Ortuno et al. 2000); levamisole at low-level (<500 mg kg^{-1}) supplementation in diet enhances weight gain in fishes after three weeks while higher-level (1000 mg kg^{-1}) supplementation leads to chronic toxicity like reduction in growth and feed intake (Alvarez-Pellitero et al. 2006).

6.6.2 Mode of Action

Immunostimulants can be administered orally, injection or immersion (bathing) in fishes. For example, oral administration of immunostimulants like glucans, EF203, lactoferrin, levamisole and chitosan was described. Oral administration is suitable for mass administration irrespective of fish sizes and is non-stressful. Injection of immunostimulants can be costly, time consuming, labour intensive and applicable only for large size of fish ($>$10–15 g in body weight). Intraperitoneal injection of chitin in fish was found to be effective to enhance humoral and cellular response while intravenous injection is ineffective (Maqsood et al. 2011). Immersion produces less innate immune response comparatively. It is cost effective in comparison to injection method and effective for intensive system, acclimatisation of juveniles; however, it can give handling stress to fish. Bathing fish in levamisole solution was reported, namely, carp immersed in levamisole solution showed activated phagocytosis and chemotaxis and enhanced protection against *A. hydrophila* (Baba et al. 1993). Immersion treatment of rainbow trout in glucan or chitosan solutions can impart protection against *A. salmonicida* (Anderson et al. 1995).

6.6.3 Particle Size

Immunostimulatory effects of chitin and chitosan were reported to be affected by particle size, that is, in vitro study showed that leucocytes of *S. aurata* can phagocytose chitin particles of size less than 10 μm but unable to do so with particle size more than 10 μm (Cuesta et al. 2003).

6.6.4 Species Being Tested

The efficacy varies according to the species tested, for example, chitin-supplemented diet at 25, 50 or 100 mg/kg body weight was able to enhance immune response in *S. aurata* while chitin supplementation at 25 and 50 mg/kg was unable to bring any alteration in *L. rohita* (Choudhury et al. 2005).

6.7 Efficacy of Immunostimulants

- Immunostimulants are widely used for fish health management.
- It provides protection to fish and shellfish prior to exposure of a pathogen and improves the survival post infection of a particular pathogen.
- Immunostimulant activity is broad-spectrum, that is, it protects fishes from several infectious diseases and provides protection against infectious bacteria (*V. anguillarum*, *V. salmonicida*, *A. salmonicida*, etc.), virus (IHN, infectious haematopoietic necrosis; YHV, yellow head virus) and parasite (white spot disease and sea lice).
- It stimulates activities of phagocytic cell, NK cells, complement system, lysozyme activity and antibody response in fish and shellfish and thus imparts protection against infectious diseases.
- It can stimulate both specific and non-specific immune system in fishes.
- Some immunostimulants are often used to increase efficacy of vaccine.

6.8 Limitations of Immunostimulants

- The use of immunostimulants is costly in few cases.
- They are not effective against all diseases, namely, some bacteria like *Renibacterium salmoninarum*, *Pseudomonas piscicida* or *Edwardsiella ictaluri* can resist phagocytosis by host cells.
- Overdose of immunostimulants can induce immunosuppression.
- Some immunostimulants failed to render protection against diseases.
- Immunostimulants are effective for short duration on non-specific immune mechanism; therefore repeated administrations are required.
- An immunostimulant may not be effective for all life stages of an aquaculture species; rather it requires different immunostimulants for different life stages.

6.9 Conclusions

Fish disease is a major concern of crop loss in aquaculture, and in order to combat this, many therapeutic and prophylactic measures are available. Development of effective vaccine requires time and cost while the use of antibiotics possesses concern for development of antibiotic-resistant bacteria. Moreover, fishes rely

more on their non-specific defence mechanisms. Under such circumstances, immunostimulants are better option as they have indefinite sources, primarily target non-specific defence system, are inexpensive in comparison to vaccines, are biodegradable and are reported to be effective against a wide range of fish pathogens. Immunostimulatory effects depend on the species tested, particle size, dose and timing of application and mode of action. Thus, optimisation of the above mentioned conditions is important. Research focus should be on safety level of immunostimulants, mechanism of action, stability in aquatic environment and synergistic effects while they are used in combination with other biological response modifiers and toxicological examination. For shrimps, efforts need to be given in compounds that do not give inflammatory reaction.

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Molecular Markers and Their Application in Fisheries and Aquaculture

7

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Abstract

Overfishing, pollution and human activities are making it harder to manage the fisheries. Recent developments in molecular technology and genome projects have produced a plethora of knowledge that may be used for genetic enhancement, decreasing resources and genetic variability. Assessment of genetic diversity and genetic structure is essential for exploring the conservation measures and management policies of populations. Over the past ten years, molecular markers have become more important in aquaculture due to DNA polymorphism. The quick analysis of fish germplasm has been supported by the use of DNA markers. When selecting a more useful marker for successful management, it is important to carefully analyse the principles, methods and applications of the many molecular markers that are now accessible. This chapter covers the basic ideas, specifications, advantages and disadvantages of numerous markers as well as their use and restrictions in fisheries and aquaculture.

Keywords

Molecular markers · RFLP · RAPD · AFLP · Microsatellites · SNP · Mitochondrial DNA · Microarray · RNA sequence · Fisheries · Aquaculture

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7.1 Introduction

Fisheries play a major role in world's protein consumption. Fish provides an average of 15% per capita of animal protein for 4.5 billion people. Unique health and nutritional benefits for fish consumers are gaining importance in both developed and developing countries. More than 10% of the world's population benefits directly or indirectly from fishing and related activities. Fishery and aquaculture activities are gaining importance globally due to the demand among fish consumers. Fish and fishery resources are declining with the growing population and urbanisation. Various strategies were planned and executed by fishery managers and policymakers with the aim of sustainable fishing and biodiversity conservation. Basic and applied researches in the field of fishery stock management and improved varieties are in line with the motive of enhancing the fishery. Stock management strategies are smoothly functioning with the intervention of emerging technologies in the fields of biotechnology and bioinformatics.

The stock management plan and hatchery management are highly interested in enumerating the stock variation and stock structure information. The identification of genetic variation among stocks or fishes helps in finding an inbreeding rate, information on migration events, mutation rates, bottlenecks, population expansion and selection. Population genetics is the distribution of genetic variation among and within populations at a particular time and space. Mutation, selection, migration and genetic drift are all important evolutionary factors in population genetic variation (Hansen 2003). The measurement of genetic variation could provide information on population history and phylogeography. Variations in alleles, genes, chromosomes and gene arrangements on chromosomes are examples of how genetic diversity manifests itself in populations.

In wild fish populations or aquaculture stocks, measuring genetic diversity is critical for sustainable management of these populations. The study on phenotypic variables for breeding and performance of animals helps to determine the genetic diversity indirectly. The study on physiology, behaviour, morphometrics, meristics, scales, otolith, skull and spinal cord are widely used for stock structure research (Ihssen et al. 1981). The study on phenotypic expression and its relationship with genes is tedious process, because environmental effect acts the larger impact. Laboratory-oriented researches on Mendelian traits in the studied species are not widely practised for wild stock or natural stock.

Molecular genetic studies are recognised after the evolution of DNA structure in the 1950s until those studies were limited with protein and enzyme level. Later in the twentieth century, fresh approaches of classifying, measuring and analysing genes emerged. With the advancement of next-generation sequencing and bioinformatics, the research in molecular genetics is blooming. The introduction of the PCR (polymerase chain reaction) method for DNA amplification has created the opportunity to study genetic changes in fish populations (Ferguson et al. 1995). Molecular techniques are utilised to explore the performance of brooders from wild stock and the interaction with cultured stock. The selection of markers for specific applications depends on the expertise of the resource person, the availability of lab space and

financial resources. As a result, periodic evaluations of the advancements in methodologies, applications and data interpretations are required. This chapter made an effort to give a summary of the molecular markers and the application of markers in the field of fisheries and aquaculture. The objectives of this book chapter are to examine the fundamentals of frequently used genetic markers and assess the prospective of molecular markers in fisheries.

7.2 Summary of Molecular Markers in Fisheries

Molecular marker studies are the milestone in the development of fisheries and aquaculture field. Allozyme is a conventional molecular method for examining genetic variation and diversity based on Mendelian theory at co-dominant loci. The method was discovered in the 1960s and widely used until the early 1990s. The application of mitochondrial DNA analysis in population and quantitative genetic studies appeared in the early 1980s (Avise et al. 1979). Due to the invention of the polymerase chain reaction (PCR), a variety of new technologies arose, ranging from methods for analysing length polymorphisms, such as microsatellites, to methods for sequencing the DNA of interest (Hansen 2003). In 1953, F. Crick, J. Watson and M. Wilkins discovered the double helix structure of DNA. Since then, it has become clearer how DNA and gene structure and function work, and this information is now being used to estimate genetic diversity. Technologies such as cloning, hybridisation and selective breeding were developed with the application of molecular markers in the 1970s. Sequencing technology and its automation were established with the information of molecular markers in the 1980s.

A molecular marker, also known as a marker gene, is a DNA sequence that is employed to "mark" or track a specific position also called locus on a certain chromosome. The study of how a characteristic or gene is inherited is made easier by genes that have a known location, a distinct phenotypic expression that can be identified using DNA sequencing methods. The markers must regulate an easily observable trait or be easily detectable by molecular methods, such as a microsatellite marker, in order to be easily recognised in the phenotypic expression (Williamson 2001). There are various molecular techniques available today for researching fish populations, but they may be broadly divided into two categories: protein markers and DNA markers. Allozymes, mitochondrial DNA and nuclear DNA are the major criteria utilised in stock structure and phylogeography investigations (Fig. 7.1) (Table 7.1).

7.2.1 Allozyme

The process of locating genetic diversity at the intensity of enzymes, which are directly encoded by DNA, is known as allozyme electrophoresis. Allozymes, which differ slightly in electrical charge, are protein variants created as a result of allelic variations. Allozymes are co-dominant Mendelian traits that are predicted to be

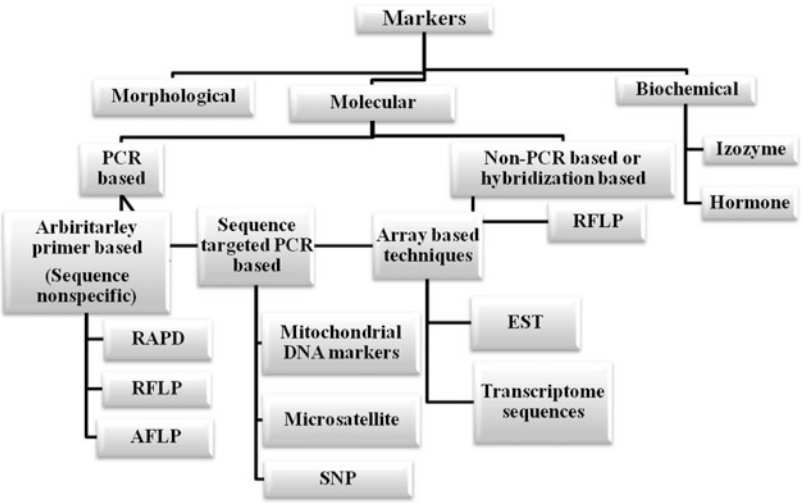

Fig. 7.1 Classification of molecular markers

transferred from parent to offspring. Allozyme variation gives information on single-locus genetic variation, and these data on locus genetics enable us to respond to a wide range of fundamental inquiries regarding fish and fish populations. Only those genetically distinct versions of an enzyme that are generated by various alleles at the locus and often identified by protein electrophoresis are referred to as allozymes. It is feasible to distinguish between various alleles using electrophoresis, in which tissue extracts are placed to a gel and an electrical current is applied, since allelic variation reflected in an enzyme may result in distinct qualities. Then, at a pace set by the enzyme's net charge and conformation, several allelic variations of the enzyme may transit over the gel. Finally, various alleles are recognised from different banding patterns using enzyme-specific histochemical staining, which is utilised to visualise specific enzymes.

Allozyme variation detection generally involves extraction, electrophoresis and detection. At the origin, tissue extracts are added to a gel-like solid support medium. A variety of media, including acrylamide, cellulose acetate and hydrolysed potato starch, can be employed as the gel for electrophoresis. The most effective resolving power is often achieved with acrylamide, but it is a hazard chemical and difficult to handle (May 2003). In contrast, cellulose acetate electrophoresis is the quicker and easier but also extremely sensitive and offers good resolution (Easteal and Boussy 1987). This type of molecular variation is the most extensively researched due to its ease of use, rapidity, relative affordability, minimal need for specialist equipment (Park and Moran 1995) and universal application of the approach. Allozyme analysis is theoretically appropriate for any source of soluble proteins, including bacterial cultures and animal fluids. It is simple to adapt the electrophoretic separation and staining methods from one species to another. After field samples have been gathered, there is no need for a marker development step, and analysis may begin

Table 7.1 Commonly used molecular markers

Marker type	Requires prior molecular information	Mode of inheritance	Type	Locus under investigation	Likely allele numbers	Polymorphism or power	Major applications
Allozyme	Yes	Mendelian, codominant	Type I	Single	2–6	Low	Linkage mapping and population genetics
Mitochondrial DNA	No	Maternal inheritance	–	–	Multiple haplotypes	High and conserved (based on mitochondrial gene)	Population genetic, phylogeny and DNA
Restriction fragment length polymorphism	Yes	Mendelian, codominant	Type I or type II	Single	2	Low	Linkage mapping
Random amplified DNA	No	Mendelian, dominant	Type II	Multiple	2	Intermediate	Species discrimination and population genetics
Microsatellite	Yes	Mendelian, codominant	Type II	Single	Multiple	High	Linkage mapping, population genetics, parentage analysis and pedigree analysis
Expressed sequence tags	Yes	Mendelian	Type I	Single	2	Low	Linkage mapping, physical mapping, population genetics, comparative genomics and pedigree analysis
Single nucleotide polymorphism	Yes	Mendelian	Type I or type II	Single	2–4	High	Linkage mapping and population genetics
Insertions/ deletions (Indels)	Yes	Mendelian	Type I or type II	Single	2	Low	Linkage mapping

for any species right away. It is also simple to evaluate allozyme profiles genetically. It enables the screening of several loci, sometimes more than 30–40. The method does, however, have significant drawbacks.

One significant problem is that allozymes cannot be used for small organisms due to the difficulty to read genotypes from small amounts of tissue. Freshness requirements for tissue samples are significant, as many loci manifest themselves in specific tissues. Therefore, the necessity for cryogenic storage to maintain enzyme function and the intrusive tissue sample preservation approach, which necessitates the sacrifice of the fish, are significant limitations. Specific alteration in the nucleotide sequence cannot affect the amino acid and hence cannot be reflected in protein electrophoresis. Additionally, a mutation in nucleotide sequence that affects one amino acid may not also affect the protein's total charge (Park 1994). Allozyme marker had a significant impact on fishery field and management measures, despite these limitations. Numerous studies that identify variations in protein allele frequencies between stocks have shown that genetic markers can be helpful in stock identification. Allozyme analysis is widely used in fisheries; it includes inbreeding and hybridisation, individual identification and inheritance, conservation measures, stock analysis and genome mapping.

7.2.2 Mitochondrial DNA (mtDNA)

To examine the gene directly or indirectly by finding variations in the DNA molecule was possible in the early 1980s. A minor fraction of the DNA found in cytoplasmic organelles called mitochondria. The following are the main characteristics of mtDNA:

(a) It is a haploid single molecule that is inherited maternally.
(b) The whole genome is transcribed as a unit.
(c) It does not recombinate and results in homologous markers.
(d) It is basically neutral and occurs as many copies in every cell.
(e) There is no clear modifying or repair process.
(f) Replication is constant, unidirectional and symmetric and has an appropriate size, without the presence of introns (Billington 2003).

The limitations of mtDNA's specific neutrality and non-recombining nature result in significant preconceptions in phylogeny regeneration (Hansen 2003). The mtDNA may be isolated from any tissue or blood and the genome has coding and non-coding areas (Avise 1994). The evolutionary changes in the mtDNA are comparatively faster than nuclear genome. Because of maternal transmission, mtDNA has a smaller effective population size, making mtDNA variation a more sensitive predictor of population events like selection, migration, bottlenecks, mutations and hybridisations. By analysing nuclear and mitochondrial DNA, sex-specific changes in gene flow might be discovered.

Because of the mtDNA's rapid pace of evolution, maternal mode of inheritance and tiny size, RFLP (restriction fragment length polymorphism) analysis is one of the methodologies for stock/population investigations (Ferguson et al. 1995). Variation in mtDNA may be studied primarily using two methods: (a) RFLP analysis of mtDNA acquired from fresh tissue followed by digestion with restriction endonucleases and (b) short segment mtDNA molecules produced by PCR amplification (Billington 2003). The first procedure entails the isolation of mtDNA, digestion and subsequent electrophoresis. The entire procedure required comparatively huge volumes of DNA material, which might be intrusive and fatal to tiny aquatic creatures. The second method employs DNA bands acquired by the southern blot process from total DNA digests. The approach gives the highest resolution and information in genetic variation easier and faster (Ferguson et al. 1995), but the key downside is that it is costlier and time-consuming process and huge amount of sample is required for accuracy in analysis.

Numerous studies have been done on mtDNA, and it has been shown that certain of the molecule's sequences are conserved between species. In order to analyse the identical mtDNA segments in several species, numerous sets of "universal primers" have been developed. *Cytochrome c* oxidase subunit I (*COI*), ATPases 8 and 6, *cytochrome b* (*cyt b*), NADH dehydrogenase subunits 4 and 5 and NADH dehydrogenase subunit 4 are the most frequently utilised mtDNA genes (ND5). COI and *Cyt b* among the four genes change significantly more slowly than ND4 or ND5. Because the universal primers for COI are so strong, it is possible to recover the gene's 5′ end from members of the majority, if not all, animal phyla. This gene offers two additional benefits. For finding inter- and itra-species prediction, barcoding and population genetic analysis of COI gene was widely used. Cytochrome *b* is widely used for population studies and genetic stock structure analysis (*Clupisoma garua*, Saraswat et al. (2014); *Tenualosa ilisha*, Behera et al. (2015). In fishes, ATPase genes have repeatedly been identified to have a high evolution rate of 1.3% per million years (Bermingham et al. 1997). In several fishes, ATPase8 and ATPase6 regions have been successfully used for phylogeny and phylogeography (*Osteobrama belangeri*, Singh et al. (2014); *Silonia silondia*, Mandal et al. (2016) Cronin et al.'s (1993) universal primers for ND1 and ND5/ND6 genes in mtDNA were employed in preliminary experiments with fish. Smaller segments of the mitochondrial genome, D-loop, have also been probed or PCR-targeted, and results suggest that for species comparisons, it is appropriate to focus on slow and rapid evolving' coding segments with six and four base cutters, respectively. The D-loop region is a non-coding segment of mtDNA, which is highly variable in mammals but has less variability in fish species. Studies using a variety of fishes (*Salmo trutta*, Hall and Nawrocki (1995); *Silago japonica*, Gao et al. (2019) have revealed that D-loop region has less diversity than the rest of the mitochondrial genome.

The use of mtDNA in animals, especially fish, is fraught with complications. The key drawbacks are the rigorous demand for fresh or frozen tissue, the requirement for large sample than other DNA approaches (Park and Moran 1995) and the low degree of allelic variation in some species and populations (Ferguson et al. 1995). The discovery of mitochondrial pseudogenes of species is an unwelcome fact for

population research (Zhang and Hewitt 2003). This finding has significantly reduced the utility of utilising mtDNA in population genetic investigations. Furthermore, mtDNA data has several significant drawbacks. For starters, mtDNA only represents a single locus. It represents solely the maternal lineal history, which may differ from that of the population or species as a whole. As a result, the inferences we make about stock or specimen history are sometimes extremely skewed, emphasising the importance of independent, genetic markers to carry out the mtDNA analyses. The effective population size of mtDNA is just one-fourth that of nuclear autosomal DNA, implying that mtDNA lineages sort significantly quicker and have a greater allele extinction rate.

There are several applications of mtDNA in demographic and evolutionary research for fishery science has paid close attention. For more than a decade, it has dominated genetic investigations meant to address issues about phylogeny and population organisation in fish. mtDNA studies in stock identification and mixed fishery analysis, hybridisation and introgression give crucial information for management and ranching measures.

7.2.3 Random Amplified Polymorphic DNA (RAPD)

The invention of PCR techniques makes the evolution in the genetic marker application in the field of phylogenetics. Using PCR, the technique known as random amplified polymorphic DNA (RAPD) can be used to detect variation in gene sequence. It entails using a single, arbitrary primer in a PCR reaction, which amplifies numerous distinct DNA products. The RAPD technology was independently applied in plants by Williams et al. (1990) and Welsh and McClell (1990). The RAPD and DNA amplification-based assay by a single primer used to detect nucleotide sequence polymorphisms and produces a discrete nucleotide product. Polymorphisms between individuals are observed by the differences between sequences and represented as the presence or absence of a specific band. The polymorphisms from the process are noted as dominant genetic markers.

The polymerase chain reaction underpins the RAPD technique (PCR). A target sequence of genomic DNA is amplified exponentially by using arbitrary primers, a DNA polymerase, dideoxy nucleotide triphosphates, magnesium and reaction buffer. The reaction is repeated in cycles that include denaturation, primer annealing and elongation. In the first step, the DNA is get denatured and proceeds as a single strand at 94 °C. In the next step, the temperature lowered between 40 and 65 °C causes the primers to anneal to their target sequences on the template DNA. In the last cycle, the extension step at 72 °C, the thermostable Taq DNA polymerase is most active. The nucleotide sequences from polymerisation were observed using Agarose gel electrophoresis.

The RAPD technology enables the rapid and efficient detection of DNA sequence-based polymorphism at a large number of loci. The merit of RAPD is that it does not require pre-sequencing of DNA. The RAPD analysis has been widely used for a variety of purposes, such as accession identification and classification,

breed identification and genetic diversity analysis. RAPDs have several advantages over RFLP and fingerprint because of their technical simplicity and without prior DNA sequence information. Requirement of template DNA is 10 ng for enumeration of polymorphism. A disadvantage of RAPD markers is that polymorphisms are detected only as the presence or absence of a band of a specific molecular weight, but the heterozygosity other than dominance results is lacking, and also there are some issues with data reproducibility.

The application of RAPDs in fish research has been hampered by these concerns. The major areas are population and quantitative genetics, identification of species and hybrids, phylogeography, linkage map, mapping of chromosome and genome and breeding analysis. Fisheries have used RAPD on a variety of species such as guppy by Foo et al. (1995), largemouth bass by Williams et al. (1998) and brown trout and Atlantic salmon by Elo et al. (1997). In *Oreochromis niloticus*, the effectiveness of identifying variation within and across strains was demonstrated using RAPD (Naish et al. 1995).

7.2.4 Amplified Fragment Length Polymorphism (AFLP)

AFLPs act as dominant, numerous markers, and alleles are confounded which is visualised using polyacrylamide gel (PAGE). It coalesces the advantages of RFLP and RAPD markers while overcoming their shortcomings. This is PCR-based and does not require a probe or prior sequence information, like RFLP does. In contrast to RAPD's problem of low repeatability, it is trustworthy due to rigorous PCR. The main benefit of this marker is that they may score high amount of polymorphisms through PAGE and does not require the information from the earlier study. AFLP appears to be very effective than microsatellite loci in determining an individual's origin among probable populations (Campbell et al. 2003).

AFLP analysis, like RAPD, provides for the detecting large number of loci throughout the genome in a very small period of time and at a cheap cost. The AFLP marker's dominant nature and inheritance aid in genetics for population or stock. The approaches are perfect for gaining genomic support for mtDNA investigations. The disadvantages are dominant markers; therefore only about half of them are usable for a particular backcross family. The technology is particularly challenging to examine due to the abundance of discrete fragment visibility along the polymorphic fragments.

Direct analyses of nuclear DNA (nDNA) sequence variation are the most current techniques to obtaining data important to fisheries and aquaculture (Brown and Epifanio 2003).

7.2.5 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technology developed by Alec Jeffreys in 1984 while researching genetic disorders. It is not commonly used

now, although it was the first genetic marker technology utilised for forensic and other uses. RFLP is defined as the presence of different alleles in particular locus coupled with restriction fragments of varying sizes. The molecular foundation of RFLP is that within a complete genome, nucleotide base substitution, insertion, deletion, duplication and inversions can remove or establish restriction sites. Genetic polymorphism is defined as inherited genetic variances among people that account for more than 1% of the normal population.

The RFLP approach takes use of these changes in DNA sequences to identify and analyse intraspecies and interspecies variation. The differences between two or more samples of homologous DNA molecules are caused by differing restriction site locations. The size of fragments depends on the cleavage with respect to endonuclease and differs between various organisms. The resulting dissimilarity pattern can be utilised to distinguish between species. The number and changing length of these pieces define the polymorphisms. DNA polymorphisms will be demonstrated to be a natural extension of Mendelian principles at the most fundamental level. Endonucleases are enzymes that break long DNA strands into small bits. Individuals differ in the distance between the cleavage sites of a particular restriction endonuclease and generate different size fragments.

RFLP is carried out in a number of steps, which are briefly described: First, DNA is extracted and purified from blood, saliva or other materials. Restriction endonucleases are used to digest the pure DNA, and the enzymes' identification sites are usually 4–6 base pairs long. The more fragments created by digestion, the shorter the recognised sequence. For example, suppose a short sequence of CTCG appears frequently in a DNA sample. The restriction endonuclease that recognises the CTCG sequence cuts the DNA everytime the pattern is repeated. If one sample repeats the CTCG sequence four times and another sample repeats it twice, the length of the fragments created by the enzyme will differ between the two samples. Gel electrophoresis is used to examine the restriction fragments produced during DNA fragmentation. Because the fragments are negatively charged, electrophoresis, which separates molecules based on size and charge, may readily separate them. The resulted fragments of DNA are placed in the electrophoretic gel and two electrodes. The fragments move towards the positive electrode when an electric field is introduced. Smaller pieces travel quicker across the gel, leaving bigger fragments behind, sorting out the samples into different bands. To make the DNA bands visible, the gel is treated with luminous dyes.

RFLP has been employed for a variety of genetic analytic applications. Some of the most important RFLP uses are the following:

1. To assess an individual's risk of hereditary disorders such as cystic fibrosis.
2. To map the entire genome as well as to offer disease information.
3. To identify particular genes associated with certain functions that are present on chromosomes.
4. Genetic-type detection -fingerprinting profiling and testing.
5. To ascertain or confirm the origin of a DNA sample, as in paternity testing or criminal investigations.

6. In genetic mapping, recombination rates are calculated to illustrate the genetic distance between loci.
7. Identifying a disease-causing mutation carrier in a family.

RFLP has been widely utilised in genome analysis techniques in forensic science, medicine and genetic research since its introduction. However, with the development of relatively easy and less expensive DNA profiling methods such as polymerase chain reaction, it has nearly become obsolete (PCR). The RFLP approach has several stages and takes weeks to complete, whereas techniques like PCR may amplify specific DNA sequences in a matter of hours. Furthermore, RFLP necessitates a large DNA sample, the isolation of which can be a time-consuming and arduous operation. PCR, on the other hand, may amplify minute quantities of DNA in a matter of hours. Because of these and other factors, the PCR approach has essentially superseded RFLP in most applications that require DNA sequencing, such as paternity testing or forensic sample analysis. Moreover, the Human Genome Project's discovery of single nucleotide polymorphisms has nearly eliminated the requirement for RFLP in disease status assessments.

7.2.6 Variable Number Tandem Repeats (VNTRs)

DNA sequences are replicated hundreds or even thousands of times throughout the genome of eukaryotes. These repeating segments are present in a specific pattern in the genome. They occur in tandem, differ in quantity at various loci and are distributed across the genome by different people. The replicate sequences are identified: (a) minisatellite DNA, with shorter repeat units of 9–65 bp, and (b) microsatellite DNA, with 2–8 bp long. They vary in that the repeat unit in minisatellite is relatively simple, but the overall length of the "locus" in microsatellites is significantly shorter. Most critically, microsatellites are substantially more abundant in vertebrate genomes. Minisatellites can either be multilocus or single locus. A multitude of aliases are used to describe these two markers. Based on the repeats, they are classified as short tandem repeats (STRs) and simple sequence repeats (SSRs). Mini- and microsatellite markers are the most significant population genetic techniques used in fisheries and aquaculture research.

Multilocus minisatellites: They are sequences made up of 9–65 bp tandem repeats with a size of 0.1–7 kb (Jeffreys et al. 1985). Many minisatellite loci are extremely variable and can be used to determine paternity or to identify particular families. Aside from the high amount of polymorphism, there are other significant benefits, including the creation of several relevant bands per reaction and great repeatability. Large sample numbers are employed; highly variable loci are less effective for population discrimination. Large numbers of alleles might further complicate data scoring and interpretation. The intricate mutation processes are another restriction.

Single-locus minisatellites: Single-locus minisatellite probes for fish were created for the species of Atlantic salmon and tilapia to make up for the difficulties in interpreting multilocus fingerprints (Bentzen et al. 1991). Blotting-based single-

locus minisatellite studies require a sufficient amount of superior DNA. The investigation begins with amplification of the repeated sequence, followed by visualisation through gel. After staining the gel with ethidium bromide, the results are scored (Galvin et al. 1995). Cloning nDNA bands from incomplete digestion using restriction enzyme is another option. The cloned sequences are robustly hybridise to Jeffreys probes and these are employed to probe entire DNA. Despite having preliminary technical difficulties, minisatellite probes have proven to be very effective in detecting genetic variations within and between fish populations, microgeographical population differences and reproductive success of farm escapees. Minisatellites are used in fisheries for genetic identity, parenthood and forensics and to evaluate the mating success and detect gynogenesis.

7.2.6.1 Microsatellites

A microsatellite is a basic nucleotide sequence that is replicated in numerous periods in the DNA of an organism. Because such repetitions are very variable, they can be tagged or utilised as a marker. Microsatellites are highly informative than allozymes, and mtDNA yet provide the similar analytical benefits. The knowledge required for analysis is essential based on the occurrences of the polymorphic loci across all approaches. Microsatellites are predicted to occur once per 10 kbp in fish species, whereas minisatellites occur once every 1500 kbp, implying that microsatellites may be more valuable for genome mapping investigations.

The idea that the anticipated amount of sequence divergence between units of interest is exactly proportional to the time since divergence is permissible since they are members of a group of highly variable, non-coding proteins that are assumed to be neutral. Co-dominant and mendelianly transmitted microsatellites are especially short repeating sequences of 2–8 DNA bases that are repeated up to 100 times at a locus. They serve as a notable marker for genetic analysis and PCR-based study because of their extensive polymorphism characteristics. Microsatellite length polymorphisms may now be scored in large numbers of people for genetic analysis within and across populations using current molecular technologies. Some microsatellite loci have a large number of alleles (>20), making them useful for purposes like parentage analysis from the mixed stocks. Other microsatellite loci contain fewer alleles and may be more appropriate for genetic and evolution studies. Primers designed for a certain species frequently cross-amplify microsatellite loci in closely related species.

Microsatellite markers offer several benefits compared to other molecular genetic markers, and they have increasingly supplanted allozymes and mtDNA. Because microsatellite loci are often small, the amplification using PCR is not tedious, and the amplified products are analysed by gels or automated sequencing. When compared to minisatellites, microsatellites are comparatively straightforward to isolate; sample DNA can be separated rapidly since labour-intensive phenol-chloroform processes may often be avoided with the simple DNA extraction procedure. Microsatellites' substantially greater genetic variation leads to improve a variety of applications (Luikart and England 1999). Microsatellites require only a little quantity of sample from the preserved or fresh sample without sacrificing animal; the small portions of

fin will also serve the purpose (McConnell et al. 1995). Furthermore, automated fluorescence sequencers have the ability to significantly increase frequency in analysis. Microsatellites are a strong alternative to existing marker systems for applications that need a high frequency of loci for quantitative trait loci (QTL) identification.

There are two methods for analysing microsatellites. The first requires investigating entire nDNA digests with simple repetitions (di-, tri- or tetra-nucleotide repeats). Instead, they are genotyped using polymerisation with specific oligonucleotides that are specific to the flanking regions as the microsatellite markers. Semiautomating PCR is simple. Gel electrophoresis is used to separate the resultant PCR products for better resolution by size in agarose gels or polyacrylamide gels. This approach has a considerable benefit than non-PCR methods since it can be applied with the tiny quantities of samples. The occurrence of "null alleles" is one of the major issues. The appearances of null alleles depend on the mutations in the primer binding sequences, not in the microsatellite DNA. The existence of null alleles at a locus presents serious complications, especially in individual-based studies like relatedness estimates and parentage analysis; therefore removal of loci with null alleles is generally practised (Hansen 2003).

Individual marker development for specific organisms/species is time consuming and expensive. However, in the cross amplification, the primers designed for one organism could also applied for other related organisms. Another significant drawback of microsatellite alleles is that PCR amplification frequently results in 1 or 2 bp variation in accuracy during separation in gel, and these extra bands are denoted as stutter bands due to incomplete denaturation process during polymerisation. The effect of PCR stuttering results in difficulties during ranking alleles. Trinucleotide and tetranucleotide microsatellites, on the other hand, often display little or no stuttering.

SSR markers were discovered using both traditional and high-throughput sequencing techniques. The traditional method, which involves Sanger sequencing tens of thousands of clones to find microsatellite repeats, is time consuming and more expensive. It uses enriched genomic library screening to produce species-specific microsatellite markers. High-throughput sequencing has replaced traditional methods as the predominant method for developing microsatellites (Fernandez-Silva et al. 2013). In several species, including rohu (Sahu et al. 2012), catla (Sahu et al. 2014) and ray finned fish (Luo et al. 2012), next-generation sequencing (NGS) is widely utilised to quickly detect molecular markers, including microsatellite markers.

Nuclear DNA has the most variability of any genetic marker and with specific area in both wild and farmed populations. Microsatellites are also growing extremely prevalent in forensic investigations, paternity and genetic similarity determination, mapping and ideal population size analysis. The effective portrayal of stock character and genetic variability among various stocks and populations is made possible by the use of SSR markers. Linkage maps are essential genetic tools for learning about the diversification of species. Song et al. (2012) created a sex-specific genetic linkage map utilising 567 SSR markers. Several studies have employed the SSR

markers for pedigree analysis and parentage assignment (Norris and Cunningham 2004; Borrella et al. 2011). The parentage analysis is crucial throughout the aquatic animal selection process since it improves the precision of the selection response, which is why it is employed for broodstock management.

7.2.7 Single Nucleotide Polymorphisms (SNPs)

SNPs are caused by either the transition or transversion events resulted in nucleotide substitutions; insertions/deletions of nucleotide also cause the polymorphism in single nucleotide. It is a type of single-copy DNA analysis (scnDNA) that detects individual nucleotides. PCR, microchip arrays and fluorescence techniques can be used to detect these variations. Genomic research and disease diagnostic markers are two major applications of SNPs in fisheries. They are called next-generation markers in fisheries since they constitute a major component of many gene chips. SNPs exhibit polymorphism within locus, which results in distinct alleles as a result of point mutation induced by insertion/deletions of a one base. SNPs are widely practised among other genetic markers since they are the most abundantly recognised markers in any species genome, exposing hidden variation that would otherwise go undetected using other markers and approaches (Rasal et al. 2017).

SNPs are abundant in non-coding areas across the genome. Within the coding areas, SNPs are either non-synonymous, causing an amino acid change or silent, resulting in phenotypic alterations through affecting mRNA splicing (Sunyaev et al. 1999). Because the majority of potential species in aquaculture lack comprehensive genomic sequences, SNP creation can be accomplished utilising advanced sequencing capabilities, such as restriction site-associated DNA sequencing (RAD-seq). SNPs have the potential to be exploited for linkage mapping.

Techniques for genotyping by sequencing (GBS) have created the groundwork for breakthroughs in improved seed production and stock management in fishery field. The decrease of genome complexity has aided in the identification of a vast number of molecular markers, particularly single nucleotide polymorphism (SNP). SNPs have been discovered using a variety of ways. SSCP (single-strand conformation polymorphism) and heteroduplex analysis, PCR product sequencing, random shotgun and expressed sequence tags (ESTs) are examples of smaller-scale approaches. SNPs have been utilised to identify viable brooders, characteristics of stock and strains. It also can be used to identify candidate genes for characteristics and quantitative trait loci (QTL) that are helpful in aquaculture. Genome-wide association studies (GWAS) look for relationships between SNPs and attributes in individual traits. However, a variety of QTLs can influence a variety of complicated characteristics. Using high-density markers, genomic selection (GS) includes predicting the breeding values of potential selection candidates regardless of the importance of their association studies. Effects of escapees on wild and hatchery populations are compared. Fishes raised in hatcheries are the result of intense selection for desirable features within a species.

After being released, they may cross with individuals who were born naturally, endangering the genetic stability and fitness of natural populations. Growth is the most commercially significant attribute in many aquaculture selection programmes; hence there have been many researches on the topic. *Salvelinus alpines* source populations utilised to establish aquaculture stocks have various ecological traits (Tao and Boulding 2003). Fishes have a wide variety of sex determination mechanisms. Sex may change during the course of a life in many animals, and this change can be influenced by social interactions and environmental factors. The growth rates of men and females might vary, which has effects on aquaculture. The Nile tilapia, *Oreochromis niloticus* (Garcia et al. 2016; Sansuwan et al. 2017), *Sparus aurata* (Loukovitis et al. 2012) and *Dicentrarchus labrax* (Martinez et al. 2014) are just a few examples of aquaculture species that regularly engage in sexual manipulation. Understanding the genetic underpinnings of stress reactions can enhance fish welfare and the quality of aquaculture production. Using GWAS, 26 SNP markers connected to the cortisol reaction in relation with high stocking were studied in trout (Liu et al. 2015). Like other agricultural practices, aquaculture raises epidemiological concerns and can hasten the spread of infections to freshwater ecosystems and new geographic regions. Understanding the genetic underpinnings of stress reactions can enhance fish welfare and the quality of aquaculture production. In genomic selection and domestication, compared to terrestrial farmed animals, most cultured fishes have relatively little domestication progress, although genomic selection can hasten this process. Norway has been actively engaged in selective breeding of Atlantic salmon since the 1970s.

SNPs are mostly utilised in aquaculture for genetic research and the discovery of diagnostic markers for different illnesses. SNPs have been found to be very promising in breeding programmes such as genome mapping, marker-assisted selection, strain documentation and testing, response of different haplotypes in relation to environment and pedigree and parentage analysis. The single nucleotide polymorphism is the most recently identified type of marker. These are changes at a single nucleotide on the chromosome, as the name implies. For example, a locus on one chromosome may have a C, whereas another chromosome may have a T. An SNP locus, like RFLPs, almost invariably has just two alleles. They are widely used and extremely easy to genotype. The advantages of SNPs are more abundant in the genome. The amplicons are very tiny using PCR. The identification is possible with degraded ancient samples. Polymorphisms are present in both coding and non-coding areas. Because of the abundance nature and the simplicity, quantification in genetic changes is accurate. Polymorphisms in codons have the potential to change the amino acid result in changes in protein structure or expression. Numerous samples can be proceed through a single plate or chip and the entire process might be mechanical. There are no stuttering produces.

The demerit are as follows: It is bi-allelic; its PIC (polymorphic information content) is smaller than that of microsatellite markers. Each marker provides less information. As a result, many more SNPs must be genotyped to provide the same degree of information about a DNA sample. It is more difficult to comprehend

mixtures. Multiplexes do not yet function. Currently, it consumes more of the DNA materials than STRs.

SNP markers are likely the most effective for discovering genes for QTL. Accurate genotyping also necessitates the use of specialised equipment. Regardless of the expense, SNPs are expected to be the upcoming markers of interest in omics era owing to their practically infinite potency and adaptableness to robotics. SNP markers are widely applied in identifying and analysing their associated quantitative trait loci (QTL) with trait-associated genes, a critical component of aquaculture genetics/genomics. QTLs are mainly unknown genes that influence performance factors important to breeders (such as growth rate and disease resistance). The process begins with creating a genetic linkage map which may be used to identify the relative chromosomal sites of QTL in a genome. The SNPs that are related to QTLs constitute the foundation of MAS in a selective breeding programme for effective aquaculture production.

7.2.8 Array-Based Platforms

Microarrays are properly built set of probes placed on an apparent for hybridisation of targeted cDNA. This technique gives detailed information on gene expression in different time and space scale with reference to environmental stimuli of species. This is one of the frequently applied procedures for concurrently measuring the expression pattern of thousands of genes in an organism, which eventually aids in understanding the transcriptome and proteome.

This method may reveal global patterns of expressed genes as well as find new genes connected with phenotypic traits. The array technique is a useful tool in applied science research in fishery field for quick identification of genes or protein and also helps in identifying economically relevant attributes such as genes associated with growth, feed conversation and disease management. DNA microarray occurs in two basic platforms in fisheries and/or aquaculture: expressed sequence tags (EST) or high-density arrays (HDA) comprise gene fragments identified on the chip, offering a "ready-to-use chip" to assess the contemporaneous expression of a reasonably frequent genes. Amplicons of cDNA fragments or synthesised oligonucleotides from ESTs are printed on glass slides or nylon membranes (Ju et al. 2007).

cDNA microarrays are widely used in various species such as European flounder (Cohen et al. 2008) and an African cichlid fish chip with approximately 4500 features (Renn et al. 2004). Approximately 16,006 cDNA were used to study the response in muscle damage for gravid trout (Salem et al. 2006). All of them are customised microarrays. Microarray technology has also been utilised to investigate the response chemical pollutants, toxicogenomic profiling and gene expression in atrophied muscles. The use of DNA microarrays in fish biology and aquaculture might be extremely beneficial due to the identification of new genes, their expression patterns and responses to diverse ecological stimuli.

7.2.9 Expressed Sequence Tags (ESTs)

The knowledge on the genomes of aquaculture-relevant fishes is crucial for getting thorough information regarding performance and production features. Despite the availability of techniques such as sequential analysis of gene expression (SAGE), scientists continue to choose ESTs as a preferred way for finding target genes in aquaculture. Several innovative sequencing technologies have recently been created that enable the production of expressed sequence tags using de novo sequencing of the whole genomic transcriptome. This has been one of the most widely used approaches for transcriptomic study in a broad variety of species, as well as fishes (Liu et al. 2006). In species such as Atlantic salmon (*Salmo salar*) and oyster (*Crassostrea gigas*), EST databases have been built (Venkatesh et al. 2005).

7.2.10 RNA Sequencing (RNA-Seq)

RNA sequencing (RNA-Seq) technology with significantly greater resolution than standard Sanger sequencing and microarray-based techniques was created, in addition to overcoming many of the issues given by microarray technologies, such as the limited range of detection. Next-generation sequencing tools are used in this procedure to directly sequence cDNAs synthesised from the RNA of interest.

The acquired reads are complemented to the known or reference genome to create a transcriptome map of the whole genome. The availability of high-throughput next-generation DNA sequencing (NGS) technology has completely transformed transcriptomics by allowing RNA study via cDNA sequencing on a huge scale (Liu et al. 2013). RNA-Seq data analysis incorporates two techniques based on the various sequencing technologies, sequencing yields and varied read lengths. The first method involves reference genome for mapping to reads, whereas the next method uses de novo assembly for organism of interest that do not have a reference transcriptome. As a result, a genomic scale map that includes both the transcriptional organisation and expression level for each gene may be created (Wang et al. 2009).

RNA-Seq is being widely used in zebra fish, catfish, Atlantic cod and rainbow trout for functional genomic studies. Using RNA sequencing, Salem et al. (2012) discovered 22 SNP markers and 1 mtDNA haplotype associated with growth characteristics in rainbow trout. Using RNA sequencing, Liu et al. (2013) investigate the whole genome transcriptome of rainbow trout subjected to diverse stress situations.

7.3 Practical Applications in Fisheries and Aquaculture

The issue of having a big amount and size of fish on hand during harvesting is what led to the use of genetic markers in aquaculture-based fishery management (Fig. 7.2). Although this strategy appears to be economically advantageous, it might ultimately lead to the eradication of the targeted group. The loss of fish genetic resources is a

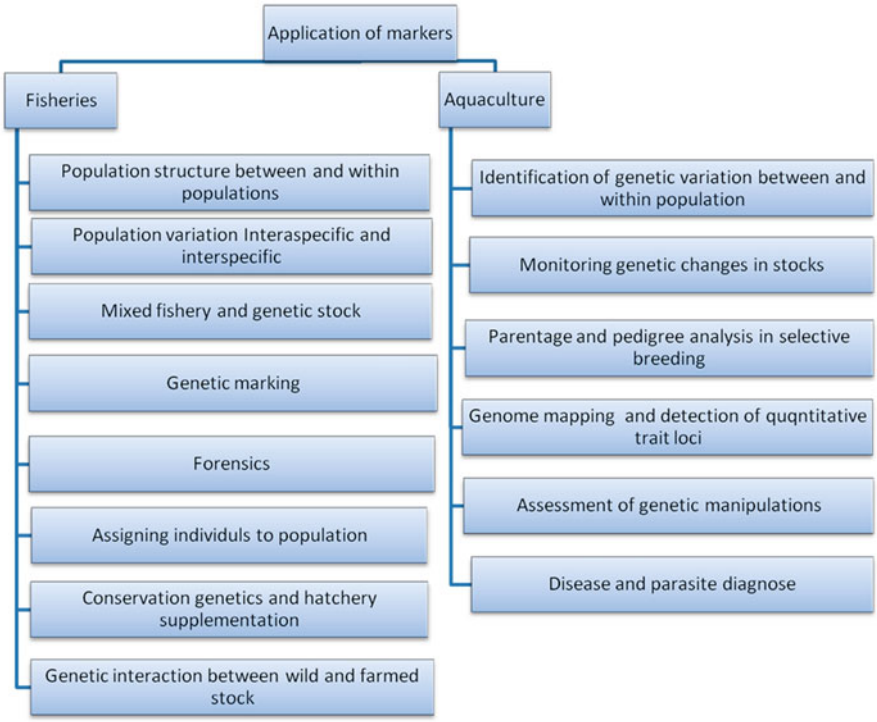

Fig. 7.2 Application of molecular markers

worldwide issue because it has the potential to reduce the genetic diversity of the biosphere. Supporting molecular genetics is consequently essential due to its essential role in the sustainable management of fishery genetic resources.

7.3.1 Fisheries

7.3.1.1 Interspecific Variations

The variation due to genome and phenome might be disconnected. The occasionally poor harmony among conventional taxonomic features is based on morphometry and the level of diversity due to gene discovered by biotechnology tools (Bernatchez 1995). Moreover, the neutrality of markers to selecting powers sometimes restricts the capacity to discriminate newly divergent taxa. But the features, such as morphological or meristic taxonomic appeals, depend on the environment, adaptability and predation. Thus, molecular genetic markers must be understood in reference to particular variance in evolutionary rates, between taxa and various sets of features (Carvalho and Hauser 1999). The information gathered through molecular marker about species or population should be validated with phenomic information.

It is critical to recognise the numerous species that coexist in mixed captures in order to successfully manage individual species. This may be difficult to recognise morphologically. Identification by exterior traits becomes more difficult when the value added, such as filleting, smoking or salting. In nutritional investigations, same considerations apply when seeking to determine food particles of fish predators. Another issue is identifying early life stages of fish, such as in egg, larvae and fingerling stages. The latter is critical in order to prevent the exploitation of endangered sturgeon species. Genetic variations across species are substantially greater than genetic differences among populations within a species. As a result, relatively tiny sample sizes may be employed based on application. Although the species are physically identical, they are identified as different species using molecular marker.

The majority genetic markers were employed to distinguish between inter- and intraspecific differences. Markers have been used to identify stocks; taxa, in different species such as RAPD employed in identification of taxa in tilapia and isozyme, were used in identification of species in Sparidae family (Bardakci and Skibinski 1994; Alarcón and Alvarez 1999). Preferably, more than one molecular marker should be pursued to drastically reduce the possibility of mistake caused by selecting animals of the similar taxa/species. Previously, allozymes were frequently utilised in identification of species, but these required more sample for analysis. A plethora of markers such as mtDNA, microsatellite loci, minisatellite loci and transcribed sequences are now available, to identify the genetic variation or alleles of interest from fresh or ancient samples. Due to the haploid form of inheritance, mitochondrial DNA is ineffective for recognising F1 hybrids. However, after identifying F1 hybrids with nDNA markers, the maternal parent may be determined.

7.3.1.2 Phylogeography and Phylogenetic

The major goal of quantitative analysis for genetic stock characterisation is to give objective groupings of people or samples. Making conclusions about historical processes impacting group connections (phylogenetics) and geographical patterns (phylogeography) might be the aims. With the beginning of mitochondrial genome studies to population and stock structure in the mid-1970s, it becomes popular. Lineage distribution analysis and interpretation often involve input from genetic marker-based analysis (Avise 1994). Phylogeography categorisation attempts to demonstrate links by recreating the evolutionary map of groupings or distinct genealogy. A hypothesis for phylogeography analysis is that gene or nucleotide sequence transfer across generation has been uncommon.

Molecular genetic data have become a common method for analysing species evolution and interactions (Avise 1994). Emerging applications include conservation and the utilisation of molecular information to supplement the conclusions of ecosystem and evolutionary processes. Mitochondrial DNA analysis has shown to be an effective approach for determining intraspecific phylogenetic trends in a variety of animal taxa. When compared to allozyme or nDNA, mtDNAs have numerous levels of insertion, deletion and slippage with lower number of individual

base in population, and primarily maternal inheritance is predicted to give more capacity to determine stock/population structure.

Furthermore, because of the absence of the recombinant process, the repair in nucleotide sequences is minimum, but changes in nucleotide sequences are frequent than single nDNA, making this molecule particularly helpful for phylogenetic analysis. In evolutionary studies, Avise (1998) noted that majority of research conducted thus far used analysis of mtDNA. mtDNA variation can determine connections between breed/strain/species that have separated for more than 8–10 million years (Peacock et al. 2001). The variation in sequences resulted in new speciation or stock variation with ancient relation. The histories of demography are analysed by evaluating different molecular markers. As a result, mtDNA is ineffective for reconstructing connections among population or species that split more than ten million years ago (Peacock et al. 2001). Nuclear DNA also provides the relationship of phylogeography.

Brown trout is one of Eurasia's most researched fish in terms of evolutionary lineages. The classic research by Bernatchez et al. (1992), using D-loop gene of mDNA, resulted in the recognition of evolutionary history using phylogeny linkages.

The evolutionary connection of the Pacific salmon and Pacific trout groups was also investigated utilising allozyme marker and mtDNA (Kitano et al. 1997). Williams et al. (1998) employed the combination of mitochondrial RFLP analysis to investigate the phylogeography of wild population of cutthroat trout, and phylogenetic trees were constructed using genetic distance matrices.

7.3.1.3 Population Structure of Population

The identification of stock of endangered or exploited fishes is crucial in both fishery conservation and management measures. The primary goal is to identify different stocks or meta population within a species that are reproductively separated population with others. The researches also focus to determine the breeding population and analyse the inflow of gene and gene pool contamination among genetic stocks and relationship on seasonal changes for alleles (Carvalho and Hauser 1995). There are several conventional stock discriminating techniques available, with varied degrees of effectiveness in separating breeding stocks.

Natural selection and evolutionary history can form physical characteristics, but environmental variations across populations, subspecies or species can also be impacted or determined. Stock identification based simply on morphological and meristic variations has become uncommon with the development of genetic approaches. The changes in phenomic traits based on external factors and disparity may not have a genetic foundation; thus these qualities may not give the inference on genetic and evolutionary links. Genetic approaches should be particularly successful, when genetic, morphological and meristic data are integrated; trustworthy information on genuine genetic differences and major environmental impacts on phenotypic traits may be provided (Cross et al. 2004).

To enable for statistical validation of observed differences, at least 50 number of animals should sampled from each site. It is also suggested that minimum

20 different loci should be explored. The life cycle and maturity stage of collected specimen also play a role in distinguishing populations. Anadromous salmon populations, spawn in different rivers, frequently combine the feeding grounds in sea. However, for marine fishes, different species spawn in the same broad region; surveying feeding aggregations is recommended instead. This indicates that when sampling is planned, the breeding ground and species biology should be aware (Cross et al. 2004). The stock of migratory fishes and its migratory behaviours should be taken into consideration, as well as related individuals by descent who may be kept away from sampling. Cross et al. (2004) recommended the collection of Atlantic salmon fry/parr along a significant portion of river (1 km in length). This also applies to sea trout and other fishes with a similar life cycle.

Since the early 1970s, allozymes are commonly employed to examine the structure of stock, and they continue to play a crucial part in the characterisation of intraspecific genetic variability. Allozymes have been mostly supplanted by DNA approaches, particularly SSR marker analysis (Hansen 2003). Although the DNA assumption has been proven right in numerous occasions, there is still no agreement on the best DNA procedure. Migratory fish populations were easily identified by protein-based markers (Utter 1991), but until the mid-1980s, there was minimal distinction between nearby marine species groups (Ward et al. 1994). Allozymes and microsatellites, for example, show comparable genetic variation in Atlantic salmon populations from two different locations (Sanchez et al. 1996). Microsatellite loci in cod have shown genetic structure of population from neighbourhood populations (Ruzzante et al. 1996).

The use of mtDNA in population genetic research has grown in popularity, notably in salmonid species (Nielsen et al. 1998). Because mitochondrial DNA (mtDNA) is very varied in most animals, it is an excellent marker for detecting probable genetic difference. Furthermore, because mtDNA is haploid and maternally inherited, it provides information that could not be retrieved using simply nuclear markers. RFLP analysis of mtDNA acts as a standard tool for population genetic investigations since the introduction of PCR (O'Connell et al. 1995).

Because the flanking primers have conserved sequences, cross amplification is possible for SSR markers in related species. The primers from one loci of microsatellite from bony fishes, for example, create bands in dogfish and lampreys (Rico et al. 1996). Enrichment techniques are used to improve the cloning efficiency of SSR sequences from target species. Minisatellites have been successfully created for salmonids and utilised to analyse population variations. Microsatellite loci are effective indicators of within-species genetic variability due to their substantial allelic variation. Microsatellites outperform mtDNA in essential regional scales for stock divergence and determining the sources of individual organisms in mixed pools of migratory fish. Microsatellites help to analyse closely related populations, including population belonging to same waterbody. If earlier samples are available, it is feasible to do a retrospective examination utilising PCR-based methods. This is especially relevant when fisheries have collapsed and populations have lost genetic variety as a result of a bottleneck (Ruzzante et al. 2001).

When examining the population of very abundant, extensively dispersed and high gene flow fishes, such as marine fishes, it is particularly critical to combine genetic with physiological, ecological and hydrographic information. Ruzzante et al. (1999) emphasised the role that oceanographic characteristics and known spatiotemporal variations in the spawning period may play as barriers to gene flow among neighbouring and frequently adjacent stocks of cod.

7.3.1.4 Identification of Genetic Stock

It is commonly understood that species are often separated into distinct subpopulations or stocks, which are parts of species which are exploited in fisheries. These components might appear as mixed populations in some situations. For fishery managers, long-term maintenance of mixed fisheries can be a serious challenge. These fisheries may include a variety of species, life stages and/or individuals from various populations. The primary goal may be to match individual fish to their population of origin. In the last decade, the information generated through molecular markers were extensively employed to analyse population structure and mixed stock in open water resources. A technique for identifying species or populations is known as genetic stock identification.

When a fishery consists of a couple of stocks, it is easy to calculate the involvement of each stock from mixed stock. For example, the Atlantic salmon stock from Europe and North America mingle to forage, because both stocks are geographically distinct with lot of genetic variations. Microsatellite markers are attributed to find the stock from more number of stocks involved in the mixed fishery with maximum precision minisatellite, Taggart et al. (1995), and microsatellite, McConnell et al. (1995).

7.3.1.5 Genetic Marking

Individual fishes have been marked by fishery scientists and managers for a range of attributes, such as inflow or outflow of species, determining effective population numbers and quantifying the involvement in mixed stock. The physical marking is the traditional practice, which is effective in recognising the individuals but not heritable individuals. As a result, a significant number of marks and marking efforts would be necessary for each generation. However, there are numerous situations in which we would like a heritable marker. Fish can be genetically marked in a variety of situations. For example, we would be interested in the influence of a breeding plan, the impact of each stock and its contribution in the next generation during mass breeding plan, the performance of progeny and the performance of wild and domesticated stock. In situations such as escapes, a method of recognising domesticated or farmed fish or their offspring would be extremely desired, because only heritable markers allow identification of offspring. But individuals possessing the relevant alleles are used in genetic marking, even though the rare alleles or novel alleles are also selected and heterozygote crosses are performed.

7.3.1.6 Forensics/Disputative Species Identification

The major difficulties that researches confront are managing open water stocks that are subjected to harvesting stress from both recreational and commercial anglers. Those physical features required for species identification must be present for examining and inspection personnel to independently verify individual species and populations. However, when considering distinct populations of a species or preserved individuals or processed fishes, the phenomic characteristics may be of limited help. In certain situations, the processed portions of fish are thought to be of a different species, and markers were utilised for precise identification. Forensics is the application to draw conclusions about historical occurrences, particularly in the context of fisheries, wildlife and conservation law enforcement. In appropriate utilisation of fish catch and resources, misreading of the composition of fish value-added products, illicit trafficking in fishery foodstuffs and inadvertent or planned undesired discharges or invasions into open waterbodies are examples of such activities. The question might be regarded one species, population or individual identification depending on the use. Forensics is concerned with major instances or hypothetical circumstances.

Identification of species: Forensic science requires species-specific diagnostic molecular markers for species identification. Allozymes, mtDNA and other nDNA markers have been utilised as molecular markers.

Identification of the population: A number of fish stocks are subject to stringent maintenance and conservation plans. Overexploitation of fishery populations is limited or prohibited, and recognition of specific stock is required. Protein markers, mtDNA, microsatellites and the major histocompatibility complex (MHC) are commonly employed as possible markers in this context.

Individual identification: Individual species identification may be required in some instances. The fishes are fishery products that may originate from an illegitimate catch or misuse of resources. In such circumstances, genetic markers with polymorphic alleles are required to directly and discreetly link the poached product or illegal practices.

Identification methods based on DNA are becoming more frequent and have won recognition in court processes. For a wide range of creatures, including commercially significant fish, methods for identifying fresh or ancient samples to species based on sequencing the portion of interest in genomic DNA have been developed.

7.3.1.7 Assigning Individual Fish to Populations

The assignment of individuals to specific population evaluated based on differences in alleles between populations has wide application in resource management and stock conservation. As a result, researchers have been able to determine the comparative role of individuals in original population and adopted population, stock status during migration, the contamination in gene pool, sex composition in population, hybridisation between population and novel speciation and the short-term and long-term changes in alleles. Assignment tests have also aided forensic studies since they may be utilised to determine whether an animal came from an illegal source. Microsatellite loci have mostly been used in assignment tests.

Although the resolution attained with these markers is frequently excellent, a significant limitation of using advanced markers in designation of individuals particular to population or stock depends on the accuracy of statistical analysis. The combination of advances in software with genetic markers allows for the determination of parent population from mixed stock and migratory stock for fishery biologist to explore the management practices. Due to greater genetic diversity across populations in freshwater and anadromous fishes than in marine fishes, assignment tests are most effective.

7.3.1.8 Interaction Between Fisheries and Aquaculture

Aquaculture, often known as "fishery augmentation," can help wild populations via restocking and/or sea-ranching. The idea of improving wild populations by stocking/ranching has received a lot of attention. Systematic conservation practices for migratory fishes or stocks by seed production and ranching in the natural environment and the feasibility study on ranching are long-established methods. Enhancing fisheries through ranching and recapture has to significantly impact the increased fish yield. Culture and wild stocks, on the other hand, share the same habitat, and interactions in various forms are nearly inescapable. The impacts of importation through augmentation and escapes from hatcheries of fish farms are genetically significant.

7.3.1.9 Conservation Genetics and Hatchery Supplementation

Stocking and ranching are the practices for release of fingerlings into the open water for stock enhancement as well as for conservation. The issue with decline species abundance in wild could be rectified by ranching or stocking programmes with the motive of restoration. Stocking hatchery-raised fish and purposeful introductions are critical elements of fishery programmes. Inbreeding, performance, dominance and adaptability of introduced populations are the main concerns during restoration. The impact of native population in such restoration programmes should be studied to explore the wellness of wild stock. The changes in gene pool are because of genetic alterations in captive animals in a variety of ways during the raising period. The primary sorts of genetic issues stated in relation to stocking include extinction, reduced heterozygosity in within and between population, uniqueness of stock, adaptation of species and viability and fixation of alleles. The well-known fundamental ideas for dealing with these challenges are related to identifying and maintaining genetic resources. Environment is a critical complement for wild fish with native stock. Following the selection of the donor population, a suitable number of founder fish are examined. Molecular approaches enable the monitoring of genetic diversity within and between wild and hatchery populations, assisting in the discovery of deleterious impacts on receiving wild stocks. Genetic studies that regulate gene flow, mapping of population distribution, create captive breeding programmes, deal with taxonomically challenging or complex grouping, estimate fitness variation, and help with management plan.

Using allozyme markers, the knowledge on the patterns of gene flow from hatchery population to native populations was obtained. For broodstock

management, DNA markers are an excellent monitoring tool. In this case, mtDNA can be especially valuable (Hansen et al. 1995). However, mtDNA exclusively traces maternal gene flow, and conclusions are based on only one haploid marker locus. Microsatellite markers, which are highly variable and repeated nuclear DNA loci, appear to be better indicators. Hansen et al. (2000) investigated the identification of the performance of hatchery stocked and native population in wild using mtDNA and SSR markers in Denmark's Karup River. Hansen et al. (2001) established the utilisation of VNTRs for tracking the gene flow and migration of native stock due to introduced population in a broader genetic context. In summary, utilising microsatellite and mtDNA markers combined appears to be the best method addressing the aforementioned problems in hatchery-supplementing programmes.

7.3.1.10 Genetic Interactions Between Wild and Farmed Fishes

Native species are subjected to genetic risks when they are exposed to the release of fish from hatcheries. The most often mentioned hazards are genetic pollution and loss of natural stock characteristics. Relationships between hatchery and native fish can be troublesome for a variety of reasons, including the following:

Gene pool contamination: Specificity of brooders based on hatchery demand, breakout of farmed stock into wild during natural disaster or purposeful introduction from aquaculture facilities and mate with native population can reduce genetic variety.

Competition: The feeding biology and behaviour of hatchery or harm reared population can harm natural populations by competing for food, place, habitat and mates.

Disease: The culture stock can raise disease and parasite levels, which can be passed on to native fish through escapees.

Genetic markers may be useful for detecting variations among farm and native populations, as well as resolving concerns regarding aquaculture farm escapes or releases into natural populations. Every year, it is estimated that millions of farmed fish escape from fish farms and end up in the wild. As a result, the topic of escapes in Atlantic salmon has received considerable attention. According to Clifford et al. (1998), the farm fishes reach breeding ground and inbreed with native salmon. Genetic markers like mitochondrial DNA and VNTRs revealed significant frequency differences between agricultural and wild populations. Farmed populations also had lower mean heterozygosity at the three minisatellite loci studied. The presence of mitochondrial DNA and VNTRs markers in juvenile population samples led to the conclusion that escaped culture salmon interbred with native wild stock. According to Skaala (1994), despite the fact that the number of escapees in Norwegian rivers exceeded the wild population, the occurrence of swamp was not recorded on a genetic basis.

7.3.2 Aquaculture

Molecular markers have great potential for aquaculture applications. Genetic markers could provide the information on performance of cultured and wild

populations, the identification of hatchery population in the wild, the detection of genetic contamination of wild population due to interbreeding, the parentage analysis and the detection of QTLs for marker-assisted selection programmes.

7.3.2.1 Identification of Genetic Variability Between and Within Stocks

It is critical to evaluate the information on genotype of farm-based strains and its wild donor populations, as well as the genetic diversity within and across hatchery strains. Several studies have shown a significant loss of genetic variety in hatchery stocks as a result of several variables such as a less number of brooders, the stress on adaptability of species from wild to cultured environment, selection pressure and hormonal manipulation during induced or other breeding technologies in farm condition and mating design. As a result, farmed stocks must be observed in order to identify the genetic alterations from naïve wild donors and to avoid or minimise any future modifications. Majority of cultured species have already published heritability and genetic correlation values for important features like growth and maturity. Molecular markers, also known as DNA fingerprinting, can be important tools for population identification and tracking of the prospective of farmed stock.

The molecular markers, from protein to SNPs, have been employed to determine genetic diversity across and within hatchery stocks. The use of mini- and microsatellite markers to solve aquaculture problems was recently presented, and salmonid fish were once again the first to be researched (Estoup et al. 1993). Sekino et al. (2002) used microsatellite and mtDNA sequencing to examine genetic diversity of farmed and wild Japanese flounder populations. Bártfai et al. (2003) used RAPD assay and microsatellite analysis to examine the Hungarian common carp brooders from farms. VNTR markers are widely utilised to detect the genetic diversity than the RAPD markers. They also assessed the genetic makeup from the populations of hatcheries and two rivers.

7.3.2.2 Monitoring Genetic Changes in Stocks

The presence of genetic variety is a necessary prerequisite for every breeding effort. As a result of the lower number of brooders in the base population, cultivated stocks are more susceptible to inbreeding and genetic variety loss. Even in vast populations, inbreeding can be triggered by behavioural, physiological and other causes. If stock enhancement is done with related species and if a less number of individuals monopolise, the breeding biology becomes substantially skewed and leads to random genetic drift and loss of diversity.

Allozyme tests have proven to be quite effective in determining the genetic influence of culture. Studies have showed that substantial levels of enzyme heterozygosity and the loss of variation are retained in farmed rainbow trout (Ferguson 1994). mtDNA markers and their diversity may be a more sensitive predictor of maternal history than allozymes (Ferguson et al. 1993). Because mtDNA is more sensitive to processes like genetic drift and the founder effect, it is excellent for detecting the repercussions of aquaculture founding and propagation. In rainbow trout, microsatellite markers have been utilised to reduce inbreeding (Fishback et al. 1999).

7.3.2.3 Parentage and Pedigree Analysis in Selective Breeding

Selective breeding has been extremely effective in enhancing productivity in a variety of farm species. The plan on different selection programmes required the details on their relatives, to improve selection accuracy and hence selection replies. As a result, optimum exploitation of farmed livestock frequently necessitates understanding of family ties (parents, sibships). Individuals should be individually identifiable when they are born, and their pedigrees should be traceable between generations.

Due to limited raising area in tiny commercial facilities, such constraints are frequently enforced. Information on pedigree structure can lead to a better rate of genetic improvement in this case since it is feasible to identify descendants of parents with desirable or undesirable qualities. Matings between related individuals must be avoided. Genetic knowledge can potentially enhance the strength of selection for sex-limited features. In reality, a molecular pedigree system should be able to differentiate between at least a few hundred families, and ideally, it should also be able to discriminate between individuals within families.

Mass spawning: Identifying the contribution of probable parents in a mass breeding is widely practised with the uses of genetic markers in management of brooders. Typically, only a small number of broods are employed in breeding, and some potential brooder parents do not appear to be spawn. It is impossible to evaluate the relative success of potential parents in these instances without the use of genetic markers. After spawning, parentage can be determined using minisatellite or microsatellite markers.

Communal rearing: As previously stated, the significant barrier to implementing efficient selective breeding for fish is that young animals are too tiny to be physically tagged. The family--based selection programmes required individuals to keep in the respective families until the fish are large enough to be individually tagged. Maintenance of families in respective rearing system is expensive, reduces the number of families accessible for selection and may result in environmental impacts shared by members of the same family. The use of DNA-based genetic markers can help to tackle this challenge. As a result, more families may be retained for selection programme without the requirement for separate rearing tanks at a young age. Genetic markers can be applicable to assess family/parentage identification and can be used to distinguish fish in mixed family groupings. Herbinger et al. (1995) were the first to show that using a modest number of microsatellite markers, it was possible to determine pedigrees in a mixed family rainbow trout population.

Walk-back selection: The individuals from the non-selected farmed stock in a breeding programme are the aim of walk-back selection approach. In general, when individuals are large enough to be marked, they will be physically tagged and biopsied, with SSR estimates based on the biopsy used to allocate people to families. During mass breeding plan, a more number of individuals in the similar age group were succeed using genetic markers. The largest fish is typed for numerous microsatellite DNA loci utilising non-destructive sampling. The next biggest fish is examined and only included in the brooders if it is more distantly related to the first than half sib. This process is repeated, with complete or half sibs discarded, until

a suitable number of possible brooders are discovered to minimise inbreeding. As a result, modified mass selection may be used without previous pedigree knowledge or the usage of numerous tanks. Over the last decade, most species' molecular pedigreeing approaches have concentrated on mtDNA, minisatellites and microsatellites.

7.3.2.4 Mapping of Genome and Quantitative Trait Loci (QTLs)

The genome maps help the mapping of undiscovered genes and markers, as well as the identification of genes in mapped species with comparable sections in another. They give clear proof of gene and marker placement. Thus, genetic markers may and have been employed to locate commercially significant quantitative genes. Genes are identified based on the inheritance as a same gene using a Mendelian manner. Alternatively, they might be sections of the genome that have been identified as accounting for a considerable fraction of the variance in a quantitative characteristic.

Quantitative trait loci (QTLs) are discovered by the genetic makeup and genetic marker in relation to specific traits or phenotype which are challenging to measure such as fecundity, feed conversion and quality of flesh. The aim of QTL mapping is to analyse the genetic variation and its relationship among stocks, to enhance the related economically viable traits.

The construction of a genetic map facilitates the identification of QTLs and is an expensive and skill-required process. The strategy entails extracting a large number of highly variable markers and then looking for correlations between each allele, alone and in combination and numerous production variables. Hypervariable microsatellite loci are anticipated to be the most suitable candidate of molecular markers. Based on existing mapping studies, they appear to be abundant in most fish species and extensively scattered in fish genomes. SSR markers are time-consuming and skill-required process but superior than RAPDs and AFLPs for genome mapping and QTL search (Sakamoto et al. 1999), because of co-dominant inheritance (Cross et al. 2004). A rainbow trout genomic map with roughly 200 microsatellites has been constructed and published (Fjalestad et al. 2003).

The application of molecular markers linked to QTLs in breeding programmes is known as marker-assisted selection (MAS). Although the word QTL technically refers to any gene that has an impact, in reality, it usually refers to significant genes because only these are big enough to be found in mapping. MAS will be most useful for measuring qualities that are challenging and costly to quantify. The resistance of disease and survival can be tested not only in selected individuals but also from the progeny. The quality of flesh attributes can only be assessed after the fish has been scarified. The markers are used as fixed effect during the analysis in the breeding programmes such as the standard genetic assessment methodology that yields BLUP (best linear unbiased prediction) EBVs for the residual breeding value and an EBV for the QTL impact.

7.3.2.5 Assessment of Genetic Manipulations

Polyploidy and gynogenesis are two strategies for genetically manipulating fish. Genetic markers have shown to be effective in conforming intended alterations.

Microsatellite loci, for example, are useful for verifying triploidy because their high polymorphism allows for the detection of ploidy individuals by the monitoring of respective allele genotypes at specific microsatellite loci. The discovery of male parent inheritance in gynogenesis is crucial and may be achieved most successfully by utilising genetic markers. Allozyme markers have been used in a few situations, but microsatellite loci, particularly single-locus ones, are well suited for such tests (Ferguson 1994). Highly polymorphic genetic markers can also be used to differentiate gynogenesis process by analysing the amount of homozygosity, which in the case of misogynes is predicted to be full. Finally, nDNA-based approaches are used to examine the integration, expression and germ line transmission of inserted genes in transgenic fish. This cannot be accomplished using allozymes or mtDNA markers (Ferguson 1994).

7.3.2.6 Disease and Parasite Diagnose

The molecular approaches for illness identification in aquatic species have grown in popularity. In many diagnostic laboratories, PCR tests have now become reasonably affordable, safe and user-friendly technologies. Molecular approaches are one of the widely accepted methods for demonstrating the exclusion of a particular disease pathogen for the goal of health certification in connection with life fish and fishery product marketing in international market. Many commercially relevant viral diseases of farmed finfish and shrimp have been diagnosed and detected using DNA-based approaches. Pathogens such as channel catfish virus (CCV), infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicemia virus (VHSV), viral neurological necrosis virus (VNNV) and *Renibacterium salmoninarum* have been tested for in finfish. In Japan, PCR was used to screen striped jack broodstock for VNNV, the selection of disease spawners as an efficient method of limiting vertical transmission of this deadly virus to the larval progeny (Muroga 1997). DNA-based detection approaches for penaeid shrimp viruses are now frequently utilised in a number of laboratories worldwide. Probes for illnesses such as white spot syndrome virus (WSSV), yellow head virus (YHV), infectious haematopoietic and infectious hypodermal and haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV), which represent the biggest danger to global shrimp culture output, are among these (Lotz 1997). The genes of the major histocompatibility complex (MHC) can potentially be selected. MHC molecules' functionality is determined by structural polymorphism, which is determined by variability in the DNA that codes for MHC molecules.

7.4 Choice of Markers

As molecular markers are becoming more widely available, they provide a universally applicable and objective method for comparing species, populations/stocks and individual identities. However, the selection of markers contains a large subjective component. As a result, the marker used will have a substantial impact on the divergence estimations acquired. A study's genetic marker and technique of analysis

must be adequately matched. Thus, it is critical to consider the following factors when selecting a genetic marker: (a) the time of evolution, (b) the rate and mode of evolution of the genetic marker and (c) the mode of inheritance (e.g. maternal, biparental) and expression (dominant, codominant). Because of the rapid rate of evolution, phylogenetic history will be erased and no common alleles. In contrast, slow-evolving genetic markers are ineffective for resolving connections between more recently separated populations and recently divergent subspecies or species. When dealing with contemporary gene flow, population isolation and recent speciation events, a highly variable marker with a rapid rate of evolution can considerably improve resolution. No one molecular approach has been shown to be ideal for resolving significant genetic challenges in fisheries and aquaculture stock management to date. Each approach has advantages and disadvantages depending on the balance of repeatability, cost and development time, as well as the detection of genetic variation. The following are the main factors to consider when selecting a marker (Cross et al. 2004):

- Sample availability: Protein/RNA analysis requires fresh or − 40 °C preserved tissue samples. Most tissues may be used to harvest DNA. Sample quality criteria are substantially less severe, particularly with PCR. PCR may be conducted on alcohol-preserved materials as well as dry samples such as scales or otoliths, although it is more challenging.
- Sensitivity: A marker must be sensitive enough to answer the inquiry. Choices of markers with adequate resolution can be made on more sensible grounds.
- Marker availability: The markers of choice should be easily accessible at the laboratory level.
- Rapid development and screening: Genetic markers were previously produced or may be transferred from previous work, and the ability to conduct rapid screening can result in significant resource savings.
- Multilocus vs. single locus? There is usually a trade-off between the practicality and accuracy of molecular markers. There is a contrast between multilocus DNA methods and single-locus approaches. Multilocus techniques are technically handy, but they have several significant flaws and limitations, such as the fact that the majority of the variation found is not heritable. Dominant inheritance is a major constraint. Because single-locus markers may be analysed as genotypic arrays, haplotypes with frequencies and gene genealogies, they are significantly more versatile, informative and connectible.
- Relative costs: The relative cost of developing and employing various markers should be evaluated. It is frequently claimed that multilocus approaches are more cost-effective, although this is questionable, especially when considering per unit information. When the benefit of comparing data sets is included, single-locus markers are even more cost-effective.
- Organelle and nuclear DNA: As previously said, mtDNA has a less population size than nDNA markers; hence mtDNA variations become taxonomic more quickly. A comparison of nuclear and mitochondrial genotypes can aid in the

identification of hybrid individuals, mating preferences and stochastic effects on variations whose ancestral taxa were polymorphic.
– Computation: With the use of recent NGS technologies, enormous amounts of data were produced. Utilising particular data relevant to our interests requires skilled personnel for bioinformatic analysis and interpretation (big data analysis). Prior to choosing a marker with high computing capacity, it is crucial to take knowledge person into account.

Choosing a certain DNA method generally includes a set of trade-offs. Some strategies are simple to use with little or no prior knowledge, but they are more difficult to comprehend than others. Methods for targeting mtDNA are very simple to create and understand; however, the information obtained only reflects a single locus. Other types of markers give more information from more loci but require considerable cloning and sequencing, which adds significantly to the lead time. Another trade-off is the creation of non-destructive markers. Unfortunately, most fish must be sacrificed, at least in the early stages (larva and juvenile). This might be especially important for species of particular concern when lethal sampling is not possible.

7.5 Concluding Remarks

The biological sciences, including fisheries and aquaculture, have been transformed by molecular markers. The above-discussed markers demonstrate a variety of applications in applied aquaculture and fishery research. Identification of stock or species, diversity, intra-specific relationships, stock enhancement, hatchery stock management, selective breeding, systematic and phylogenetics and legal applications are the areas in fisheries that all can benefit from the use of these markers as valuable tools. These molecular markers might be useful in areas like illness management plans and fish nutrition. Once again, whatever technique is best suited for a given case is determined by the level of polymorphism, the inference and analysis capability of different approaches and the time and resources availability. Markers that are widely used in research field are frequently used in fisheries and aquaculture for comparison of performance and cross amplification from one species to others.

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Applications of Electronics in Fisheries and Aquaculture

8

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Abstract

Aquaculture and fisheries have been showing constant year-on-year growth worldwide. The increasing human population on the other hand has been demanding more of it. Further intensification of catch and culture of fishes is becoming cumbersome as this requires constant monitoring, precise handling, timely executions and above all assuring sustainability. Thus, it is the very right time for adopting different modern electronical technologies and information technology (IT) devices to better handle fish farming. Because of the sector's labour-intensive nature, it has been difficult to regulate the ideal characteristics necessary for a good habitat for fish. As a result, in today's management system, the primary emphasis has been on constant monitoring of water quality measures, which is critical in fish development, well-being and illness prevention. To perform precise quality control, it is also necessary to understand the many chemical and physical properties of water, as well as other environmental elements and their interrelationships. Application of electronics may be

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indispensable in different aspects of aquaculture and fisheries like water quality monitoring, disease detection, early warning system, automated and demand feeding, maintaining aquaculture value chain, monitoring offshore open sea fish farms, optimizing catch and reducing bycatch, etc. In present time it is imperative to understand and link technological advances with fisheries. This chapter gives a brief view of how the various technologies can be integrated with aquaculture and fisheries for a holistic betterment.

Keywords

Cloud computing · Aquaculture · Internet of Things · Artificial intelligence · Drone and robotics · Sensor

8.1 Introduction

Aquaculture and fisheries play a crucial role in feeding the increasing population. Omega-3 fatty acids and high-quality proteins have significantly contributed to humans through aquaculture. This industry supports the livelihoods of billions of people. World's fish production, which is estimated around 179 million metric tonnes in 2018, is very essential for food supply systems. Out of this, 156 million metric tonnes were directly used for human utilization, giving 20% of the average global per capita animal protein intake to more than 3.3 billion people. There is a disengagement between the supply and demand chains due to the high demand for fish and fishery merchandise and the incapacity of gaining control of fisheries to provide that need. Fish and shellfish can be raised and produced through aquaculture for food and non-food uses. The majority of aquaculture production occurs in controlled or semi-controlled environments. Therefore, the only current way to guarantee production efficiency is through technological applications that manage population densities and improve culture management.

While this is happening, aquaculture's contributions to the world's fish production have grown over time, reaching 46% in 2016–2018 compared to 40.1, 42.6, 43.7, 44.7 and 45.1% from 2011 to 2015. Despite this expansion, technical developments are still required to address issues including disease outbreaks, water pollution, handling stress of fingerlings and broodstock and inadequate establishment techniques (O'Donncha et al. 2021).

It is necessary to utilize cutting-edge techniques to overcome some of these problems. The adoption of technologies to improve efficiency and sustainability in farm management, as well as using information technology (IT) devices and tools like autonomous tractors, drones, robotics, sensors, data analysis, etc., is surprisingly more significant in the agrarian sector than in pisciculture (Shamshiri et al. 2018). New firms in precision agriculture are also working to create technologies that will maximize production by managing every aspect of crop farming, including pest stress, moisture levels, soil conditions and microclimates (Abdullahi et al. 2015). It is impossible to overstate how quickly technology is developing, particularly when

finding solutions to issues by accelerating processes, eliminating the need for manual labour, automating repetitive tasks, etc. In the fields of artificial intelligence (AI), the use of IT, cloud computing and Internet of Things (IoT) makes a variety of technological aspects available. With the advancement of web-enabled smart gadgets, data on their use and the surroundings may be gathered and shared without the need for anthropomorphic interaction (Mattern and Floerkemeier 2010; Mustapha et al. 2021).

The latest sustainable aquaculture trends have shifted towards being preventative rather than curative, which relies on intelligence and automation (Lopez-Marcano et al. 2021). Fish death causes considerable losses to the farmers as it is nearly impossible to avoid altogether, but early warnings can be very helpful to act on (Billah et al. 2019). Due to the labour-intensive nature of the sector, it has been hard to control the optimum parameters required for a suitable environment for fish. Thus, in the modern management system, the prime focus has been on continuous monitoring of the water quality parameters, which is indispensable in fish growth, well-being and disease prevention. It is also imperative to know the different chemical and physical parameters of water along with the other environmental factors and their co-relations to perform accurate quality control (Xiuna et al. 2010).

This study looked for the potential benefits of technological breakthroughs for aquaculture. It is centred on cutting-edge technologies, that is, the IoT, cloud computing, AI, drones and robotics in aquaculture.

8.2 The Role of Cloud Computing, the IoT and AI

8.2.1 Cloud Computing

Cloud computing is mainly utilized in aquaculture to gather and store data created during production, processing and sales so that it can be processed and analysed later (Yongqiang et al. 2019). During the production of fish, aquaculturists frequently adhere to sets of rules and guidelines. Thanks to cloud computing and big data technology, massive amounts of data may be collected for process optimization and traceability. Cloud computing provides a good platform for application system integration, combining with CIA (confidentiality, integrity and availability), for creating an intelligent system of aquaculture to promote the best execution, feasibility and adaptability (water quality monitoring system, fish pathogen knowledge base and data intelligent processing system).

8.2.2 Internet of Things (IoT)

Building a network of actual computing devices that can receive and share information is known as the “Internet of things”. It is a web-enabled “smart device” (Internet of things (IoT) models: AWS, Amazon web service; IaaS, infrastructure as a service; PaaS, platform as a service; and SaaS, software as a service (Atzori et al. 2010)

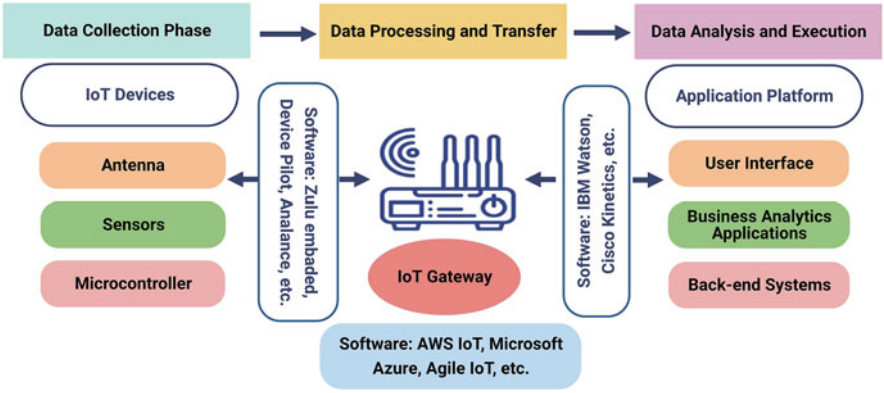

Fig. 8.1 Web-enabled smart devices

network that can gather and share data about the environment and usage without human input). An IoT system's typical composition is shown in Fig. 8.1. IoT is quickly being adopted for use in agriculture to attain high yields and greater levels of precision farming. Its application within the aquaculture industry is gradually becoming more well known. The peril tangled in the aquaculture sector is the difficulty which occurs due to the greater degree of uncertainty in water bodies and the amalgamation of climate factors, including sea level rise, ocean acidification, global warming, drought and flood. Due to this, it can be hard to supplant human intercession with an automatic IoT system entirely. According to new studies, artificial intelligence and machine learning algorithms can pre-specify aquaculture operations to prevent or reduce peril when IoT devices are connected (Yang et al. 2020). Thus, implementing the same in the aquaculture sector through the use of advanced IoT devices like remote cameras, bionic robots, intelligent sorting, energy-saving processing equipment and micro-nano sensors concerning aquatic items would benefit in areas like monitoring fishery, operation production, automatic inspection, keeping an eye on the environment, etc., to reduce manpower cost and boost productiveness.

8.2.3 Artificial Intelligence (AI)

One branch of computer science that seeks to enable robots to mimic living beings' cognitive functions and make decisions based on past experiences is AI. AI and machine learning are two academic disciplines that replicate natural behaviour.

The key to AI is the resolution of difficulties by interpreting an automated intelligent task. The usage of modern techniques of data science (AI models) is being rapidly accepted in various fields. At the same time, within the aquaculture segment, they have not been adequately controlled (Yang et al. 2020). Now a days, in aquaculture, digitalization, big data and deep learning (DL) allow machine

learning proficiency in emerging models to venture unexpected manifestations and secure productivity in the outcome. DL uses more extensive data sets to evaluate and interpret smaller data sets (Bengio et al. 2013; Schmidhuber 2015; Yang et al. 2020). DL in intelligent fish culture meticulously studied beneath species classification, behavioural analysis, feeding decisions, live fish identification, water quality prediction and size estimation and render high precision outcome than conventional approaches (Naddaf-Sh et al. 2018; Qin et al. 2016). Using machine learning and AI, issues were found and delivered into methods such as intelligent work and then interpreted into understandable forms after being processed for decision-making. Nevertheless, utilizing machine learning instead of depending on professionals can process and comprehend a variety of jobs more quickly. Research and development are required to enhance statistical algorithms and techniques used in AI and machine learning to provide great precision in prediction through eradication of outside influences.

8.2.4 Deploying and Areas of CIA Applications in Aquaculture

Aquaculture is expanding from traditional farming to mechanized aquaculture and eventually to automated processes due to the sector's recent rapid expansion and the invention of new technologies (Li and Li 2020). To obtain adaptive processing of data and lead to the appearance of intelligent aquaculture, the CIA can solve poor performance limitations and inadequate data in understanding massive and diversified big data in aquaculture (Yang et al. 2020).

Deep leaning (DL) has been used to assess better fish growth parameters, including width, length, abundance, quantity and other areas. Assessment of these factors is essential (Li et al. 2020; Saberioon and Císař 2018). Sampling through old-styled methods has shown themselves to be difficult and time-taking. To provide precise, immediate and consistent fish population census for offshore salmon farming, Yang et al. (2021) suggested an automated fish counting approach based on resolving the existing challenges. A co-convolution neural system was created as the front end for gathering feature data from different receptive fields in the hybrid neural network model they made. A deeper and broader expanded complexity of neural network is utilized as the back end to lessen the loss of physical and structural data during data transmissions. According to experimental findings, the suggested hybrid neural network model's counting accuracy can reach 95.06%.

8.2.4.1 Monitoring

Regulating the environment is crucial due to huge fluctuations, particularly in the open sea, which might significantly threaten aquaculture. Culturing certain species is time-consuming, difficult and dangerous if water quality irregularities are not timely identified. Concurrent surveillance of surroundings, quality of water parameters (pH, DO, ammonia, chlorophyll, temperature, nitrite, nitrogen, etc.) and fish behaviour are essential for aquaculture supervisors to act fast and minimize the threats. Online remote surveillance equipments, drones, sensors, underwater robots, buoys and other

devices can all be used to observe the aquatic environment. Wind direction, wind speed, speed of ocean current and wave height can also be measured away from shore (Anusuya Devi et al. 2017). Keeping track of water quality draws a lot of interest, and numerous new technologies have been introduced (Wei et al. 2020).

Moreover, fish behaviour reveals a lot about how fishes react to their surroundings. Fish behaviour changes are a sign of the simultaneous impact of environmental changes and stress on fish. Fish activity including swimming, feeding, stress response, population fluctuations, etc. can be observed by machine vision, acoustic or sonar technology (Bae and Park 2014; Føre et al. 2018).

8.2.4.2 Feeding

In aquaculture, feeding is a factor that significantly determines output costs and water quality (Li et al. 2020). The majority of large-scale farming use mechanized feeding techniques to minimize costs and save time. Regardless of the state of the water, automatic feeding machines function periodically; they only feed at the particular time on which they have been programmed. Thus, an automatic feeding device may lead to overfeeding and affect water quality (Liu et al. 2014). Deep learning and data fusion applications have great potential to improve fish feeding behaviour detection (Li et al. 2020). In a scenario where fish feeding is automated, the result of environmental monitoring technology is connected with an automated feeding control system. As a result, feeding in culture systems can be managed and changed or stopped entirely depending on the fish's behaviour or the water's quality.

8.2.4.3 Disease Detection

There is a wider variety of pathogenic species, including bacteria, fungi, parasites and protozoa. Some sick fish exhibit various outward symptoms, such as ulcers, exophthalmia, emaciation, lethargy and apparent granulomas, or no symptoms. Nematodes can also be found outside (e.g. hookworms). Alteration in skin colour, head swelling, protruding eyes, eroded fins, gills and ulcers are a few examples of outward indications of illnesses. It has been demonstrated that using CIA techniques may help identify most exterior fish abnormalities, which can indicate fish illnesses. The majority of illness diagnosis methods are based on pattern recognition theory, with three primary tasks delineated: (1) segmentation, which seeks to isolate the lesions from the surrounding portions of the image; (2) feature extraction, which seeks to gather as much data as possible about the region of interest (typically lesions); and (3) classification, whose purpose is to combine the data from the features into a precise identification of the illness (Barbedo 2014).

8.2.5 Contribution of CIA in the Aquaculture Value Chain

The market structure of any industry needs a perfectly functioning network (supply chain) with services that offer traceability and transparency to implement robust technology-based solutions. An efficient network is required to improve real-time data-driven decision-making that provides users with all the necessary information.

Fig. 8.2 CIA in smart farming to upsurge productivity

Technology-based solutions, like CIA, address problems with the product tracking and transparency supply chain network, including discrepancies, maintenance, contamination, audits and quality. IoT devices and AI combined with a safe and dependable cloud storage solution can enhance the capacity to precisely track and monitor product status at specific points all through the aquaculture value chain (Fig. 8.2). CIA in aquaculture has made a substantial impact on the entire aquaculture value chain, particularly with the creation of cloud-based traceability systems which make it simple to obtain customer awareness and product quality requirements. These involve the ongoing measurement of product transportation and storage conditions, climate forecasting, disease transmission, surveillance of dietary recommendations and market data at a broader global scale. Transparency across the whole value chain of food items, including fish, is now something customers and institutions expect. In addition to protecting public health, accurate traceability is required to deliver crucial details on the source of fish, the group and whether it was correctly kept and transported, as well as the present position at any given time. The fisheries value chain can be followed back and forth due to the traceability system.

8.3 Drones and Robotics in Aquaculture

Production through aquaculture is quite expensive when accounting for the need for feeds and labour. Aquaculture farms may produce large quantities because they are situated offshore in deep, open ocean waters. Only boats and ships can access most offshore fish cages because they are immersed in water. Farming methods vary in terms of their techniques, routines and infrastructures. Changing climate significantly impacts the standard of aquaculture produce (e.g. temperature and acidification of water), and it is currently a concern for the sustainability of world's fish production (Ahmed et al. 2019). Significant issues and worries also surround global food loss and waste. To avoid the problems stated and maintain the caliber of the production, it is also vital to handle things properly from production and harvest to consumption. High levels of regularity in stocking intensity, water quality observation and fish welfare, net cleaning and structural upkeep are essential to achieve profitability and sustainability in production (Bjelland et al. 2015). Large-scale

offshore aquaculture farms require significant manual work and close human contact to monitor and manage. Farm management needs to be monitored, observed and recorded regularly. Collecting data from the aquaculture site is essential for monitoring and integrating artificial intelligence (AI) for a smarter fish farm using technology like sensors and unmanned systems. For instance, when it comes to feeding management issues, feed expense accounts for the most significant percentage of the production period (Bakİ and Yücel 2017). Bait machines assist in automating the feeding process, but for it to be appropriately optimal, knowledge of the fish's degree of satiety or hunger is needed. The amount of fish appetite or the intensity of their eating behaviour can be inferred from information such as disturbance on the water's surface. The UAV (unmanned aerial vehicle) may gather this data using its camera sensors and transmit it to the storage in cloud for analysis of data utilizing AI services like deep learning methods to gauge the level of intensity of fish feeding. The baiting machine will get the analysis data to decide how much food to administer (Ubina et al. 2021a). Aquaculture production faces the difficulties mentioned above, necessitating the identification and implementation of different techniques.

Intelligent fish farming as a fresh scientific approach helps maximize and effectively utilize resources. The IoT, big data, cloud computing, AI and other modern technological advances will be fully integrated to enable sustainable development in aquaculture. With real-time data collecting, quantitative decision-making, intelligent control, accurate investment and individualized service, it achieves a new way of fish production (Yang et al. 2021). Numerous technology advancements are already available to enhance aquaculture productivity and administration (Cai and Juang 2020). For site surveillance, it is well known that unmanned vehicles with overhead cameras, sensors and processing power are much more viable (Ubina et al. 2021a). Goals of precise fish farming are to increase farming operations' accuracy, precision and consistency.

Unmanned planes or vehicles are now frequently employed in agriculture and aquaculture to maintain and monitor fish (Murugan et al. 2017). UAVs were initially created at the turn of the twentieth century with military uses in mind (Choudhary et al. 2019; Ko et al. 2021). But in recent years, drone technology has developed to the point that it can carry out multiple duties simultaneously. Aerial photography (Liu et al. 2014), shipping and delivery (Cokyasar 2021; Rahman et al. 2021; Wang et al. 2021), data collecting (van Nhan et al. 2018; Yao et al. 2019), search and rescue efforts during emergencies or disasters (Kurt et al. 2021), agricultural crop monitoring (Reddy Maddikunta et al. 2021) and watching over and tracking natural disasters (Avanzato and Beritelli 2019) are a few illustrations of these abilities. UAVs were also successfully used for marine science and conservation.

Drones have been effective at the aquaculture site today in obtaining environmental data and fish behaviour for monitoring (Chang et al. 2021). An autonomous drone conducted visual surveillance during the investigation of Ubina et al. (2021b) to monitor fish feeding activities; spot nets, moorings and cages; and spot anything suspicious. Depending on the directions or instructions, the drone may fly over the area of aquaculture to carry out perimeter monitoring and can also navigate automatically.

8.3.1 Unmanned Vehicle System Platforms

Unmanned vehicles can save operating costs and increase the safety and repeatability of missions (Verfuss et al. 2019). The duties carried out by unmanned vehicles are frequently too risky or expensive for people to complete. They are also given straightforward yet repetitious tasks and are less costly to perform without people (Nichols et al. 2020). Off-the-shelf, low-cost systems are gradually becoming more prevalent, but many still need to be customized (Verfuss et al. 2019) to fit the precise needs of aquaculture management and monitoring. The work of Verfuss et al. (2019) details the most advanced autonomous technologies for observing and detecting marine animals.

This work considers three categories of unmanned vehicles – autonomous underwater vehicles, unmanned surface vehicles and unmanned aircraft systems – for aquaculture monitoring and management. However, by working together, they can achieve the objective of aquaculture monitoring and control.

8.3.1.1 Unmanned Aircraft Systems (UAS)

An alternate platform that addresses the drawbacks of manned aerial surveys is provided by UAS or unmanned aerial vehicles (UAVs). According to Jones et al. (2006), using a UAS to conduct surveillance does not cost hundreds of thousands of dollars and is the most effective for ensuring the geospatial correctness of the data gathered and survey repeatability. Lower operational expenses, uniformity of flying routes and image capture are potential benefits of UASs. To avoid data loss or deterioration during transmission, UAS should be compact, powered by an electric motor, simple to use and reasonably priced (Verfuss et al. 2019). They should also record and store data onboard. UAS should communicate data using its wireless capability for real-time monitoring. They can operate in hazardous conditions that are unavailable to people because they are pilotless aircraft (Otto et al. 2018). They are composed of sensors, like cameras that soar into the air to observe the desired targets for monitoring and surveillance (Savkin and Huang 2019). UAVs with cameras fitted can also collect data and transfer it to a repository system. Recent advancements in UAS technology also offer safer missions and longer flying times. They are capable of flying in dangerous areas that are unreachable for people, because they are unmanned aircraft (Otto et al. 2018). They have sensors, such as flying cameras, to monitor the target interests (Savkin and Huang 2019). Low-cost or multirotor drones can fly and land vertically and are simple to handle and control. The wingspans of multirotor UAS vary from 35 to 150 cm, and lightweight materials like plastic, aluminium or carbon fibre are used to boost efficiency.

8.3.1.2 Autonomous Underwater Vehicles (AUVs)

Since AUVs and remotely operated underwater vehicles (ROVs) are outfitted with cameras for capturing pictures and movies, as well as other sensors to gather data on the condition of water, they are waterproof and submersible in water. In an ROV, sensors with specific capabilities can collect data on water temperature and depth and chemical, biological and physical characteristics. Lithium-ion batteries are installed,

allowing longer or extended periods (Panda et al. 2021) for navigation or data collecting. To do underwater inspections, AUVs are now favoured because they are less expensive and offer better safety when compared to human divers. They can carry various payloads or sensors and provide a four-dimensional perspective of the changing underwater. Differential global positioning system (DGPS)-based UV navigation has a high degree of accuracy. Using an acoustic Doppler current profiler, its position is calculated when it is submerged in water by gauging its relative speed over the current or seafloor (ADCP).

8.3.1.3 Unmanned Surface Vehicles (USVs)

Autonomous surface craft, also known as unmanned surface vehicles (USVs), operate on the water without the assistance of a human. They were created to facilitate unmanned tasks, including data collection and environmental monitoring (Jung et al. 2018). USVs need to be robust in the field environment and are simple to use. USVs can quickly approach targets or objects to get up close and collect high-quality photos. USVs can collaborate with other UVs like UAVs to establish vast heterogeneous communication and surveillance networks. The simultaneous communication with more vehicles on or beneath the water's surface is one of the USV's unique capabilities.

Additionally, USVs can serve as relays for other vehicles that are airborne, underwater, on land or in space (Breivik et al. 2008). A USV-UAV collaborative technique can perform duties, including payload transfer and mapping. This allows it to manage more complicated jobs with higher robustness due to redundancy, increased efficiency due to work distribution and lower operational costs (Zhu and Wen 2019). To achieve extensive monitoring, these diverse vehicles can cooperate.

8.3.2 Unmanned Vehicles and Sensors

The ability of unmanned systems to navigate and monitor various environmental variables depends solely on their sensors (Yeong et al. 2021), measurement infrastructure and data processing algorithms. To guarantee security and dependability, sensor failure detection is also crucial. UVs use different quantity, variety and arrangements of sensors installed in numerous configurations to compute data utilizing specialized, varied and unique algorithms. The sensor's primary function is gathering information for missions other than platform navigation. The sorts of information that sensors can collect include sound profiles, radar and infrared signals, electro-optical images, localized ocean depth and turbidity, to name a few. Sonar, radar, environmental and light or optic sensors are examples of the primary sensor subtypes (Martin et al. 2019). Aerial systems typically use electro-optical imaging sensors, whereas surface and subsurface vehicles primarily use acoustic techniques (Verfuss et al. 2019). Accelerometers, tilt sensors and gyroscopes are combined to calculate UAVs' flight location and orientation (Balestrieri et al. 2021). To address the issues influencing image quality due to weather conditions, some

options include wave information perception, picture stabilization, image defogging and multi-camera methods.

When choosing underwater quality sensors, physical, chemical and biological aspects should be considered (Kruse 2018). Do not employ sensors that emit ultraviolet light, acoustic beams that the fish can sense or magnetic fields that impede the fish's regular activity; additionally, sensors should not interfere with a fish's ability to swim or feed. Therefore, sensors must require less upkeep, be inexpensive, power-efficient, durable, water-resistant, able to endure biofouling and unaffected by living things. Modern real-time water quality sensors, like optical and biosensors, are more sensitive, selective and responsive and can analyse data in real time (Parra et al. 2018).

8.3.3 Framework of the Aquaculture Monitoring and Management Using Unmanned Vehicles

The structure shown in Fig. 8.3 establishes a foundation for how a drone interacts with sensors like water quality monitors and underwater cameras. These sensors are placed in the fish tank to gather data and communicate it to a cloud computing system via a Wi-Fi communication channel. The cloud database acts as a repository and contains artificial intelligence-based data processing and analytics capabilities (e.g. computer vision and deep learning). A discreet and non-intrusive solution is provided by the vast volume of data gathered from the undersea environment

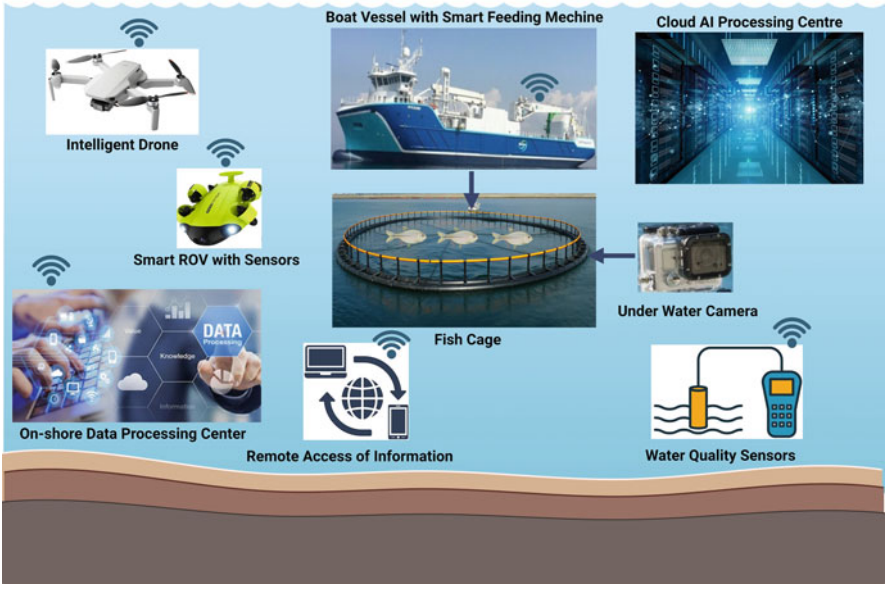

Fig. 8.3 Architecture for aquaculture monitoring and management using drones

employing sensors. For aquaculture operators, this method can achieve real-time image analysis (Chang et al. 2021). These sensors can be used to collect information from the aquaculture site to monitor the water quality and fish behaviour. The data collection gives the aquaculture sector the necessary information, enabling the farmers to make quick adjustments to the farm to ensure that the procedures and products are high quality and optimized to boost output. The level of fish meal satiety is used as an example to illustrate how the data is assessed and transformed into helpful information to distribute food from the novel feeding system. Food is continuously delivered when satiety levels are high, when satiety levels are low and when feeding is lowered or halted. Aquaculture performance will be at its best with real-time information and these procedures.

With the use of this feature, users, such as aquaculture farm owners, can remotely monitor their facilities and assess the fish stock and welfare. Thanks to the vast and varied amount of data gathered from the aquaculture site, management based on data of fish production is now feasible. In order to better understand how environmental factors affect fish welfare and development, farmers will be able to monitor, control and record biological activity on their farms with the use of this strategy (O'Donncha et al. 2021).

8.3.4 Unmanned Vehicles as Communication Gateway and IoT Device Data Collector

The Internet of Things (IoT) is helpful for farmers in wealthy nations where Internet connection is not a problem. This improved connectedness contributes to higher output, lower operational expenses and more productive workers. Combining UAVs and IoT is a beautiful way to extend coverage to outlying or remote places now that 5G technology is available (Moheddine et al. 2019). The drone functions as a flying gateway and is outfitted with an antenna to gather data and LTE cellular networks to base stations. UAVs serve as the intermediary node that collects data from sensors and transmits it to the intended recipients.

IoT devices can now send data to the cloud for processing via wireless connectivity; the drone can also collect data. When collecting data in difficult-to-reach locations like offshore aquaculture farms, UAVs are more affordable and practical than employing crewed aircraft. When used in conjunction with deep learning, UAVs can significantly advance the administration of aquaculture farms**.** The use of drones as a communication route (Sharma et al. 2020), along with cameras and sensors (such as sonar devices and stereo camera systems), to record the underwater environment, has much potential. The data is gathered by the drone and sent to the cloud, which is used by computer vision and deep learning applications. Users can learn about the present state of aquaculture farms from the processed data. Fish behaviour detections, such as schooling (Banerjee et al. 2021; Manna et al. 2018; Xu et al. 2006), swimming (Jakka et al. 2007; Pinkiewicz et al. 2011; Zhao et al. 2017), stress reaction (Banerjee et al. 2021; Israeli 1996; Kane et al. 2004), tracking (Alzu'Bi et al. 2015; Ben-Simon et al. 2009) and eating (Liu et al. 2014; Wang

et al. 2021; Zhao et al. 2017), are among the fish survey activities that can be carried out (Niu et al. 2018). The fish feeding intensity evaluation (Parsonage and Petrell 2003; Ubina et al. 2021b) and the lookout for uneaten food pellets (Parsonage and Petrell 2003; Skøien et al. 2016) are two methods used to gauge the satiety or feeding level of fish utilized in demand feeders, to communicate this data to the cloud servers for processing and data analysis to generate forecasts (Lopez-Marcano et al. 2021; Måløy et al. 2019). The drones used to capture footage from the aquaculture site can aid in estimating fish growth (Azzaydi et al. 1998; Difford et al. 2020), fish count (Ditria et al. 2020; Fan and Liu 2013) and fish length and density estimation (Almansa et al. 2015; Shieh and Petrell 1998).

8.3.5 Aquaculture Site Surveillance Using Unmanned Vehicles

Illegal capture of fish is a widespread issue that jeopardizes the aquaculture industry's future and costs money. The typical method of observing or reducing this practice is on-the-ground observation (Provost et al. 2020), although this has a very high operational expense. UAVs and submersible drones can now identify illicit fishing activities (Bloom et al. 2019) and are less expensive (Saska et al. 2012; Wong et al. 2015). Illegal fishing vessels having a size and speed advantage that enable them to remain undetected when undertaking surveillance were found using autonomous surveillance system made up of fish stocks, vessels and fish farmers. The automatic classification of ships is essential for maritime surveillance to find illicit fishing operations, which significantly impact aquaculture producers' income. According to Marques et al. (2014), naval vessel detection algorithms are used to identify vessels in aerial picture sequences acquired by sensors installed on a UAV. To stop illegal fishing, a surveillance system framework was presented (Prayudi et al. 2020), employing drone aerial photos, drone technology and deep learning. By the use of its built-in camera, the drone gives visual information. Crabs are also valuable commercial goods, and to stop illicit activity, drones equipped with infrared cameras are employed to find crab traps and floats.

8.3.6 Aquaculture Farm Monitoring and Management

Farmers will be able to check farms, gather additional information necessary to take decisions and accomplish and engage farms effectively due to the continuous automation and mechanization of farm monitoring utilizing drones, sensors and artificial intelligence (Yoo et al. 2020). An aquatic platform made up of buoys and USVs can self-organize and carry out mission and path planning in the aquatic environment. Using data gateway stations, this platform can communicate with other devices, detect the environment (e.g. water or air) and operate as a communication channel (DTS). Workers in the aquaculture industry can access the data collected by USVs and buoys utilizing the associated sensors via a server to enhance or maintain the operation of the aquaculture industry. A cutting-edge electric marine ASV

(autonomous surface vehicle) has been created with a streamlined sail system with electric actuator control. This vehicle has exploratory capabilities and patrols the area.

Drones that fly above and below the ocean have a lot of potential for monitoring offshore kelp aquaculture fields. A small unmanned aircraft system (sUAS) can convey a light optical sensor. Then, using time and space scales, it is capable of calculating tissue nitrogen content canopy area and density to track changes in kelp. sUAS has sensors, including colour, multispectral and hyperspectral cameras, to give a raw image of the kelp forest canopy (Bell et al. 2020).

8.3.6.1 Fish Feed Management

Enhancing quality and standards for fish production technology and aquaculture products contributes to fish welfare in aquaculture. The health of the fish directly affects productivity and sustainability. Fish in healthy settings show better growth and a higher rate of food conversion, which results in better-grade fish food ("The State of World Fisheries and Aquaculture 2020. In Brief" 2020). The behaviour and traits of fish are just a few indicators used to measure fish welfare.

Fish growers can help in feeding by using machine feeders. However, if such a process were not carefully observed, it would result in food waste and a loss of revenue. It is essential to pay attention to when to stop or resume feeding while using floating pellets. According to Ubina et al. (2021a), a drone equipped with an RGB camera monitors the water's surface while detecting fish feeding levels using optical fluxes, as seen in Fig. 8.4.

8.3.6.2 Fish Behaviour Observation

An interactive fish monitoring AUV bio-interactive (BA-1) monitors fish and may stay in the fish's environment. An aquaculture system without pens offshore may swim alongside the fish to observe their movements. The vehicle can stimulate the fish and watch how that influences their behaviour. The UAV was created with the capacity to hover and cruise bio-interactive features and an LED lighting system.

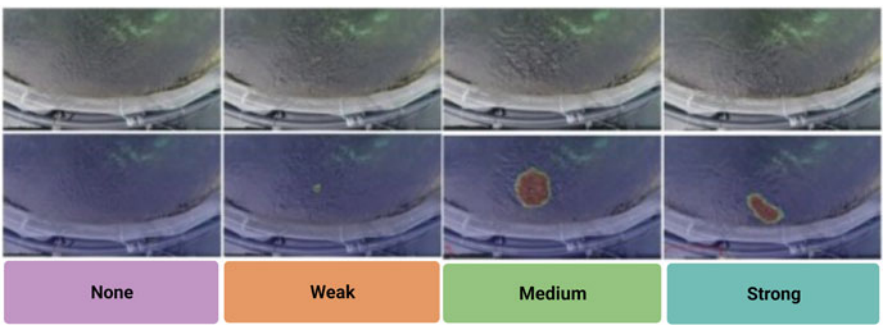

Fig. 8.4 Using four distinct degrees of feeding intensity and the measured optical flow, a drone image was taken to assess the intensity of fish feeding (Ubina et al. 2021a)

Additionally, it has the potential to operate multiple BA-1 AUVs simultaneously. The BA-1 has sensors for payload, localization, self-state monitoring, collision avoidance, navigation and localization. The device was validated using sea bream species in tanks and aquaculture pens. Once the fish is used to the vehicle, it may approach the demand-feeding system to eat the bait and assist the smart feeding process (Kondo et al. 2010).

8.3.6.3 Water Quality and Pollutant Detection and Assessment

One of the most vital elements is that fish have a close touch with water, which is among the most crucial factors as regards to fish welfare and necessitates meticulous observation. Water quality must be at its best because it can cause short-term and long-term health and welfare issues. The climate change also significantly impacts aquaculture, causing changes in abiotic (oxygen level, sea temperature, *acidity* and salinity) and biotic conditions that drive expressively and impact size ("The State of World Fisheries and Aquaculture 2020. In Brief" 2020). Conductivity, temperature, oxygen concentration, pH and nitrogenous component concentrations like nitrate, ammonia and concentration of nitrite are among the variables that indicate water quality. To maintain water quality, many aquaculture farms depend on mechanical devices, such as independent rescue power systems, oxygenation pumps and oxygenation/aeration apparatus. Even though they are beneficial, they have limits when used in offshore or open sea cages for aquaculture and demand additional setup and adjustment. Drones have significantly improved the onsite performance of water sampling, monitoring and testing owing to their excellent portability, dependability and flexibility to carry sensors to measure water quality.

A floating structure unmanned aerial vehicle (UAV) in the shape of a T that can land on and take off from the water surface carries an electrochemical sensor array to anticipate data on water quality evaluation utilizing the dissolved oxygen, ammonia, nitrogen and pH of the water. The sensor can detect in real time and transfers its findings to a cloud server backstage using a wireless network (Yao and Ansari n.d.).

The work of Lally et al. (2019) provides a thorough analysis of how drone technology aids in water sampling to collect physiological chemical and biological data from the water environment. Table 8.1 lists the parameters for monitoring pH Level, turbidity, temperature and electrical conductivity water quality, pH level including dissolved oxygen, turbidity, ammonia nitrogen, nitrate, water temperature, chlorophyll-a, fluorescent dye, phytoplankton counts, salinity, coloured dissolved organic matter (CDOM) and redox potential. Additionally, the most popular brand of commercial drones is DJI (Da Jiang Innovations).

8.4 Conclusion

Technology development is essential for the aquaculture sector's ability to conserve wild fish stocks, regulate fish prices and boost production, given the current population growth rate. Population growth puts more pressure on wild fish stocks by increasing fish prices due to rising demand and a lack of seafood. Technology research and development could prevent wild fish species' extinction, improve social

Table 8.1 Different types of sensors for testing the water quality

Measurement indicators	Type/brand of UV used	Sensors/devices installed	Sampling location	References
pH level	Six-rotor UAV	pH nitrogen sensor	High sea	Yao et al. (2019)
	AUV Tantan	Conductivity, temperature and depth (CD) sensors	High sea	Kumagai et al. (2002)
	Customized multirotor UAV	Water sampling cartridge and sensor nodes	Lakes and ponds	Koparan et al. (2020)
	Customized multirotor UAV with hovercraft	pH level sensor	Lake	Esakki et al. (2018)
Temperature	UAV Tantan	Conductivity, temperature and depth (CD) sensors	High sea	Kumagai et al. (2002)
	DJI Octocopter UAV	FLIR T450sc thermal camera and infrared camera	Coastal water	Lee et al. (2016)
	Customized multirotor UAV	Sensor nodes and water sampling cartridge	Ponds and lakes	Koparan et al. (2020)
Turbidity	DJI M600 pro	k4 multispectrometer camera	Inland	Wang et al. (2020)
	AUV Tantan	Conductivity, temperature and depth (CD) sensors	High sea	Kumagai et al. (2002)
	Customized multirotor UAV	Sensor nodes and water sampling cartridge	Ponds and lakes	Koparan et al. (2020)
	Quad-copter (DJI Phantom 2 Vision Plus) and hexa-copter (DJI Spreading Wings S800	RGB Camera	High sea	Ferraro (2016)
	Customized multirotor UAV with hovercraft	Turbidity sensor	Lake	Esakki et al. (2018)
Dissolved oxygen	DJI M600 pro	k4 multispectrometer camera	Inland	Wang et al. (2020)
	Customized Six-rotor UAV	Dissolved oxygen sensor	High sea	Yao et al. (2019)
	AUV Tantan	Conductivity, temperature and depth (CD) sensors	High sea	Kumagai et al. (2002)

(continued)

Table 8.1 (continued)

Measurement indicators	Type/brand of UV used	Sensors/devices installed	Sampling location	References
	Customized multirotor UAV	Sensor nodes and water sampling cartridge	Ponds and lakes	Koparan et al. (2020)
	Customized multirotor UAV with hovercraft	Dissolved oxygen sensor	Lake	Esakki et al. (2018)
Ammonia nitrogen	Six-rotor UAV	Ammonia nitrogen sensor	High sea	Yao et al. (2019)
	Customized UAV	Ammonia nitrogen sensor	Lake	Cheng et al. (2021)
Nitrate	Customized AUV "Dorado"	Gulper water samples	Bay and offshore water	Pennington et al. (2016)
Chlorophyll-a	UAV Tantan	Conductivity, temperature and depth (CD) sensors	Open sea	Kumagai et al. (2002)
	Customized AUV "Dorado"	Gulper water samples	Bay and offshore water	Pennington et al. (2016)
	Remo-M UAV	Sequoia multispectral sensor with four cameras to capture spectral images (algae blooms)	Streams	Kim et al. (2021)
	Customized UAV	Portable fluorometers	Streams	Kim et al. (2021)
	Quad-copter (DJI Phantom 2 Vision Plus) and hexa-copter (DJI Spreading Wings S800)	RGB camera	High sea	Zeng et al. (2017)
Redox potential	UAV Tantan	Conductivity, temperature and depth (CD) sensors	High sea	Kumagai et al. (2002)
Coloured dissolved organic matter (CDOM)	Quad-copter (DJI Phantom 2 Vision Plus) and hexa-copter (DJI Spreading Wings S800	RGB camera	High sea	Zeng et al. (2017)
Salinity	DJI Octocopter UAV	FLIR T450sc thermal camera and infrared camera	Coastal water	Lee et al. (2016)

(continued)

Table 8.1 (continued)

Measurement indicators	Type/brand of UV used	Sensors/devices installed	Sampling location	References
	DJI Phantom 3 Professional UAV	MicaSense RedEdge-M Multispectral camera	Lagoon; (shallow water)	Taddia et al. (2020)
Phytoplankton counts	Customized AUV "Dorado"	Gulper water sampler	Bay and offshore water	Pennington et al. (2016)
Fluorescent dye	Clearpath Robotics Kingfisher M200 USV (dye detection and tracking) and DJI Phantom UAV (image capture)	Fluorometer (fluorescence sensor)	Freshwater lake	Powers et al. (2018)
Electrical conductivity	Customized multirotor UAV	Sensor nodes and water sampling cartridge	Lake and ponds	Koparan et al. (2020)
	Customized multirotor UAV with hovercraft	Electrical conductivity sensor	Lake	Esakki et al. (2018)

welfare and lessen poverty. Even if the substantial effects of current information technology advancements on aquaculture are becoming apparent, this industry has a significant amount of scope for improvement in comparison to the considerably wider use of IT in the manufacturing and agricultural sectors. However, it is believed that the expansion rate of the aquaculture industry will significantly expand due to the ongoing adaptation of information technology. In a nutshell, innovative techniques are crucial for the aquaculture industry's future growth and should be considered in all contexts.

The use of unmanned systems to administer and watch over aquaculture farms is also evaluated in this study, along with its advancement and its prospects and difficulties. The different drone features were acknowledged as a data gateway for communication, a monitoring system for aquaculture sites and a management and supervision of aquaculture farms. This report also included some difficulties in managing and maintaining offshore aquaculture sites. Technical innovation was used with unmanned vehicle systems to overcome these challenges and attain the goal of precision aquaculture.

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9 Advanced Metatranscriptomic Approaches for Exploring the Taxonomic and Functional Features Relevant to the Aquaculture Industry

Sangita Dixit, Mahendra Gaur, and Enketeswara Subudhi

Abstract

Microbiome researchers have resorted to the use of large-scale metatranscriptomic-based exploration to understand how a microbial community reacts to varying environmental factors over time. Aquaculture, a rapidly expanding area of agriculture, has the potential to meet future protein needs of the population as nutritional supplement. But the industry is subjected to several drawbacks which includes the absence of genetically enhanced strains or varieties and species-specific or functional feed as well as the year-round unavailability of high-quality fish seed, ecosystem contamination and increased disease frequency. High-throughput sequencing technology i.e. next-generation sequencing (NGS), has been continuously improving in recent years, revolutionizing our understanding on biological sciences and supplying essential tools. Here, we give a thorough analysis of different metatranscriptomic methods used for investigating the behaviours of microbial populations in particular environmental conditions. We examined the planning of experiments, various fish sample types and the specific metatranscriptomic workflow in this expanding aquaculture sector. We also discussed the pipeline, workflows, available tools and their uses and limitations of these approaches. In our report, we described the use of metatranscriptome profiling in aquaculture research to identify critical microbial transcripts and expression analysis of potential genes involved in growth, development, stress,

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immunity, reproduction, toxicology and disease under changing environmental conditions.

Keywords

Aquaculture · Fish · Metatranscriptomics · Microbial community · Function

9.1 Introduction

Microbes are found throughout the environment and are crucial to the metabolism of nutrients and energy. The immune system's growth, healthy digestion and absorption and host tolerance to harmful infections and sickness are all influenced by fish-related microorganisms. To increase the sustainability and productivity of the aquaculture sector, a good balance between commensal and opportunistic bacteria must be established in the fish microbiome.

It is well acknowledged that the community of bacteria living in the GI tract of vertebrates (the gut microbiome) is essential for host development, physiology and health (Mueller et al. 2015; Llewellyn et al. 2014). Studies on mammals, which make up only 10% of all vertebrate varieties, are where most of our understanding of microbiome structure and function comes from (Sullam et al. 2012). Few researchers have looked at the gut microbiome of fish, even though this group contains over half of all vertebrates. Since only 10% of bacteria can be identified and cultivated in a lab, the earliest investigations into fish gut microbiology greatly underestimated the richness of these communities. Molecular-based, culture-independent techniques have lately been used to research the microbial communities that inhabit fishes' GI tracts, keeping with other initiatives to examine microbial diversity in various habitats.

Over the past two decades, significant advances in NGS (next-generation sequencing) technologies have changed how we perform research, mainly in diverse microbiome studies. Most NGS targeted strategies like whole genome shotgun sequencing (metagenomics) of all possible DNA samples or PCR-based 16S rRNA gene amplicon sequencing (marker genes). Biologists have used qPCR techniques to evaluate the expression of genes by using long RNA sequence. It is possible to better understand the active members by using RNA sequencing to record the transcript's expression in microbiome at a specific time and under specific environmental conditions. RNA is being subjected to next-generation sequencing (NGS) technology now possible to directly generate potential transcripts and variations in transcript from the sequence and measure existing transcript targets.

This book chapter will cover the available tools and workflows that are now in use to create and analyse the metatranscriptomic datasets. We also reviewed various already published studies that provided information on changing fish microbiota under different environmental conditions through metatranscriptomics, including sampling methods.

9.1.1 Metatranscriptomic Implementation in the Aqua System

Gene expression technology has been used to understand the responses of model organisms in a particular condition (Bailleul et al. 2010; Allorent et al. 2016; Jaubert et al. 2017). Yet, single organism studies in a laboratory environment scarcely represent natural communities that deal with unstable environmental conditions, despite being essential for the identification of various activities at the gene or protein level and comprehending fundamental biological processes. Therefore, metatranscriptomics (transcript sequencing from the entire community) is a more appropriate tool to provide a snapshot of the transcriptional patterns that correlate to distinct populations within a microbial community at sampling time. Primary active organisms and a specific community's principal activities in response to changing environmental conditions are correlated (Bizic-Ionescu et al. 2018). With this knowledge, complex microbial communities' potential behaviours and the mechanisms that hold them in check can be revealed.

9.1.2 Experimental Setup for Metatranscriptomic Study

The initial step in metatranscriptomics is deciding which experimental design best suits your needs and financial constraints. Generally, either of two approaches, that is, qualitative or quantitative, is used. The qualitative technique is highly beneficial for metatranscriptomics since even the large amount of rRNA acquired can be used to define the individuals' metabolically active and community structure. The quantitative technique is most frequently used when analysing a single organism's transcriptomes. This method enables us to identify notable variations in the total gene expression across various contexts.

Before processing the metatranscriptomic analysis, we need to know the response to a few basic questions. How many replicates we ought to employ? Which sequencing software for metatranscriptomics works best? How deep should be the sequencing? We need how many sequences, exactly? Is biological variation being captured by the experimental design? To get the biological variation between the two conditions, a rule of thumb would be to use the same number of replicas for each condition that was explored, and a minimum of two replicates per condition is required. Thus, the most straightforward and often utilized experimental design would consider one treatment group and one control group. The number of replicates would substantially increase if you used a nested experimental design (Peimbert and Alcaraz 2016). An effective technique to improve an experiment's precision is replication. As the number of observations rises, so does the precision. Replication promotes accuracy since it yields additional observations when applying the same treatment. However, various tools, such as EDDA (experimental design in differential abundance analysis), are available in web server (Luo et al. 2014) and as an R's Bioconductor package, which can assist the experiment designing and have ability to detect the differential abundance in RNA-seq data (Peimbert and Alcaraz 2016).

To study diversity, it was essential to ask questions regarding sequencing technology because culture-based identification was gradually fading. As expected, the answer to this is not simple. The primary trade-offs between each platform are costs, overall read lengths and sequencing depths (Peimbert and Alcaraz 2016). The 454 Genome Sequencer FLX systems (Roche) and the HiSeq 2000 are the two leading high-throughput sequencing platforms used in metatranscriptomic studies (Illumina, Inc.). Both technologies allow for the parallel execution of numerous independent sequencing reactions (Mukherjee and Reddy 2020). The primary players are MiSeq from the Life Technologies, HiSeq from the Illumina (X, 3000/4000, NexSeq, High-Output) and the old 454 from the Pacific Biosciences. The cost per Mb ranges from US$ 0.06 to 8.72 (Illumina 454), the length of reads ranges from 50 bp to 1.5 kb and the output yield ranges from 300 Gb (Illumina) to 40 Mb (PacBio). According to some recent studies, the expenses for detecting splice variants are fundamental discrepancies between gene expression profiles across platforms (Li et al. 2016).

A bacterial genome with an 8X coverage depth is said to be finished. To get that coverage for a typical 5 Mb genome, at least 40 Mb would need to be sequenced. In an ideal world, prior information about the researched system, such as the species abundance with 16S rRNA amplicon sequencing, would be available when discussing a metatranscriptome. Let us say a specific habitat support species 700. Assuming the genome size of one bacteria is 5 Mb, 8X coverage would require at least 28 Gb of sequencing. It makes several irrational assumptions, including that each species and gene would be equally abundant and that their genome sizes would be uniform. Although this task is difficult, Illumina's deep sequencing is anticipated to be 300 Gb of sequence production, equivalent to 85X coverage for each species in this fictitious situation. We can also use ultradeep coverage techniques for the gene that is not expressed and can be multiplexed (Table 9.1). We are not sure about the recovery of rare species or genes even with ultradeep sequencing; as a result, the efforts of the sequencing, as the majority of meta-omic analyses, are significantly biased towards over-represented characteristics. Generally, sequencing depth should be fair for every condition and replication tested (Li et al. 2016).

9.1.3 Sample Type, Sample Collection, Sample Storage and RNA Isolation Procedure

Many different types of samples from aquatic habitats have been subjected to metatranscriptomic analysis. Sample types may be the (1) liver, (2) kidney, (3) urinary tract, (4) heart, (5) testicle, (6) intestine, (7) efferent duct, (8) swim bladder, (9) ureter, (10) gallbladder and (11) stomach. However, the selection of samples depends on the objectives of the project. For a metatranscriptomic analysis, sample collection is crucial. The sample collection will vary according to the sample types. However, for RNA isolation, 100 mg of tissue is required. The species name, the subject's sex and the collection date must be written on the labels of cryovials. Cryovials will then be placed on dry ice to precool. Prepare a bowl- or wide-mouthed

Table 9.1 Metatranscriptomic experimental design

Technique	Protocol	Control library	Recommended starting material (ng)	Number of replicates	Sequencing depth	Recommended sequencing platform and run
Metatranscriptomics	RNAseq	cDNA Input Contro	Minimum of 1 ng (TruSeq®) Typically 100 ng cDNA RNA integrity, check Nanodrop UV spectrums and if possible RNA degradation with Bioanalyzer®	Two minimums for each library to be able to estimate variances	If possible, estimate species diversity (16S rRNA amplicons). Then, calculate expected average genome sizes and an 8X minimum coverage	Illumina's MiSEQ 2 × 300 bp (0.3–15 Gb, HiSeq 2500 (10–1000 Gb). First, check with your provider to use the latest technology. Considerations: Quality coverage read length sample number budget

container for the liquid nitrogen. The tissue will then be cut into pieces of approximately 100 mg (about the size of a pea, no need to weigh). Then, put an amount of tissue into the liquid nitrogen bowl using forceps, and then wait for it to freeze completely. Place the chunk in the pre-chilled cryovial after that. For future usage, store the piece at −80 °C (Table 9.1).

The three main methods that are widely used for RNA extraction are phenol-guanidine isothiocyanate (GITC)-based solutions (Chomczynski and Sacchi 2006), silica membrane-based spin column technology (Berensmeier 2006) and paramagnetic particle technology (Berensmeier 2006). The phenol-GITC-based organic extraction is one of the most widely utilized techniques. However, proteins, other biological components, salts, ethanol and chemical solvents like phenol-chloroform frequently contaminate the RNA samples obtained by this approach. These techniques also call for safety precautions (such as the fume hood use), which extend the process. They also use liquid-liquid extraction, which results in imperfect phase separation and high genomic DNA contamination carryover. Harmful chemical solvents are not necessary for paramagnetic particle or silica column-based RNA separation technologies, which are both simple, effective and reasonably priced. These systems also contain intact RNA with little protein or contamination of other cellular materials (Bizic-Ionescu et al. 2018). These techniques, however, frequently cause high quantities of genomic DNA contamination. NanoDrop 1000 can be used to measure the RNAs' concentration (A260) and purity (A260/A280, A260/A230) (Thermo Fisher Scientific). The RNAs will be occasionally diluted to a concentration acceptable for RNA integrity analysis (25–500 ng).

9.1.4 Library Preparation

The concept is to create cDNA of 50–400 bp flanked by adapters, regardless of the sequencing platform employed. Therefore, RNA fragmentation, cDNA synthesis, second-strand cDNA synthesis, adapter coupling and library validation are required for library preparation. cDNA fragments must be a specific size depending on the platform to improve sequencing (Table 9.1). Enzymes, metals, heat and ultrasonication are all fragmentation methods (Head et al. 2014). Since each sample's integrity is often unique, optimizing the incubation durations for fragmentation in each situation is necessary. A reverse transcriptase synthesizes the initial cDNA strand, and random hexamer primers are typically utilized. DNA polymerase is used to create the second strand of DNA (Fig. 9.1). Since reverse transcriptase leaves a polyC overhang in this situation, primers containing guanines at position three are typically utilized. If numerous samples are combined in the same run, they can also have a barcode to help identify the sample (multiplexing).

The complementary strand may be sequenced with Illumina, allowing longer reads (pair-end) (Table 9.2). PCR or ligation reaction can connect the adapters. Illumina platform is used the most, and kits like TruSeq R and SMARTer R are available. Compared to TruSeq R, which requires at least 100 ng of enriched RNA, the latter allows you to start with 1 ng (Alberti et al. 2014). The library must be validated as the

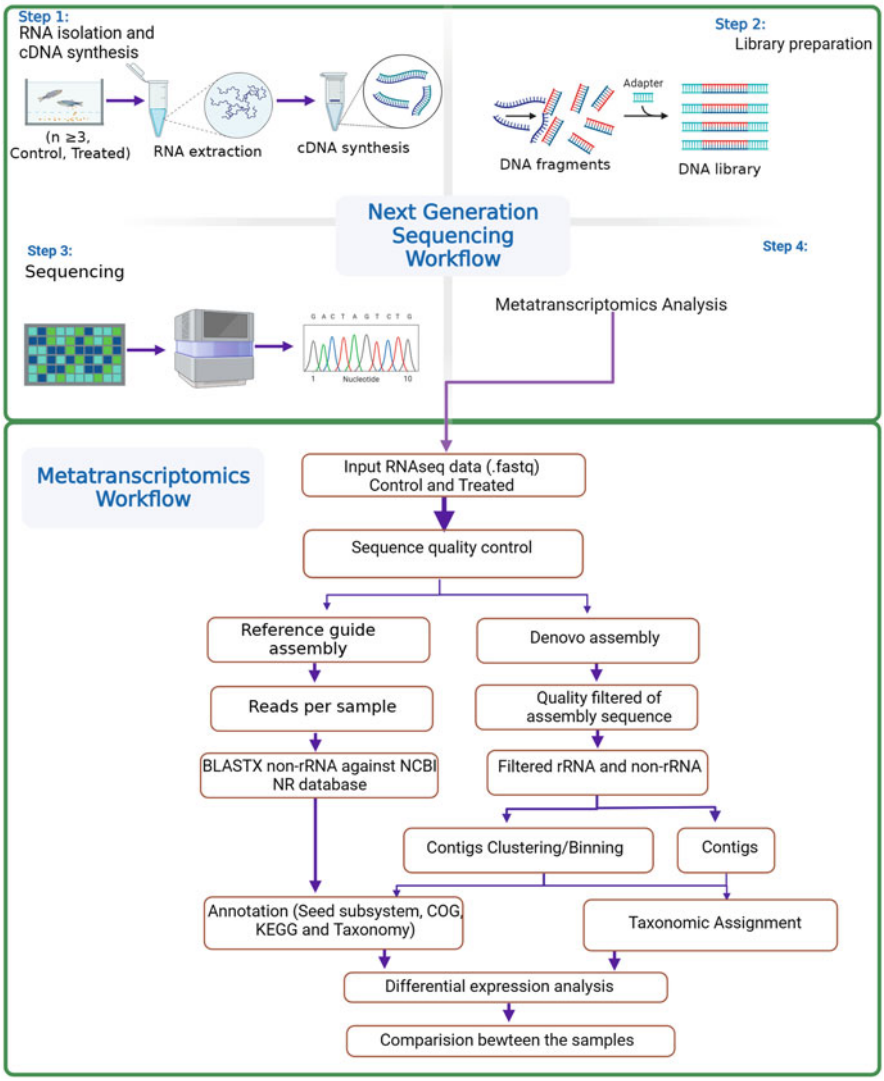

Fig. 9.1 Typical metatranscriptomic workflow for analysis of RNA-seq data

final step. The 2100-Bioanalyzer R can measure DNA size and concentration when connected to a chip like the Agilent DNA 1000 (Škereňová et al. 2016).

9.1.5 Bioinformatic Analysis of Metatranscriptomic Sequencing Data

Due to the complexity of the microbiome, metatranscriptomic studies using next-generation sequencing, typically short-read sequences produced by Illumina, have

Table 9.2 Comparison of next-generation sequencing platform

	Platform	Library preparation	Mean read length (bases)	Reads per run	Output per run	Accuracy (%)	Advantages	Disadvantages	Run time
Sequencing by synthesis	Roche/454 GS FLX+	Frag, MP/emPCR	750	~1 million	700 Mb	99.99	Long read, FASTA's run time, good for de novo assembly	High reagent cost and high error rates in homopolymeric region	23
	Illumina Hiseq 2000	Frag,MP/ solid-phase	2*100	1 billion	600 Gb	>80	Currently the most widely used platform	Short read length and less feasible for de novo	8.5 days
	IonTorent PGM	Frag/ emPCR	200	5 million	1 Gb	99.99	Very fast run, cost effective and open source	Not as much throughput	2 h
Sequencing by ligation	Life technology SOLiD 5500	Frag,MP/ emPCR	2*60	1.4 billion	90 Gb	99.99	Two-base encoding provides inherent error correction	Long run time	7 days
Single molecule sequencing	Helicos Bioscience HeliScope	Frag, MP/single molecule	35	~1 billion	35 Gb	99.995	High multiplexing ability and no template amplification needed	Short read length and high error rates compared with RT-based platform	8 days
	Pacific Bioscience PacBio RS	Frag/single molecule	1300	~45,000	50 Mb	99.999	The longest read length at present and fast run time to result	Less high-throughput compared to other NGS platforms	~1 h
Sequencing by hybridization	Oxford Nanopore technology	Frag/single molecule	Ultra long (mirror fragment size)	NA	Tens of Gb	>99.99	Low cost. Ultra long read length and high accuracy	Limited application to RNA-seq	Within 24 h

Frag fragment run, *MP* metapair run, *emPCR* emulsion PCR

become increasingly popular. Multiple sampling and sequencing deep coverage are needed, for expression studies. Finding the appropriate experimental parameters for metatranscriptomics, such as depth of sequencing, is challenging because most information about samples is unknown at the beginning, including their genome sizes, microbial community composition, relative abundance and relative expression within and among the genomes. Here, we concentrate on the short read sequence processing and workflows, pipelines and tools for metatranscriptomic data analysis (Fig. 9.1).

9.1.5.1 Sequence Pre-processing

As with other NGS datasets, one of the initial processes for processing metatranscriptomics is quality control (QC), which involves trimming or removing spurious or inaccurate reads to reduce mistakes. For short read sequence of some of the available QC tools are Trimmomatic (Bolger et al. 2014), FastQC (Andrews et al. 2015), fastp (Chen et al. 2018) and FaQCs (Lo and Chain 2014).

As they frequently make up to 90% of all data, they do not contribute any pathway characterization or expression of genes. So the physical removal rRNA transcripts should be considered from the samples. Before sequencing, these rRNAs are frequently eliminated utilizing molecular methods, but because of their predominance in samples, some rRNA is still sequenced. Tools such as barrnap (Seemann 2013) and SortMeRNA (Kopylova et al. 2012) can identify rRNAs after sequencing, so they can be eliminated from the subsequent analysis (Seemann 2014).

9.1.5.2 De Novo Assembling Tools

De novo assemblers can now build together putative transcripts from high-quality, pre-processed RNA sequence reads. A series of fragments involving multiple parts of various transcripts can be found in RNA-seq reads. These data include transcript isoforms produced by alternative splicing (Wang et al. 2009), sequencing artifacts (Hansen et al. 2010), transcriptional noise (Cavallaro et al. 2021) and other factors. Accurately determining the read's origin and accurately reconstructing the parent sequences are assembly goals. Reference scaffold is used for de novo assembly, longer genomic segments and gene expression, which can provide a reference database since most microbiomes cannot be fully described with reference genomes. As a result, users can more easily locate homologs, determine the origin of a taxa and utilize this as a benchmark for mapping during expression analysis.

Most assembly algorithms employ k-mers (substrings) rather than complete readings as assembly units, and length of k was created from a specific read (Amit et al. 2013). A dictionary containing all potential k-mers and the readings from which these k-mers can be derived created as the initial step in the assembly process. In this step, the De Bruijn graph represents the k-mers as nodes. Most contemporary assemblers are De Bruijn graph-based. Shorter k-mer lengths increase the false-positive results (nonexistent/incorrect) of contigs assembled (Bushmanova et al. 2019; Liu et al. 2016), but they also help retrieve lowly expressed transcripts. A higher k-mer length, on the other hand, would result in fewer contigs being constructed overall, but it would also inhibit the recovery of the high and low

expressed transcript. As a result, choosing the k-mer length in metatranscriptomic analysis plays a significant role in the assembly steps (Bushmanova et al. 2019).

Transcriptome sequencing problems are attempted to be taken into account by assembler tools like Trans-ABySS (Robertson et al. 2010), Trinity (Grabherr et al. 2011), Oases (Schulz et al. 2012), SOAPdenovo-Trans (Xie et al. 2014), BinPacker (Liu et al. 2016) and IDBA-Tran (Peng et al. 2013) even though they are focused more for transcriptomic datasets than metatranscriptomic ones. IDBA-MT (Leung et al. 2013), IDBA-MTP (Leung et al. 2014) and Transcript Assembly Graph (TAG) (Ye and Tang 2016) are explicitly made for metatranscriptomic de novo assembly analysis and take into characteristics of transcripts and the intricate structure of the microbial communities. Building on IDBA-MT, IDBA-UD employs several k-values in a de Bruijn graph to account for mRNA properties, including unequal sequencing depth and common repetition patterns among various mRNAs, reducing the rate of mis-assemblies. In the same way, IDBA-MTP, which can assemble mRNAs with low levels of expression, was developed from IDBA-MT. It builds mRNA sequences from smaller k-values and then includes them based on their known set of protein similarity, using the knowledge of known protein to direct the assembly. To assemble the complementary metagenome, the de Bruijn graph is used as a relatively new assembler by utilizing TAG, which is subsequently used as a reference to map and rebuild sequences of mRNA by navigating the graph with transcriptome-mapped reads. This method is useless in microbiomes containing eukaryotes since it presumes contiguous genes (without splicing). Furthermore, it is implicitly assumed that the metagenome sufficiently encompasses the community to allow for mapping all expressed genes.

In metatranscriptomic sequence, de novo assembly is still very new in its infancy, and few tools, particularly for metatranscriptomics, have been created; however, their effectiveness on different datasets, computer memory and the hardware need over different microbial community complex has not been well defined.

9.1.5.3 Post-Assembly Quality Control

Assemblies may include intronic sequences and other "transcriptional" artefacts due to the noisy nature of RNA-seq data. Additionally, there are errors throughout the assembly process (Freedman et al. 2021). A poor-quality assembly can result in incorrect interpretations in various scenarios, including gene expression analysis. A quality of an assembly can be evaluated from several angles like sequence fragmentation and length. A fragmented assembly contains a lot of short contigs. This may be the result of bad sequencing or improper assembly. Sequence length statistics can be computed using tools like SeqKit (Shen et al. 2016). The N50 measures the length of sequence at which half of the nucleotides in the assembly genome are of the same size or greater (Lander et al. 2001). The second factor is the read percentage that remap to the assembly. Most of the reads that went into a high-quality assembly will have been utilized. Additionally, fewer reads would map to multiple sequences than usual (yet, given a gene may have several transcript isoforms, this cannot be assured). The de novo assembler's trinity lists several additional techniques for evaluating the quality of an assembly, such as computing the N50 statics of sequence

and examining the assembly's strand specificity (Hölzer and Marz 2019; Bryant et al. 2017).

Examining the assembly's composition can be used as a substitute for checking the assembly's quality. Ideally, a significant portion of the sequenced transcriptome would have been recovered via a high-quality assembly. The most widely used technique is to check the assembly for orthologs of specific genes that are universally expressed, insistently expressed and nearly completely found in single copies in the genome. BUSCO and CheckM can be used to conduct this analysis.

9.1.5.4 Transcript Taxonomy

To evaluate active community members, a different and distinct approach is only to consider rRNAs, although, as was already indicated, these are regularly eliminated (both in the in vitro methods and raw data pre-processing). For metatranscriptomes, taxonomy categorization tools can be employed, such as GOTTCHA (Freitas et al. 2015), Kraken (Wood and Salzberg 2014), MetaPhlan2 (Truong et al. 2015) and GTDB-tk (Chaumeil et al. 2020) which is employed for binning-based taxonomy categorization. These methods can only be used effectively in microbiome taxonomic analysis which have close neighbours in the reference databases that are now in existence since they operate on short read sequence and are based on matching of nucleotide.

9.1.5.5 Retrieving of MAGs

The assembling reads into contigs cannot recreate the sequence of entire genomes because the reads generated by next-generation sequencing technologies are often of short length. If the reference genome is unavailable or the coverage is low, the read alignment to the reference genome sequence is hampered. Additionally, as the sample comprises various microbial species or strains, the reads from shotgun sequencing can come from numerous taxa. Therefore, it is necessary to group contigs into bins, each for a different species. The binning problem has been studied from various angles, and they may be loosely categorized as compositional methods like tetranucleotide frequencies, interpolated Markov models and Markov chain Monte Carlo models, as well as similarity-based methods like BLAST and hidden Markov models. Compositional methods have the advantage of being able to group the contigs containing genes which is not similar to the reference datasets. This benefit is crucial because those species which are closely related only share a small part of their genomes, and the genes which are non-homologous in closely related species are the key to deciphering novel function-identity links. Given a contig of adequate length, similarity-based methods typically provide a strong signal about the taxonomic location of the population source. This is an advantage of these techniques because they are relatively robust. Therefore, the only way to evaluate the results of binning which is produced by compositional algorithm without a similarity-based strategy is with generated datasets. Thus, a complete binning approach should actually combine similarity-based and composition-based techniques.

9.1.5.6 Functional Analysis Tools/Database

A key objective of metatranscriptomics is to evaluate a microbiome's functional activity. Characterizing the transcript function is critical for metatranscriptomics since expressed transcripts serve as a proxy for the actual phenotype. Both assembled contigs and readings can be used for functional annotation. Read-based sequence functional profilers like UProC (Meinicke 2015), HMM-GRASPx (Zhong et al. 2016) and MetaCLADE (Ugarte et al. 2018) rely on tool-specific databases and demand input from other programmes like FragGeneScan that anticipate open reading frames (Rho et al. 2010). One of the most up-to-date tools is MetaCLADE, which employs a two-million probabilistic model library with above 17,000 Pfam domains; each domain has hundreds of model library, and each domain is diverse along the tree of life (Ugarte et al. 2018).

As an alternative, contigs that have been created can be used to annotate genes. As with annotation of genomes and metagenomes, the metatranscript annotation is similar. Tools like DIAMOND (Buchfink et al. 2014) are used for blast against functional databases like UniProt (Bateman 2019), KEGG (Aramaki et al. 2020), NCBI RefSeq (O'Leary et al. 2016), etc. Based on similarity search, the functional assignment is performed after gene discovery using programmes like Prodigal (Hyatt et al. 2010) and FragGeneS. A variety of other tools, pipelines and platforms, such as MG-RAST (Wilke et al. 2016), EDGE Bioinformatics (Li et al. 2017), prokka (Seemann 2014) and pgcgap (Liu et al. 2022), are used for annotation and gene calling via similarity searches. These tools, pipelines and platforms can be used to perform gene finding, annotation and other bioinformatic tasks. However, using tools like iPath or MinPath, enzymatic functions can be annotated and mapped to understand metabolic pathways.

9.1.5.7 Differential Expression Analysis

Differential gene expression studies describe the active and functional genes expressed at a particular time. The metatranscriptomic sequence is used for comparison of various environmental parameters and their impact on the community; relative expression of gene is to compare the during time-course or growth rates or to track community dynamics over time. Packages in different programming languages are available that can conduct DE analysis. They all carry out the following tasks, even though their means of doing so vary: (1) adjusting the relative read counts to take into variations in the depths of sequencing between the samples (Zyprych-Walczak et al. 2015), (2) reducing contamination (Wilfinger et al. 2021), (3) using read counts to fit a distribution to the data and test the differential expression of each gene between the relevant conditions and (4) adjusting the produced P-values for multiple testing and analysing the results.

Several numerical values that can be used to determine genes or transcripts have been expressed differentially, but only two are essential in biological interpretation. The aforementioned adjusted P-values show whether there is a statistically significant change in a gene's or transcript's expression between two circumstances. With a modest P-value, the source of the variation is likely a biological phenomenon since the likelihood that the read counts would differ between the two circumstances only

by chance is very low. The log2FoldChange defines the degree of quantity change between original and final values. As the name implies, this is the log2 value of the ratio of the mean counts between the two circumstances. Compared to the condition chosen as the comparison baseline, a log2FoldChange (LFC) having positive values denotes upregulation, and a negative LCF represents downregulation. The LFC is overstated for genes and transcripts with low levels of expression since these tend to have more significant levels of support variability. Adjusting the LFC estimations with a shrinkage algorithm (like ashr (Bickel 2019) or apeglm (Anqi et al. 2018) before using them for biological interpretation is a crucial yet frequently skipped step. It is customary to only classify genes and transcripts as being differentially expressed if they meet certain criteria for statistical significance and the size of the expression difference (P-value=0.05).

Numerous methods were initially designed for single genomes for metatranscriptomic analyses of differential gene expression. The input for these tools is abundant information for each transcript (gene) and each sample (representing expression over a time period or condition). Several approaches to achieving abundance often include reference genome mapping, assembly, read alignment or gene abundances. In addition to the abundance data, the R packages EdgeR (Robinson et al. 2009) and DeSeq2 (Love et al. 2014) are widely used to find the genes that are significantly expressed across a variety of samples (i.e. time points/some conditions). Similarly, Generally Applicable Gene-Set/Pathway Analysis (GAGE) can be used to identify pathways that are more prevalent in one condition than another (Luo et al. 2009). Non-parametric techniques, such as those used in NOISeq (Tarazona et al. 2015), should also be considered because reproducing metatranscriptomic datasets is more complex than doing transcriptome research on isolated species. A typical workflow from analysis of metatranscriptomic data was shown in Fig. 9.1.

9.1.6 Available Pipeline for Metatranscriptomic Analysis

As previously mentioned, numerous bioinformatic steps can be taken while studying a metatranscriptomic dataset, and there are multiple tool alternatives for each step. The experiment's objectives, whose particular may become more complex based on the nature of the investigation, frequently determine which procedures and equipment should be used. However, bioinformatic pipeline/workflow can take a raw sequence and process them to produce final results like taxonomic characterization, gene and pathway identification and differential expression of genes/transcripts. This automated workflow reduces the complexity of the work by integrating numerous separate tools into a workflow. In this section, we list some of the workflows that are currently in use, including MetaTrans (Martinez et al. 2016), COMAN (Ni et al. 2016), FMAP (Kim et al. 2016), SAMSA2 (Westreich et al. 2018), HUMAnN2 (Franzosa et al. 2014), SqueezeMeta (Tamames and Puente-Sánchez 2019) and MIntO (Saenz et al. 2022). We compare the many studies these workflows can carry out, determining the various biological problems they can address. A

description of these seven workflows, their capabilities (such as quality control, assembly and expression analysis) and their references are shown in Table 9.3.

9.1.7 Work Done to Date Metatranscriptomic Host-Microbe Interactions

About half of all seafood is produced by the industry, which is agriculture's fastest-growing sector (FAO 2018). Early in the new millennium, metatranscriptomics was first introduced, and in a short period, the number of initiatives involving the sequencing of RNAs from microbial communities, or metatranscriptomics, has significantly expanded. Recently, aquaculture sectors have employed metatranscriptomics extensively to effectively identify organisms in mixed microbial communities and their expression under different environmental conditions. Researchers studying the microbiome are starting to use extensive metatranscriptomic techniques to obtain how a microbial community is influenced over time or in changing environmental conditions (Fig. 9.2). A large number of the known and novel microbial transcript have been successfully identified in various non-model and model aquatic species using the metatranscriptomic approach, such as marine fish (Geoghegan et al. 2021a), divergent *Amnoonviridae* (Turnbull et al. 2020), deep-sea shrimp intestine (Lin et al. 2022), *Bathymodiolus azoricus* (Barros et al. 2018) and zooplankton communities (Lopez et al. 2022). In addition, the intestinal-related microbial transcripts corresponding to various environmental conditions have been identified in Asian seabass (Xia et al. 2014), grass carp (Wu et al. 2015) and anchovy (Jiang et al. 2020). Similarly, metatranscriptomics has been performed in fish reproduction in various fish species, such as marine fish (Geoghegan et al. 2021b) and gilthead sea bream (Naya-Català et al. 2022). In addition to the fish, infection-related metatranscriptomics corresponding to different environmental conditions have been identified in mitten crab *Eriocheir sinensis* (Shen et al. 2017) and coral reef fish community (Costa et al. 2023). All the metatranscriptomic data was deposited. The overall graph trend in the number of articles restricted only to aquaculture, published between 2012 and 2022, can be observed in Fig. 9.2. This graph was made by extracting the article through PubMed with the keywords "metatranscriptomics", "fish", "gene expression", "environment", "RNAseq", etc.

9.1.8 Challenges of Metatranscriptomic Sequencing

Although the new metatranscriptomic approaches are promising, several barriers prevent widespread use. First, since ribosomal RNA (rRNA) makes up a large portion of the RNA retrieved, the coverage of mRNA, which is the primary focus of transcriptomic investigations, may be significantly reduced due to rRNA's predominance. Effective rRNA removal has been attempted to get around this. Second, mRNA's well-known stability compromises the sample's integrity before

Table 9.3 Comparison of different metatranscriptomic pipelines and their approaches

	MetaTrans	MUFFIN	COMAN	FMAP	SAMSA2	HUMAnN2	SeqeezeMeta	IMP	MOSCA	MIntO
Data source	Paired end short reads	Paired-end Illumina short reads bd nanopore-based long reads	Short read	Short read	Short read	Paired end quality controlled short reads	Paired end short reads and nanopore based long reads	Paired end short reads	Paired end short reads	Paired-end Illumina short reads and nanopore based long reads
QC control	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
rRNA/tRNA removal	Yes	No	Yes	No	Yes	No	No	Yes	Yes	Yes
Assembly	No	Hybrid and long read assembly	No	No	No	No	Hybrid and long read assembly	Hybrid assembly	Hybrid assembly	Hybrid and long read assembly
Taxonomy assignment	Read based	Contigs based	Read based	Read based	Read based	Read based	Contigs based	Contigs based	Contigs based	Contigs based
Binning	No	Yes	No	No	No	No	Yes	Yes	Yes	
Function Annotation	Contig-based annotation	Contig-based annotation	Read-based annotation	Read-based annotation	Read-based annotation	Read-based annotation	Contig-based annotation	Contig-based annotation	Contig-based annotation	Contig-based annotation
Normalization		TPM		RPKM	No	No	No	CPM	TMM, RLE	TPM, marker genes
Gene Expression	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Visualization	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes
Web interface	No	No	Yes	No	No	No	No	No	No	No
Installation	Python	Nextflow and conda/docker	No	Perl and R	Python	Python, Conda and Docker	Conda	Python and Docker	Conda and Docker	FetchMGs and Conda
References	10.1038/srep26447	10.1371/journal.pcbi.1008716	10.1186/s12864-016-2964-z	10.1186/s12859-016-1278-0	10.1186/s12859-018-2189-z	10.1038/s41592-018-0176-y	10.1186/s12859-020-03703-2	10.1186/s13059-016-1116-8	10.1007/978-3-319-98,702-6_22	10.3389/fbinf.2022.846922

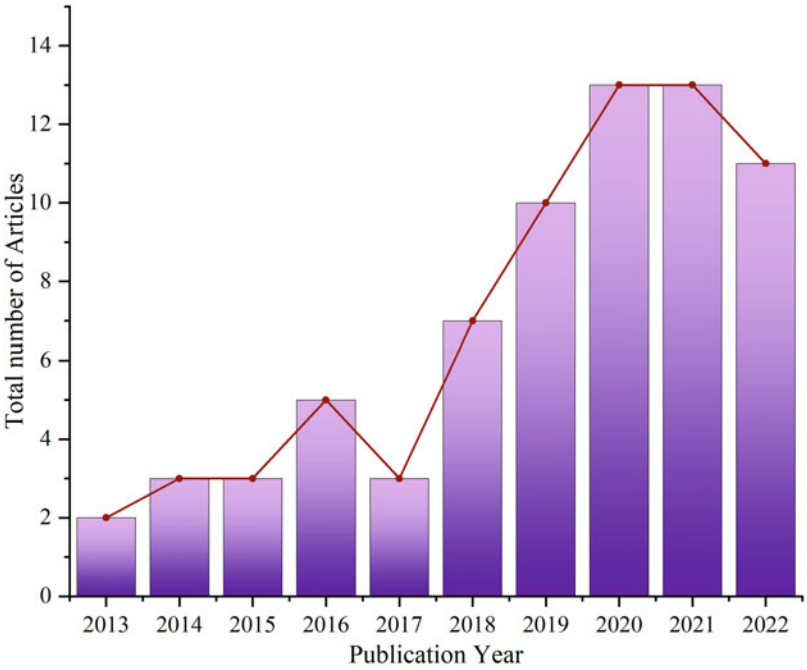

Fig. 9.2 The number of articles published in 10 years restricted to aquatic environment

sequencing. Third, even though commercial enrichment kits are offered, it might be difficult to distinguish between host and microbial RNA. Suppose a reference genome for the host is available. In that case, this can also be done in silico, as in the study by Perez-Losada et al., who looked at the influence of host-pathogen interactions on the human airway microbiome. The coverage of transcriptome reference databases is another restriction.

9.2 Concluding Remarks and Future Outlook

In conclusion, metatranscriptomics is attracting new researchers from multiple disciplines. A promising tool to define the taxonomic and functional variety present is the prospective outcome of the study and its expression under specific circumstances. The use of metatranscriptomic technology to sustainably improve aquaculture output has been underutilized compared to its application in other animal industries, despite the current excitement surrounding meta-omics and the rush to the sequence. Depending on the study's goal, the experiment's design is crucial for RNA-seq data analysis and sample collection. The Illumina platform should be used to sequence data that is size-selected to allow paired-end read merging and sequenced in the PE150 format or better with a minimum sequencing

depth of five million merge-able reads; however this number will probably be closer to ten million reads. In this review, we have highlighted the metatranscriptomic technology-specific computational tools for analysing RNA-seq data, and more sophisticated workflows for metatranscriptomic that integrate several of these technologies may answer various biological queries with little human involvement or input. Despite some of the issues with metatranscriptomics, the next generation of metatranscriptomic methods offers considerable potential in aiding our comprehension of the physiologically active portion of microbiomes and the pertinent pathways involved. This is due to the ongoing development of new methods, tools and algorithms for analysing metatranscriptomic data as well as our expanding knowledge presented by such datasets.

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Bioremediation and Its Application in Aquaculture

10

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Abstract

Aquaculture is the fastest-growing sector of food production, which has seen steady growth in recent years and is now an important part of many countries' national development and poverty alleviation strategies. Rapid advancements in aquaculture have led to serious worries about pond effluents that could harm the local ecosystem as a result of an increase in nutrient intake. Sludge generated as a result of significant amounts of unwanted organic material, primarily excess feed, and organic breakdown has negative environmental effects. The effluent must be treated before being released from the culture ponds due to its detrimental effects. The bioremediation technique, which has been regarded as an environmentally beneficial approach to treating organic waste that does not entail the use of chemicals, is one of the options that has attracted the attention of many researchers. In this book chapter, the potential for helpful bacteria in bioremediating aquaculture effluents is highlighted together with the hazardous elements in aquaculture waste.

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Keywords

Aquaculture · Effluents · Organic material · Bioremediation process

10.1 Introduction

Aquaculture is one of the fastest-growing sectors in the world. Modern aquaculture studies need high-density cultivation for commercial prospects. It also accounts for about 50% of all human consumption; by 2030, this value will jump to 62% (Abbasian et al. 2015). In another way, aquaculture contributes to elevated levels of dissolved nutrients in aquatic water bodies (Abe et al. 2002). In the bioremediation process, toxic compounds are converted into less harmful compounds such as CO_2 and H_2O. In aquatic environments, nitrogen (N) and phosphorus (P) lead to eutrophication, which also causes oxygen destruction and siltation. Nowadays, several countries have implemented integrated multi-trophic aquaculture (IMTA), which is also useful for the growth of parasites, bacteria, fungi and algae. Microbial metabolism involves a chemical reaction like oxidation-reduction (Adams et al. 2014).

Aquacultural wastewater contains nutrients such as ammonia (NH_3), nitrite (NO_2^-), nitrate (NO_3^-), organic nitrogen (N) and phosphorus (P) (Aislabie et al. 2006). The inwardness of nutrients mainly removes nitrogen in aquatic ecosystems. As per available literature, it is necessary to reduce nutrients from biological treatments (Álvarez et al. 2017). This remediation process is eco-friendly and removes nutrient that leads to a cheap cost. Bioremediation methods produce utilised products like fuels, fertilisers, fine chemicals and feed, which are also helpful in the aquaculture sector. Moreover, several studies also reported that numerous techniques used in the field of remediation technology polluted aquaculture ecosystems (Andreotti et al. 2017; Attasat et al. 2013; Behera et al. 2020). So to reach better goals, we need technology like a biological aerated filter that uses wetland technology and also needs a carbon dioxide (CO_2) absorption method (Azeez et al. 2021).

Exposure to aquaculture in animals leads to high concentrations of nitrogenous waste, specifically ammonia and nitrite (Bartoli et al. 2005). The elevated level of ammonia shows a decreased survival rate and inhibits growth rate and several physiological parameters in aquatic animals (Borges et al. 2005). Nitrite and ammonia both act as stressors, and they elicit corticosteroid hormones in the fish. High levels of circulating corticosteroids in aquatic animals showed immune dysfunction that ultimately decreased disease resistance powers (Burford et al. 2003). Ammonia accumulation is the direct result of the interaction between fish osmoregulation and the action of heterotrophic bacteria. Nitrifying bacteria transform ammonia into nitrite (NO_2) and nitrate (NO_3), which are then utilised by plants. As a result, the most prevalent nitrogen compounds in aquatic habitats are nitrate and ammonia. The neutral, unionised form of ammonia (NH_3) are extremely poisonous to fish and other aquatic species (Carolin et al. 2017).

In this study, a very desirable goal is to enhance waste management in aquaculture. Aquaculture will become a more long-term sustainable farming method as a result of enhanced wastewater treatment, a decrease in the amount of organic compounds and a reduction in biological risk due to the removing phosphorus (P) and nitrogen (N) from the water column (Chai et al. 2021).

10.2 Types of Bioremediation

The key technique of regulation called competitive exclusion, which limits the occurrences of pathogenic microorganisms in aquacultural habitats. The bacteria are naturally drawn to hydrocarbons, reducing excess nutrients and organic matter in the water, particularly nitrogen, ammonia and phosphorus (Chávez-Crooker & Obreque-Contreras 2010). The competitive exclusion of the microflora and the antagonistic effects of pathogenic microorganisms are the reasons for microbial intervals in the aquaculture field's function. Here, specific metals are removed like oxyanions and organic samples (Cheng et al. 2019). Different types of bioremediation are discussed below.

- **Biostimulation**: Enhancing the environment by introducing elements that increase the capacity of naturally occurring microbes to break down toxic substances (Fig. 10.1). By modifying the environment, existing bacteria that can bioremediate are stimulated through the process of biostimulation (Chew et al. 2017). This can be accomplished by adding different types of limiting

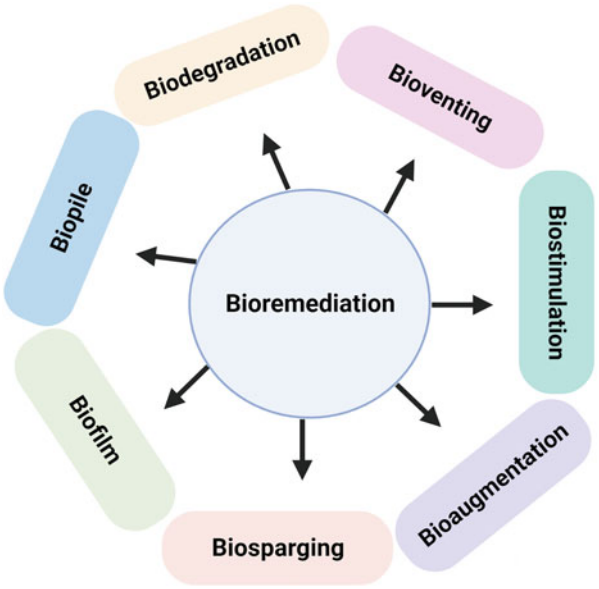

Fig. 10.1 Different types of bioremediations

nutrients and electron acceptors, such as phosphorus, nitrogen, oxygen or carbon (e.g., in the form of molasses), which are otherwise present in amounts that are too low to limit microbial activities. It was defined as adding nutrients, oxygen or other electron donors and acceptors to the coordinated site in order to boost the population or activities of naturally occurring microorganisms that are available for bioremediations (Converti et al. 2006). Biostimulation is a type of natural remediation that can accelerate pollutant degradation by enhancing factors like aeration, nutrient addition, pH regulation and temperature control. According to Davey & O'toole (2000), biostimulation can be used as a remediation technique to remove petroleum pollutants from soil, but it first needs to be evaluated for both the intrinsic degradation potential of the native microflora and the environmental factors that affect the kinetics of the in situ process. The main benefit of biostimulation is that bioremediation will be carried out by native microorganisms that are already present, well adapted to the subsurface environment, and are widely distributed throughout the subsurface (De la Torre et al. 2008). The main issue is that the subsurface's local geology makes it difficult to deliver additives in a way that makes them easily accessible to subsurface microorganisms. It is challenging to evenly distribute additives throughout the affected area due to the tight subsurface lithology that is impermeable (tight clays or other fine-grained material). A uniform distribution of additives is prevented by subsurface fractures that provide preferential paths in the subsurface that additives follow (Durborow et al. 1997). Nutrient additions may also promote the growth of heterotrophic bacteria, which do not naturally break down Total Petroleum Hydrocarbon (TPH).

- **Bioaugmentation**: Bioaugmentation is the inclusion of pre-grown microbes to enhance microbial populations in the aquatic environment to mitigate the pollution and reduce the mitigation time and cost. The microbes play important role in the distribution of nitrogen in aquatic environment (Elektorowicz 1994). The denitrifying bacterium, *Pseudomonas* sp., and the coupling and smelling salts, oxidise by employing *Nitrosomonas* sp. According to the study, *Pseudomonas stutzeri* strains were isolated from catfish lake, which was rich in the levels of dissolved nitrogen (NH_4^+, NO_2^- and NO_3^-) in fishpond water, which was approximately 10 mg/L (Francis et al. 2007). For the nitrifying microorganisms to survive, higher oxygen levels were necessary (Fig. 10.1).
- **Biofilm**: The complicated process of biofilm formation may involve a single species of microorganisms or more than two species. Microorganisms proliferate on a surface and release extracellular polymeric substances as biofilm forms (EPSs [extracellular polymeric substances]). As a result, the growth rate and gene transcription of microorganisms is altered in their phenotypes (Gao et al. 2016). The surface of this place is covered in microorganisms that adhere to one another. Extracellular polymeric substances (EPSs), which make up the slimy extracellular matrix, become enmeshed with these adhering cells (Fig. 10.1). Biofilm production starts when bacteria that are now floating freely adhere to a suitable living or non-living surface (Gomez & Sartaj 2014). Functional groups involved in the diffusion process, proteins, sugars and extracellular DNA make

up the EPS (nucleic acid). The construction of a bulbous and intricate three-dimensional structure known as a biofilm is made possible by the EPS, which lets the microbes stick to one another in a biofilm (Hall-Stoodley et al. 2004).

- **Biodegradation**: By converting petroleum hydrocarbons to energy or vital metabolites for biomass development, cold-adapted bacteria can produce both. There are various hydrocarbons that cause these bacteria to respond in various ways. Shorter water-soluble alkanes can be readily taken up by bacteria cells for biodegradation, but the absorption of surface-active biomolecules like biosurfactants and bioemulsifiers makes it easier to dissolve medium- and high-molecular-weight (MLMW) hydrocarbons. Enzymatic hydrocarbon breakdown is carried out after these substrates enter the bacterial cell to produce cellular metabolites for microbial growth (Hammouda et al. 1995). It is interesting to note that mesophilic bacteria and cold-adapted bacteria both degrade petroleum hydrocarbons in a similar manner. The primary distinction between them is the rate of reaction, which is slower in the former group due to environmental limitations than in the latter group (Hu et al. 2017). When hydrocarbons are attacked by oxygen-based attacks on electron acceptors, an enzymatic breakdown of the hydrocarbons begins.
- **Bioventing**: In order to facilitate microbial biodegradation, bioventing in a cold environment provides oxygen via air injection for native, cold-adapted microorganisms. The enhanced bioventing is done at the original contaminated site, where an air vessel installed in the subsurface soil provides the oxygen. This process transforms volatile pollutants into comparatively harmless vapours that slowly spread through the soil (Hulth et al. 2005). There are a number of variables that affect bioventing, including soil permeability, humidity, the presence of interfering compounds and the availability of oxygen. Microbes require nutrients for growth and the degradation of hydrocarbons, while ozonation is used to hasten the partial oxidation of stubborn pollutants into volatile vapours (Hussenot et al. 1998). Due to the increased oxygen supply, enhanced bioventing has been successfully used to remove small-scale, medium-molecular-weight hydrocarbon pollution from soils. Medium-weight hydrocarbons like gasoline, diesel and bitumen are volatile substances that can break down into smaller BTEX (benzene, toluene, ethylbenzene and xylene) vapours. Anaerobic bioremediation of chlorinated contaminants uses bioventing in addition to petroleum hydrocarbons. According to a study, resistant chlorinated pollutants including 1,1,1-trichloro-2,2-bis(pchlorophenyl) ethane, DDT (dichlorodiphenyltrichloroethane) and 2,4-dinitrotoluene (DNT) are significantly eliminated from the soil when oxygen is replaced with a mixture of nitrogen and a little amount of carbon dioxide (electron donor) (Jegatheesan et al. 2009; Johnson et al. 2001).
- **Biosparging**: Similar to oxygen injection, biosparging treats the condition at its natural site. When air is injected into saturated soils, volatile contaminants that contain microorganisms that degrade hydrocarbons spread from their original location to the soil surface (Junier et al. 2010; Kaloudas et al. 2021). Unsaturated soil has many pores as opposed to saturated soil, which has few or no pores. This air injection's objective is to transport volatile contaminants from a deeper soil

layer to the soil's surface, which has more oxygen and is inhabited by microorganisms that degrade hydrocarbons. According to reports, the soil permeability and the degree of pollution recalcitrance play two significant roles in biosparging treatment. While highly degradable pollutants produce higher removal efficiencies, highly permeable soil favours the transfer of contaminants. In a study by Wu et al. (2005, 2020), it was found that biosparging treatment on soils with a deeper depth resulted in significant removal of benzene pollutants (96%). Another biosparging study by Kao et al. (2008) claimed that biosparging has removed 70% of the groundwater's BTEX contaminants. According to these studies, biosparging can be applied to groundwater and deeper soil that is contaminated with hydrocarbons (Kong et al. 2021; Könneke et al. 2005).

- **Biopile**: For the remediation of hydrocarbon-polluted soils, biopile has been shown to be efficient and practical. High-rise piling of the polluted soil removed from a polluted region is used in biopile-facilitated bioremediation in cold climates (Kumar & Yadav 2018). In reaction to the cold, irrigation, organic fertiliser and treatment pile are used in the biopile treatment to improve the effectiveness of removing hydrocarbons from the air. Some advantages of biopiles have been suggested, including space savings due to elevated piles and high removal efficiency, provided that the soil temperature, aeration and fertiliser bioavailability are supported adequately (Lefebvre et al. 1996, 2004). When using a biopile to remove hydrocarbon pollutants in cold weather, temperature is crucial. Low-molecular-weight hydrocarbons' volatilisation can be controlled, and microbial biodegradation can occur in an ideal soil environments (5–10 °C) with nutrient additions. The influence of overall sunlight exposure time on its removal efficiency was recently brought to light in an Antarctica study on a half-tonne biopile. According to the study, the first biopile with 157 h of total sunlight exposure had higher hydrocarbon removal (75%) than the second biopile with 55%, which had 108 h (Li et al. 2017). According to research, increased exposures to sunlight may raise the soil's temperature and promote the growths of microorganisms as well as the biodegradation of hydrocarbons (Li et al. 2019). In another study in Antarctica, a temperature-dependent biopile was used to abate TPH pollution and the amount of TPH was significantly reduced by 75% during the summers compared to other times of the year, when temperatures are typically higher and microbial activity is more (Liu et al. 2013, 2017). The hydrocarbon removal effectiveness of a 12-month biopile with a mean temperature of 6.5–6.7 °C is higher (75.7%) than that of the control (49.5%), which has a mean temperature of 5.2–5.3 °C (Álvarez et al. 2020). All of the aforementioned studies used fertiliser to promote the development of microbes that break down hydrocarbons for effective biodegradations. These findings demonstrate the importance of temperature on biopile's ability to clean up petroleum hydrocarbons in cold conditions when nutrients are sufficient. Prior to implementation, the temperature requires should be optimised as excessive heats can kill and inhibit psychrotolerant microbes (Sanscartier et al. 2009).

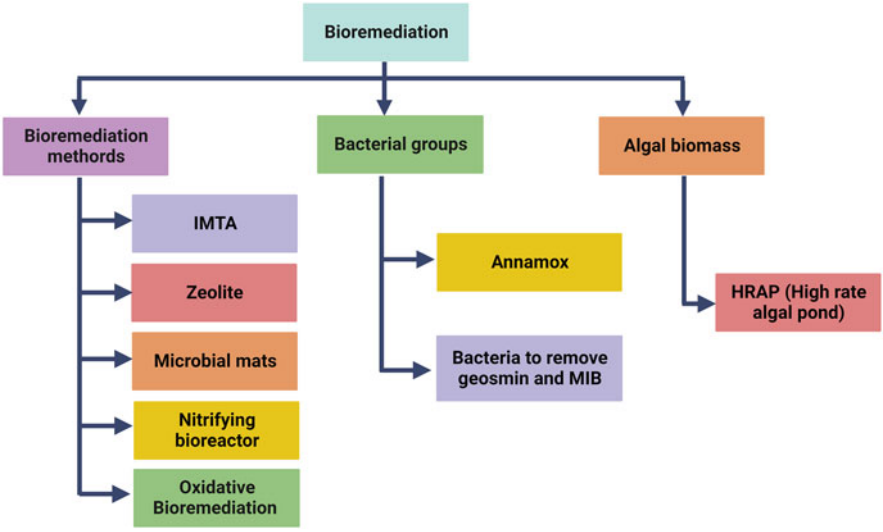

Fig. 10.2 Application of bioremediation

10.3 Applications of Bioremediation in Aquaculture

There are three major methods of bioremediation applications, all of which are biological methods. Apart from this, various technological developments are described below (Fig. 10.2) (Bartoli et al. 2005).

10.4 Biological Methods

The utility of microbial mats and zeolite was focused on the removal of ammonia in the aquaculture ponds and worked MIB (2-methylisoborneol) as natural filters in the system. In another way, microbial mats were another process for the treatment of shrimp to lower organic loading (Long et al. 2021).

The nitrifying bioreactors play an important role in removing TAN (total ammonia nitrogen). The nitrifying bioreactors play an important role in removing TAN and were developed after 16 years of research. Here, two types of reactors have been proposed as follows.

- In situ stringed bed suspended bioreactor (SBSBR)
- Ex situ packed bed bioreactor (PBBR)

The vital component discovered is known as recirculating aquaculture systems (RAS). Great efforts have been made to nitrify biofilters for aquaculture purposes (López et al. 2010).

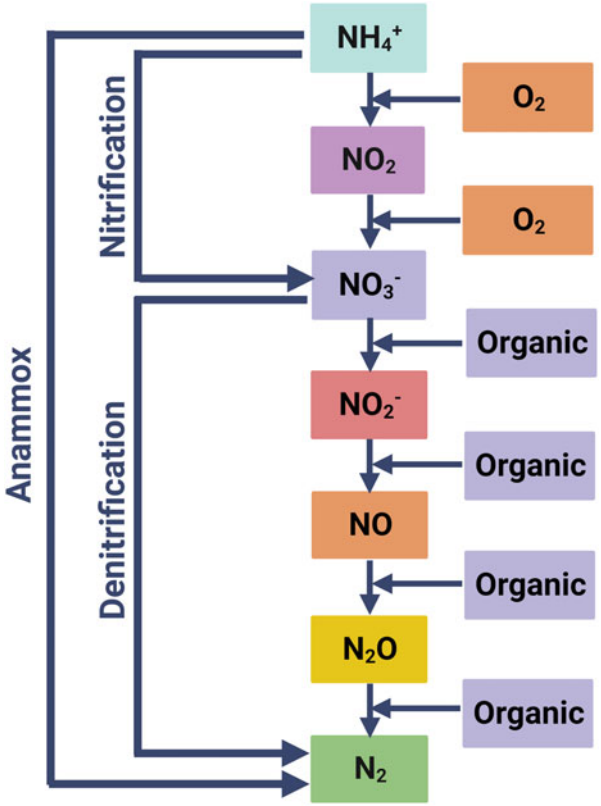

Fig. 10.3 Schematic representation of the nitrogen (N_2) cycle

10.5 Bacterial Groups

10.5.1 Aerobic Nitrification

In the presence of oxygen, bacteria transfer ammonium (NH_4^+) to nitrite (NO_2^-) and finally nitrate (NO_3^-) ions (López et al. 2010). In the presence of oxygen, ammonia-oxidising bacteria (AOB) oxidise ammonia and transform it into nitrite via intermediate compounds like nitrite transformed into nitrate by hydroxylamine and then nitrite-oxidising bacteria (NOB). Ammonia-oxidising microorganisms (AOM) are the result of the merger of the AOB and the ammonia-oxidising archaea (AOA). With the aid of the Calvin-Benson cycle, autotrophic bacteria that consume nitrogenous substrates fix CO_2 as a source of carbon (Ma et al. 2021). Nitrate and ammonia provide the main nitrogen sources for the development of microorganisms (Fig. 10.3).

10.5.2 Denitrification

Denitrification is the key process where fixed nitrogen converts into nitrogen gas and finally returns to the environment. It takes place in low-oxygen environments where nitrogen oxides, such as nitrate, nitrite, nitrous and nitric oxides, are used as electron acceptors in place of oxygen, resulting in the conversion of N_2 gas as an end product (Margesin & Schinner 2001). The process of denitrification in bacteria, archaea and eukarya can be carried out by a wide variety of microorganisms. Many genes involved in the denitrification process are found in the denitrifying bacteria and archaea (Michels et al. 2014). This gene produces four metalloenzymes that sequentially convert nitrate to N_2: nitrite reductase, nitric oxide reductase and nitrous oxide reductase (NO_3^-, NO_2^-, NO^-, N_2O and N_2) (Fig. 10.3).

10.5.3 Anaerobic Ammonium Oxidation (Anammox)

A conventional process where the removal of ammonia converts into nitrogen is known as Anammox. In the bacterial grouping, a two-step process of aerobic nitrification and anaerobic denitrification, it conjugates nitrate or nitrite with ammonia and produces dinitrogen gas (N_2) (Muhamed & Edwin 2003). The key benefit of this process is that it does not want any organic carbon source and it keeps lower costs and also less energy for aeration (Fig. 10.3).

The main problems in freshwater aquaculture ponds are the Geosmin and MIB, which are terpene-based compounds made from algae (Fig. 10.2). To escape this smell, several bacterial strains were confirmed and observed such as *Rhodococcus wratislaviensis*, DLC-cam, *Pseudomonas putida* G1 and *Rhodococcus* sp. T1 had the capability to transform Geosmin or MIB (Mulbry et al. 2007, 2008). In these circumstances, a main regulatory enzyme helps in the degradation of the terpene-like complex. There is a question about the bacterial toxicity of the fish. The study showed that *Rhodococcus wratislaviensis* DLC-cam was not virulent against fish, even in higher concentrations (Nithiya et al. 2016). Those bacterial strains are able to grow in minimal media with fish feed as the sole source of carbon. The three strains can be frozen without hampering viability which is capable of storage and transportation to a treatment area (Perfumo et al. 2007).

10.5.4 Algal Overview in Bioremediation

Generally, there are two types of algal water systems, static algal ponds and high-rate algal ponds (HRAP), both of which are useful for effluent treatment. The HRAP is a low-energy wastewater treatment process which creates the second loop for water treatment via flow in aquaculture systems (Philippot 2002). Continuous water flow makes the circulation of water in every culture tank, either a paddlewheel or strong aeration on a short time scale (in days). The main benefits of the effluent treatment of wastewater are that it improves energy consumption and harvested algal mass can be

useful for biofuel production. The perforated algal species can assimilate nutrients from the wastewater, which provides secondary- as well as tertiary-level treatment (Piehler et al. 1999).

10.5.5 Parasitic Fauna in Bioremediation

Parasites are pathogenic and cause several diseases in aquaculture. Helminthic parasites act as bioindicator that provide early warning of ecological hazards specifically low-level sensitive hazards. As a result, the host enhances the heavy metal channels (Rayner et al. 2007). The parasite-infected fish reduces the nutritional value and causes mass mortality. Bayoumi et al. (2016) opined that a few parasites including metazoan parasites, crustaceans, digeneans and monogeneans have been obtained in various Nile tilapia tissues. The monogenean parasites showed activity towards chromium, iron and nickel. Bayoumi et al. (2016) also reported the external and internal metazoan parasites in three fish species of the Arabian Gulf of Dammam. The highest parasitic load was observed in the session in spring and summer (Risgaard-Petersen et al. 2006). The monogenean and digenean fish parasites play a crucial role in Zn and Se. The intestinal flukes added high concentrations of Se, As, Zn and Cu. They performed as bioindicators for metal contamination. More recently, Hassan et al. demonstrated that contamination of marine fish with cestodan increases the bioaccumulation of heavy metals (Fe, Pb, Cd, As and Zn) in fish organs. Ashmawy et al. reported that heavy metal contamination affects *Oreochromis niloticus* in Egypt.

10.6 Composting in Bioremediation of Organic Contaminants

Recalcitrant organic pollutants are biodegraded by the various microbial communities found in composting materials. According to several studies, there are species that can mineralise these pollutants (Sales da Silva et al. 2020; Shah et al. 2001). For example, *Acinetobacter lwoffii*, *Bacillus subtilis* and *Raoultella ornithinolytica* can degrade crude oil.

10.6.1 Basics of the Composting Process

The four stages of the composting process are mesophilic, thermophilic, cooling and maturation. These phases differ from one another in terms of temperature, oxygen consumption, stability, carbon and nitrogen content and pH profiles (Shpigel et al. 1993). The beginning of the thermophilic phase is thought to be the time of greatest activity because the temperature is an indicator of microbial activity. While thermophilic microorganisms make up the majority of the microbial population, microbes that are mesophilic are temperature-sensitive and become inactive during the thermophilic phase (Sirakov & Velichkova 2014). Complex organic molecules like

cellulose and hemicellulose, which are less biodegradable, start to break down during this phase. The breakdown of organic nitrogen-containing material results in the creation of ammonia, which causes the pH to rise (Sirakov et al. 2013).

The high temperatures during this time also render most human and animal pathogens, including *Salmonella* sp. and *E. coli*. The soluble C/N ratio and germination index are two indicators used to assess the stability and maturity of the composts (Soletto et al. 2005).

10.6.2 Conditions for Efficient Composting

Like other biological processes, the availability of nutrients and the environment have an impact on composting. When necessary, this section will also cover the ideal circumstances for efficient composting in addition to the major factors that influence the process. The four steps of the composting process are monitored and managed for characteristics, such as oxygen and moisture content, that promote microbial activity (Varjani 2017).

10.6.3 Initial Compost Materials and Nutrient Balance

The bacteria in the compost require both macronutrients like metals and minerals and micronutrients like carbon, nitrogen, phosphorus and potassium. As ammonia is volatilised, lower ratios that are excessively high compared to the needs of the microbial population may cause nitrogen loss and odour pollution (Vilchez et al. 1997). On the other hand, bigger ratios cause composting operations to take longer because there are not enough nitrogen supplies available. As a result, the initial C/N ratio is often altered before composting. Sucrose, glucose, spent mushrooms and cellulose have all been utilised to increase the C/N ratio and decrease ammonia loss in compost mixtures (Wang et al. 2019).

10.6.4 Moisture Content

The microbes require a sufficient amount of moisture to exist: Moisture content is a crucial factor in the composting process. The ideal moisture percentage depends on the physical properties of the feedstock, such as the particle size and water-holding capacity, but most composting studies treating different kinds of organic materials have used a range of 55–65%.

10.6.5 Oxygen Content

The provision of oxygen is essential during composting since the aerobic bacteria in compost need it for respiration 1076 C. To maintain their metabolic processes

throughout the composting process, the bacteria must receive enough oxygen. It is advised that the compost gas contains more than 10% oxygen at all times. In addition to the composting gauge, the optimal aeration frequency will depend on the particle size and moisture content of the composting materials (Whelan et al. 2015).

10.6.6 Temperature

The heat produced by the aerobic microbial breakdown of organic materials is a by-product of the self-healing biological process of composting. Moisture and the makeup of the microbial community are affected by the heat generated. There is evidence that hot weather dries out the compost and slows down the composting processes. Only thermophile microbes, like those in the *Thermus* genus, can live and grow in high temperatures because the diversity of microbes also declines in such environments (Xia et al. 2018).

10.6.7 pH

The type of organic waste has an impact on the starting pH of the composting materials. While the fungal species in compost favour slightly acidic environments, the bacterial community prefers neutral or near-neutral pH. The pH in the thermophilic stage rises as ammonia is produced during the breakdown of nitrogen-containing organic materials. Other decomposition products like NH_4^+ and HCO_3^{2-} serves as a buffers to keep the pH high during the composting process (Xiaobin et al. 2021).

10.7 Bioremediation of Recalcitrant Organic Contaminants

Microorganisms are used in bioremediation systems to break down organic pollutants. Three main pathways can lead to this degradation process: (1) mineralisation or metabolism, in which the contaminant is used as a source of nutrients by the microorganisms; (2) co-metabolism, in which the destruction of pollutants that do not serve as a source of nutrients happens simultaneously with metabolic processes; and (3) nonspecific oxidation, on the other hand, which involves the extracellular degradation of contaminants. Composting is a kind of bioremediation offers distinct advantages over single- or multi-strain bioremediations (Yap et al. 2021).

10.7.1 Petroleum and Petroleum-Related Organic Pollution Bioremediation

Because of anthropogenic activities including petroleum extraction, processing, transportation, storage and consumption, petroleum pollutants are among the most prevalent organic contaminants in the environment pollutants connected to petroleum at various scales. Diesel, PAH and TPH are petroleum-related toxins that have been cleaned up via bioremediation. Various investigations have identified a wide variety of microbial species that are capable of metabolising petroleum and compounds associated with it. *Raoultella ornithinolytica*, *Bacillus subtilis*, *Serratia marcescens* and *Acinetobacter lwoffii* species were among the crude oil-degrading bacterial strains that Abena et al. discovered and enhanced in polluted soils, boosting the TPH degradation to 48.1%. There are a number of species that can break down petroleum, including mesophilic microbes like *Acinetobacter calcoaceticus*, *Bacillus simplex*, *Paenibacillus pabuli*, *Bacillus pumilus* and *Pseudomonas aeruginosa*, as well as thermophilic microbes like *Bacillus megaterium*, *Aspergillus* sp. and *Pseudoxanthomonas* sp.

10.7.2 Microalgal Bioremediation of Heavy Metal Pollution

There is a lack of water for direct human consumption due to the economy's rapid growth and the population explosion. Therefore, the remediation of water contamination naturally draws attention from all around the world. Heavy metals include substances like gold, silver, copper, iron and lead that have an atomic weight between 63.5 and 200.6 with a density greater than 4.5 kg/cm^3. Most heavy metals are carcinogenic, teratogenic, mutagenic and non-biodegradable which affect life. When these heavy metals enter the human body, they cause headaches, arthralgia, mental disorders, kidney damage and even cancer. Heavy metal contamination in water can be brought on by industrial pollutants as well as heavy metal pollution in the air and soil. Activated sludge and biofilm adsorption are biological methods that have better feasibility and environmental friendliness. It can effectively handle contaminated water. Since the 1950s, microalgae have played an important role in the treatment of sewage and their ability to control CO_2 emissions. In urban sustainable development, the role of microalgae was revealed recently (Zhuang et al. 2020).

10.7.3 Mechanism of Microalgae in Green Application and Bioremediation

The role of microalgae is crucial in the production of economic energy. The emission of a large amount of nitrogen oxides, carbon monoxide, sulphur oxides, etc. due to burning of fossil fuels causes an adverse impact on the ozone layer of the earth and also causes changes in climate.

The cornerstone for the production of high-quality biofuels in microalgae is the precursor to lipid and amino acid biosynthesis of cellulose, which is produced by microalgae during the fixation of CO_2. The process releases oxygen, and the bacteria in the sewage that are present then eat the released oxygen, boosting and lowering the organic load in the wastewater, increasing the metabolic activity of the microorganisms and remediating the wastewater. As suggested, microalgae are employed as a raw material to create bioenergy and store carbon.

According to Balzano et al. (2020), microalgae primarily produce metallothionein (MT) and phytochelatins: these two proteins help to minimise toxicity effects and heavy metals (PCs). Human activity and the movement of natural materials result in the production of metals, such as copper (Cu), lead (Pb), mercury (Hg), zinc oxide (ZnO-NPs), titanium dioxide (TiO2-NPs) and silver, as well as metal nanoparticles (MNPs) (Ag-NPs). Nutrients like organic matter (OM), nitrogen and phosphorus have an impact on several environmental factors, including the growth rate of microalgae, biomass accumulation and the efficiency of pollutant removal (Zumft 1997).

10.7.4 Utilising Microalgae to Bioremediate Heavy Metals in Water

Cadmium (Cd) is the most hazardous heavy metal (HM) found in industrial effluent and is also one of the most intrusive and toxic HMs. Consumption of cadmium can results in kidney illness, hypertension, peripheral neuropathy, osteoporosis and, in extreme situations, cancer, Parkinson's disease and Alzheimer's disease.

Chromium is used in many industrial processes, including the production of paint, steel, leather tanning and dyes. Several industrial operations, including battery manufacturing, electroplating and the fabrication of microelectronic goods, release lead into the environment. Lead concentrations in surface water, soil and sediment can be very high. Thus, common bacteria play a crucial role in eliminating lead from the environment.

In order to comprehend the process of lead adsorption by microalgae, Keryanti and Mulyono (2021) employed the microalgae *Aphanothece* sp. produced in a photobioreactor system for 14 days. They then used the microalgae as a biosorbent to bind lead in an aqueous solution. Mental retardation, damage heart, brain and other organs, as well as an increase in the prevalence of Parkinson's disease, Alzheimer's disease and autism, are all major health issues that mercury can bring on. *Pleurococcus* species, *Scenedesmus* species and *Chlorella* species were procured from the Andes and Ecuador by Vela-Garcia et al. (2019) to assess the impact of mercury (Hg), phosphorus (P), sulphate (SO_4^{2-}) and other pollutants that can be removed from gold mine effluent using microalgae treatment.

10.8 Conclusion

The bioremediation process reduces the contamination levels and lessens the harmfulness of abiotic stresses in oceanic ecosystems. It is eco-friendly and economically favourable. It is observed as an advantage to all kinds of water bodies. The bioremediation mechanisms grow rapidly in the field of aquaculture. It provides superior systems and good quality control of ecological biotechnology measurements. In this medium, a more practical approach is needed for screening for harmful bioremediation materials. The aquaculture divisions need to deliberate these cautiously and take part in good quality waste management in their actions as an asset that will support them to tolerate and expand their creativity while tranquilly caring for the ecosystems.

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Application of Probiotics in Aquaculture

11

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Abstract

Every action towards intensifying aquaculture practices and raising productivity has been associated with environmental consequences, such as an increase in physiological stress and a substantial rise in fish disease outbreaks which has resulted in bacterial resistance and loss of productivity, and hampered the aqua industry's ability to develop sustainably. These effects are partly brought on by management strategies used in production cycles, which result from the indiscriminate use of antibiotics or chemotherapeutic drugs. This chapter gives an overview of the application of probiotics in aquaculture, along with a realistic evaluation of the outcomes, focusing mainly on the sources, selection criteria, method of application and the mode of action which are crucial from an eco-friendly, sustainable and intense aquaculture perspective.

Keywords

Aquaculture · Microorganisms · Probiotics · Prebiotics

11.1 Introduction

Aquaculture is the practice of cultivating aquatic organisms by taking part in the procedure of breeding the stock that will eventually become the crop to increase production output. Aquaculture now is a lucrative industry, as it has begun to appear as a significant source for providing a large population of the world with cheaper and easy-to-digest animal proteins, nutritional security and livelihood safety (FAO 2018). Aquaculture methods must be intensified to increase the production rate

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since the existing fish output is insufficient to fulfil the rising per capita fish consumption demand in the world. To meet this demand, vertical expansion through intensifying and diversifying aquaculture involving high stocking densities is highly warranted, including mono- and polyculture. However, with the rising intensification and commercialization of aquaculture production, high stocking densities are liable to create havoc in the culture environment because of the release of a wide range of concentrated organic wastes, which diminishes the dissolved oxygen level, subsequently releasing poisonous/toxic metabolites (such as ammonia, hydrogen sulphide, nitrites and methane) into the culture environment (Martínez Cruz 2012). Deterioration in water quality invites many disease infestations where the aquatic creatures experience significant levels of environmental stress, rising occurrence of disorders and mortality and thereby reducing the profit by a huge margin.

Pathogenic factors vary, from ss-RNA viruses like infectious salmon anaemia (ISA) to complex parasitic crustaceans. The pathogenic agents are commonly known as bacteria, viruses, fungi and parasites. The disease-causing microbes are considered etiologic representatives of numerous illnesses like septicemia, enteric red mouth disease, haemorrhage, vibriosis and many more. Antimicrobials and other chemotherapeutic drugs were once the standard treatment for pathogen attacks in fish farming. Nevertheless, frequent use of these chemotherapy medications results in their buildup in aquatic environments, which has detrimental effects including the establishment of antimicrobial resistance (AMR) and a biomagnification problem in the food chain (Saini et al. 2014). The application of commercially developed vaccines to vaccinate the fish by injection is unserviceable for smaller-sized fish or enormous numbers of fish. Against the backdrop that when all the solutions are unsafe or expensive, the hunt for safer, new alternatives to these obnoxious products or chemical substances, has become crucial.

As a result, probiotics have been used worldwide as an alternative approach. Different components such as the origin, dosage level and period of probiotic supplementation may influence the probiotic activity differently since probiotics often exert host-specific differences in their activity as well as strain-specific differences (Van Hai 2015).

11.2 Probiotics: Definition

The term and definition of probiotic were probably coined by Parker (1974) as microorganisms or agents contributing to the microbial equilibrium in the intestines. The concept of probiotics has developed since then and is often used for associating bacteria with beneficial effects. The words ‘pro’ and ‘bios’, which both indicate ‘for life’, were combined to produce the terminology ‘probiotic’ (Gismondo et al. 1999) and are usually indicated as ‘supporter for life’, which organically improves the recipient’s general health. Fuller (1989) reviewed probiotics as ‘a living supplement for microbial feed which improves the recipient’s health by enhancing its microbial intestinal balance.’ Naidu et al. (1999) altered the concept of probiotics to accommodate the immunostimulant effect of probiotics as ‘an adjuvant to the microbial

diet that has beneficial effects on the physiology of the host by influencing gut microbiota and metabolic balances, regulating mucosal and systemic immunity'. A broader application of the term 'a living microbial adjuvant that benefits the host by modifying the host-associated microbiota, assuring improved feed utilization or greater nutritive quality, enhancing host resistance, or increasing the ecosystem's suitability for cultivation'. Thus, there is an incomprehensible display of definitions, and the 'theory of probiotics' still stands complicated as new discoveries arise. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have taken into account each of these concepts and declared that probiotics are live microorganisms that, if administered in an appropriate quantity, may deliver health benefits to the host. However, in aquatics, probiotics can be cellular/extracellular components of microorganisms, which can be readily available in live or dead preparations. Salminen et al. (1999) suggested probiotic agents to be 'any microbial cell preparation or cellular elements that have an advantageous impact on the health and wellness of the host'.

Probiotics refer to a balanced microbiome with a calibre that consistently improves their effectiveness and encourages good health benefits for the hosts. Probiotics protect the organism from pathogenic bacteria by releasing various antagonistic compounds such as bacteriocins and specific organic acids, competing for attachment or binding sites, restricting the resources available such as nutrients and space and operating as an immunostimulant and modifications in gut enzymatic activity and dietary aids, such as those that increase feed usage and feed digestion (Merrifield et al. 2010; Nayak 2010; De et al. 2014).

The popularity of probiotics has paved the way for additional ideas, including prebiotics, which is often non-digestible fibres which enhance the beneficial bacterial population in the host's intestine. Prebiotics are indigestible additives in food that have a selective and beneficiary impact on the host and promote the growth and/or development of single or more useful bacteria in the colon. Prebiotic recruitment will dramatically alter the number of specific microflora in colonies, thereby altering microflora composition. Prebiotics are carbohydrates that can be categorized as monosaccharides, polysaccharides or oligosaccharides, based on molecular size or degree of polymerization. Kocher (2004) subsequently proposed a different type of functional saccharides, such as fructooligosaccharides, mannan-oligosaccharides, insulins and β-glucans which are known as immune-saccharides, which directly stimulate the immune system instead of probiotics. Another potential strategy to enhance the adoption and survivability of live microbial supplements in the gastrointestinal process is through the simultaneous administration of pro- and prebiotics, also known as synbiotics. Recognizing all the benefits conferred by pro- and prebiotics, we can apply them symbiotically only if we are aware of their upsides in fish production. As a result, during the past few decades, fish farming has benefited from the administration of both probiotics and prebiotics that gain from their potential to inhibit pathogens. Additionally, the methods utilized to investigate probiotics and prebiotics need to be standardized, especially with respect to the accuracy of the innate and/or cellular immunological reaction. The utilization of prebiotics in the farming of fish and shellfish has received little attention, irrespective

of the fact that they may have advantages for health and productivity as seen in a variety of terrestrial species.

11.3 Sources of Probiotics

Although no one source justifies the employment of probiotics in aquatics, evidence indicates that fish and shellfish are used extensively in South America and Asia (China and India) in particular. Since the early 1990s, it has been beneficial to use microorganisms from host-derived sources for probiotics. There are claims that microbiomes existing in healthy hosts are expected to be a component of the natural defence mechanism and helpful in numerous manners (Gomez et al. 2013). The candidates who are indigenous, in particular to the ecosystem they are exposed to, will naturally survive and perform physiological functions at their best. Thus, the gut of aquatic species has drawn interest in the isolation of supposed putative probiotic production strains, including a large microbial population at high concentrations. Microbes residing in the water bodies and soil sediment may be vital as probiotics. Further, additional sources of probiotics described in several types of research include marketable products (Toyocerin®, Bactocell®, Cernivet®LBC, Calsporin®, Sanolife®MIC, Efinol®). However, the lack of data, such as species identification, composition, amount and dose level, makes the quality assurance of commercial items appear weak. Thus, host-derived microorganisms especially microbes isolated from the aquatic animal's gastrointestinal tract help to eliminate some of the above problems and also serve the host's interests in several different ways, emphasizing their great probiotic potential.

11.4 Selection Criteria of Probiotics

In order to certify a specified microorganism as a probiotic, it must necessarily satisfy the following criteria (Merrifield et al. 2010):

(a) Must resist and metabolize in the gut environment like resistance to low pH and the action of bile salts and pancreatic enzymes.
(b) Must be non-pathogenic, non-toxic and free of plasmid-encoded antibiotic resistance genes.
(c) Should possess multiplication and colonization ability in the intestinal mucosa lining of hosts.
(d) Must be viable for a long period under standard storage conditions and technically appropriate for industrial processes like lyophilization.
(e) Must be capable of producing antimicrobial substances against pathogenic microbes.
(f) Employ their beneficial effects like enhanced nutrition and elevated immune response in the host.

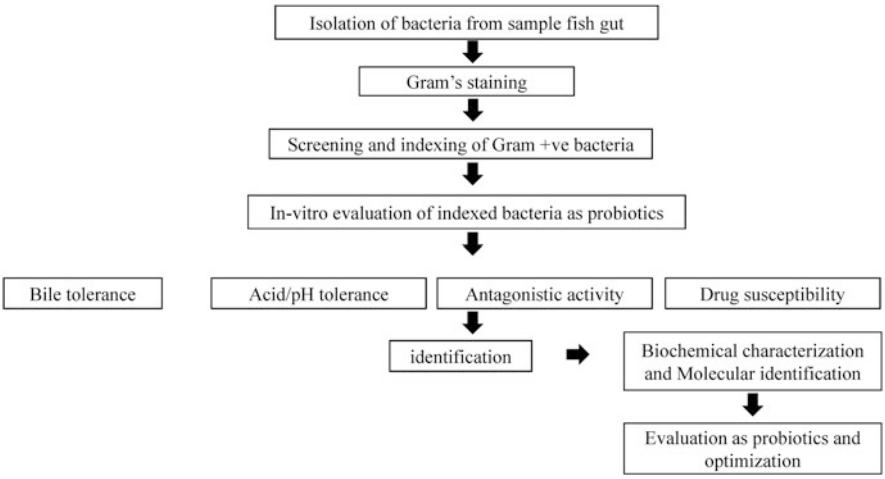

Fig. 11.1 Screening process flowchart for selection of isolated bacteria as suitable probiotic

Bacteria isolated from various sources are screened for their potential as effective probiotics employing numerous measures. The sequential test process for selecting isolated bacteria as ideal probiotic substances is shown in Fig. 11.1 where they are eligible as a potent probiotic candidate suitable to be used in aquaculture if all conditions have been met successfully.

11.4.1 Application of Probiotics in Aquaculture

Administration of probiotics is usually carried out via water immersion/bathing or injection or feed diet (Fig. 11.2). It also can be encapsulated into feeding stuff or live feed like rotifers, and artemia has proven to be a successful carrier of probiotics in fish and shellfish larvae. The majority of probiotics have been formulated to be combined with feed supplements to display increased efficacy against disease-causing pathogens. Certain approaches have also been published, for example, adding probiotics directly to cultured water (Cha et al. 2013). As soon as the probiotics are put into the culture system, it begins to grow and outnumber the infectious population already prevalent in the water which has a consequence on fish health by refining several qualities of water, since they alter the bacterial composition of the sediments and water. Administration of probiotics via injection method is a possibility where the probionts could be freeze-dried and injected as a vaccine. However, in the case of small fish or large numbers, vaccination is impracticable or not feasible.

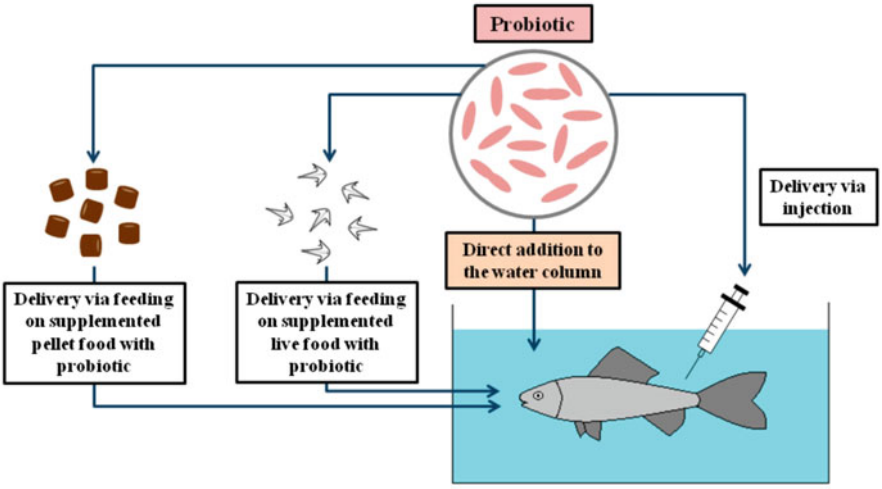

Fig. 11.2 Different routes of probiotic administration

In his groundbreaking study, Fuller (1989) wrote: 'The probiotic preparations may be single strains or can contain several strains'. Since the early 1990s, however, most probiotic research has been on the use of sole culture species in aquaculture, but in the last 10 years, attention has turned to the use of other probiotic supplements in aquatic animals (Mukherjee et al. 2019). Multi-strain preparations benefit from a variety of conditions, and the mechanism of defence against multiple infectious diseases is strengthened (Kesarcodi-Watson et al. 2012).

Synbiotics is the co-administration of prebiotics or plant products along with probiotics. From several studies, it is evident that the microbial supplementations of the host organism have been enhanced by synbiotics in the gut. Probiotics, prebiotics and synbiotics application have enhanced aquatic organisms' survival against pathogenic microbes. Dead or inactivated bacterial strain applications were also registered. For instance, heat-inactivated oral administration of *Lactobacillus delbrueckii* ssp. *lactis* and *Bacillus subtilis* is independent or in conjoint (Salinas et al. 2008).

The duration of the administration of probiotics also plays a major factor. Research has shown that the application period can be as limited as 6 days or even 5 months or even 8 months for a possible probiotic (Aubin et al. 2005; Aly et al. 2008). In maintaining the impact and function of probiotics, the frequency of probiotic administration acts as a game changer. A daily application of probiotics is better during the culture period to gain maximum results (Table 11.1).

Table 11.1 Application of different probiotics in aquaculture

Probiotic strain	Used on	Administration method	Effects on applied species	References
Bacillus circulans	*L. rohita*	Diet	Improved growth and survivability	Ghosh et al. (2004)
Bacillus subtilis	*M. rosenbergii*	Diet	Improved the survivability rate against *Aeromonas hydrophila* pathogen	Keysami and Mohammadpour (2012)
B. subtilis, *L. lactis* and *S. cerevisiae*	*L. rohita*	Diet	Increased growth with good digestibility and associated parameters	Mohapatra et al. (2012)
Pseudomonas M174	*Oncorhynchus mykiss*	Immersion	Fighting for *Flavobacterium psychrophilum* infection	Korkea-Aho et al. (2011)
Lactobacillus rhamnosus	*Oncorhynchus mykiss*	Diet	Induced immunological reaction	Panigrahi et al. (2005)
LAB and yeast	Ornamental fish fingerlings	Mixed along the basal feed	Total blood count boost	Firouzbakhsh et al. (2011)
Enterococcus faecium Z14	*Oreochromis niloticus*	Culture system	Amplified growth and immunological reaction	Wang et al. (2008)
Lactobacillus, *B. subtilis* and *B. licheniformis*	Pacific white shrimp	N/A	Enhanced growth, superoxide dismutase, catalase, lysozyme, lipase and amylase activities	Xie et al. (2019)
E. faecium MC13	*Cyprinus carpio*	Injection and oral	Resistance to *A. hydrophila*	Gopalakannan and Arul (2011)
Pediococcus pentosaceus and *Staphylococcus haemolyticus*	*Litopenaeus vannamei*	Sprayed on commercial diet	TBC increases and survival against WSSV and IHHNV diseases	Leyva-Madrigal et al. (2011)
Saccharomyces cerevisiae var. boulardii CNCM I-1079	*Oncorhynchus mykiss*	Diet	Gastrointestinal enzymatic reaction stimulation	Waché et al. (2006)
B. subtilis and *B. licheniformis*	*Oncorhynchus mykiss*	Diet	Improved resistance against *Yersinia ruckeri*	Raida et al. (2003)
Live form of yeast	European seabass	Diet	Growth and feed efficiency enhanced	Tovar-Ramırez et al. (2004)

(continued)

Table 11.1 (continued)

Probiotic strain	Used on	Administration method	Effects on applied species	References
Heat-killed LAB	Japanese pufferfish	In vitro tissue culture	Boost of Proinflammatory cytokine genes	Biswas et al. (2013)
Pseudoalteromonas aliena	Blue crab Zoeae	Culture system	Less mortality	Morya et al. (2014)
Bacillus NL110 and *vibrio* NE17	Juveniles of *M. rosenbergii*	Through feed and culture water	Improvements in growth, survival, water quality and immune reaction	Mujeeb Rahiman et al. (2010)
Saccharomyces cerevisiae	*Oreochromis niloticus*	Diet	Growth and feed efficiency increases	Lara-Flores (2011)
Strain E (*Vibrio alginolyticus alike*)	*Scophthalmus maximus* larvae	Rotifer boost	Better survivability	Gatesoupe (1997)

11.4.2 Mode of Action

The correlation between probiotic bacteria and their hosts is complicated, and therefore probiotic action mechanisms are not fully understood. However, possible mechanisms and benefits linked to the application of probiotics are illustrated in Fig. 11.3.

11.4.2.1 Competitive Exclusion of Pathogenic Microorganisms

The first requirement for a pathogenic microorganism to start a disease is an attachment to the mucosal layer of the host's gastrointestinal tract. This establishment is hindered by a process called colonization resistance or competitive exclusion, by the probiotic microbes. A well-established microflora can reduce or stop competing bacteria from colonizing the same area of the gut, a phenomenon known as competitive exclusion. The primary goal of probiotics under the theory of competitive exclusion is to achieve: competition for mucosal attachment sites, competition for nutrients and secretion of inhibitory substances by the microorganisms which destroy the troublesome bacteria and thereby reduce the likelihood of colonization. It is preferred when choosing probiotics that the bacteria colonize the intestine by adhering to the epithelial surface and by doing so prevent the binding of pathogens (Balcázar et al. 2006).

11.4.2.2 Antagonistic Activity

Defence against harmful microbial strains is a major property of probiotics to employ them for use. The defence mechanism is primarily due to the secretion of various types of anti-microbial substances like antibiotics, bacteriocins, siderophores, hydrogen peroxide, lysozymes, proteases and organic acid mainly acetic acid and lactic acid which can strongly inhibit the activity of gram-negative

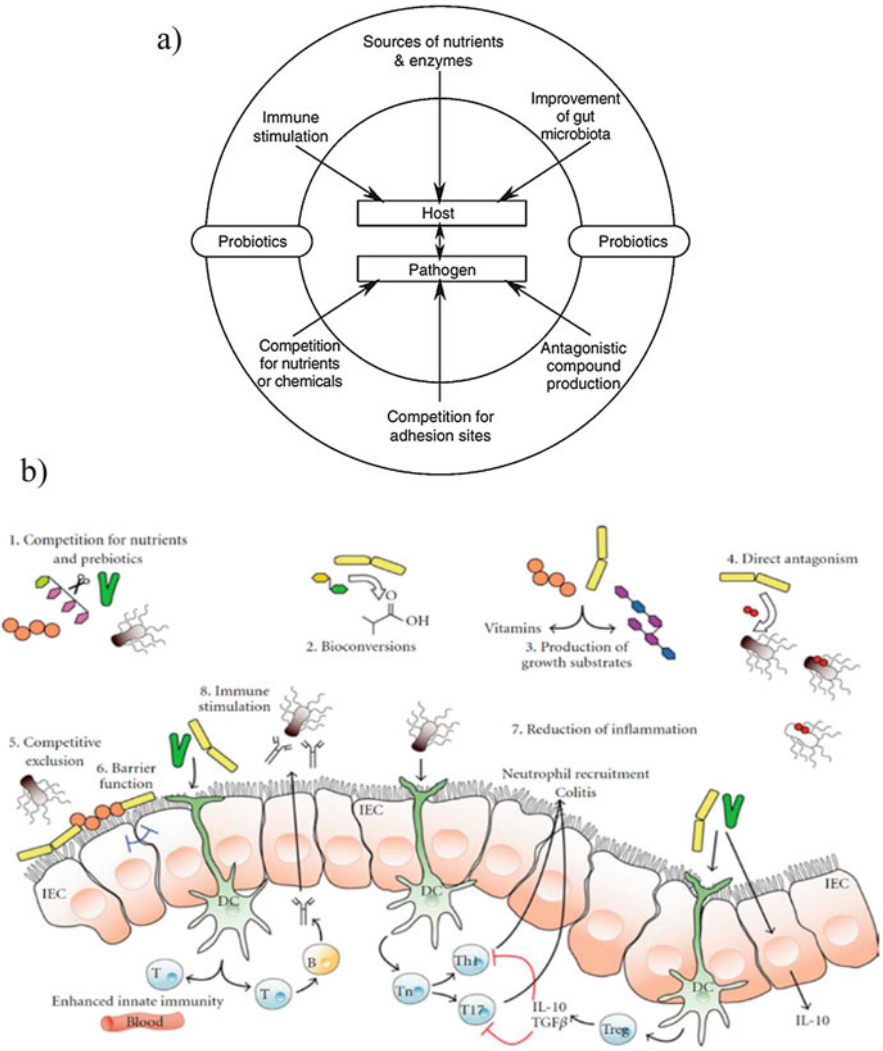

Fig. 11.3 Mode of probiotic action

bacteria by their bacteriostatic or bactericidal impacts on pathogenic strains. Thus, probiotics play a key role by secreting inhibitory complexes that act antagonistically against pathogenic microorganisms that prevent the propagation of pathogenic microbes and the occurrence of diseases in the hosts (Tinh et al. 2008). Substances produced by probiotics act as an antagonist for quorum sensing mechanisms (Ringø 2020).

11.4.2.3 Enhancement of Water Quality

By modulating water microbiota (Cai et al. 2019) and enhancing the physicochemical parameter of water (Zokaeifar et al. 2014) and control of pathogens (Kuebutornye et al. 2019), water quality in ponds can be upgraded. The aquatic organisms are vulnerable to higher concentrations of nitrogenous compounds, like ammonia, nitrite and nitrate which can increase mortality when accumulated in high quantities in the culture system. The probiotics act beneficial in eliminating ammonia and nitrate toxicity efficiently (Mohapatra et al. 2012). Moreover, gram-positive microorganisms are better converters of organic matter back to CO_2 than gram-negative microscopic organisms which could convert a greater percentage of organic carbon to bacterial biomass or slime (Thirumurugan and Vignesh 2015).

11.4.2.4 Competition for Nutrient and Energy Sources

The survival of any microbial population depends on its capability to strive with the other microbes which share the same environment for chemicals and available energy. The capacity to acquire iron for pathogenic bacteria is essential for survival in the host. Some iron-acquired genes are subsequently related to bacterial virulence. The supply of iron to pathogenic bacteria is decreased by siderophores or by low--molecular-weight substances, which were developed by probiotic applicants or beneficial endosymbionts, as siderophores have a great affinity for ferric ions (Ringø et al. 2006). This mechanism would reduce the presence of the pathogen in the intestinal tract since the bacteria cannot survive without the necessary nutrients. Microbiota can be used as an alternative source of diet and a good source of vitamins or important amino acids and for the microbial function inside the gut.

11.4.2.5 Growth Promoter

Some researchers earlier have illustrated that the beneficial probiotic microorganisms may improve development and feed utilization by stimulating the intestinal enzymatic actions like that of protease, amylase, cellulase, lipase and phytase (Banerjee and Ray 2017) and by enhancing the intestinal microbial balance, which could further improve the nutrient absorption and use (Wang et al. 2015).

11.4.2.6 Enhanced Immune Response

The last decade saw considerable attention being drawn towards probiotics that can improve host immunity and finfish disease resistance (Merrifield et al. 2010) and shrimps (Kumar et al. 2016). LAB and *Bacillus* species are most widely used as probiotics and have been shown that they promote animal health through the activation of the host's innate immune response and enhance resistance to microbial pathogenic infection (Ringø et al. 2018; Soltani et al. 2019).

11.4.3 Drawbacks

A significant problem remains under doubt over the fate of probiotics following application. In particular, concerns were raised about the probable acquisition by

gram-negative probiotics of antibiotics resistance and virulence genes through horizontal gene transfer. Although no known analysis of this concept is available, however antibiotic-resistant bacteria from aquaculture sites have often been identified, and this may lead to safety issues in an open aquatic environment while utilizing live probiotics. Probiotic formulations that are inactivated or encapsulated may be a solution to the issue of horizontal gene transfer. Probiotics can also have an impact on the gills, skin, surface mucus and intestinal tract of the host organism. This ensures that probiotic strains even have to be considered healthy for the host's cellular integrity. Additionally, there are substantial concerns with product quality in developing nations due to the lack of understanding about species or strain labelling, species concentration in mixed products and precise cell numbers on probiotic products. The best means to boost the consistency and effectiveness of business aquaculture probiotic products may therefore be regulatory oversight. The production and sale of probiotics should also be standardized and regulated to protect product quality, produce organic aquatic food and yield greater financial returns.

11.5 Concluding Remarks

The basic functions of probiotics in aquaculture are shown in this chapter where they exert the benefit of improved disease resistance, better digestibility of nutrients and growth of host animals and enhance the water quality of the culture system. However, probiotics are a crucial tool for managing it, but their efficacy depends on an understanding of the nature of competition between species and strains. There is also a lot of room for further research in determining optimum conditions for probiotic contact with the host. Furthermore, progressive molecular research on probiotic science is essential to understand the mechanisms and interpret the unique probiotic genes with new applications. Nevertheless, most of the experiments were/are usually performed in laboratory conditions or small aquarium installations. Hence, the application of probiotics under culture conditions is therefore essential for the correct assessment of their usage in aquaculture.

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Identifying Novel Antibiotic Resistance Genes (ARGs): Important Aspect of Metagenomic Research

12

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Abstract

The rapid growth of antimicrobial resistance (also known as AMR) is a major reason for concern when it comes to public health around the world. The rise of AMR is a result of the overuse and misuse of antimicrobial agents, such as antibiotics, antivirals, antifungals and antiparasitics. India is indeed one of the world's top consumers of antibiotics and has its own unique set of constraints related to its large population and diverse cultural, social and economic landscape. Major obstacles to the application of antimicrobial resistance (AMR) containment strategies include self-medication, the use of antibiotics for growth promotion in animals and the development of residual antibiotics in the environment. The use of antibiotics in various sectors, including aquaculture, medicine, agriculture and the food industry, has contributed to the spread of antimicrobial resistance. The presence of antibiotic resistance genes (ARGs) in aquatic environments is of particular concern because it increases the risk of antibiotic

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resistance in human pathogens. ARGs can spread from aquatic environments to humans through contaminated seafood, and they can also spread from humans to aquatic environments through wastewater discharge. The pollution due to antibiotics and the prevalence of antibiotic resistance genes vary greatly between low-middle and high-income countries, as well as between different regions within a given country. The presence and spread of antibiotic resistance genes are also influenced by several factors, including the presence of antibiotic residues, microbial communities and environmental variables. The presence of antibiotics and antibiotic resistance genes (ARGs) in the environment can have significant impacts on microbial communities, biogeochemical cycles and both marine organisms and human health.

Keywords

Metagenomics · Antibiotic resistance genes · Resistome · Multidrug-resistant bacteria · Microbiome

12.1 Introduction

Antibiotic resistance in bacteria has evolved naturally and long before antibiotics were manufactured in large quantities for human use. Bacteria have evolved a variety of mechanisms to resist antibiotics, including changes in the structure of target proteins, the production of enzymes that break down antibiotics and the efflux of antibiotics out of the cell (Larsson and Flach 2021). The spread of ARGs in the environment is also a major concern, as it can increase the risk of antibiotic resistance in human and animal pathogens, making it more difficult to treat infections (Galera-Laporta and Garcia-Ojalvo 2020; Sun et al. 2020; Reverter et al. 2020). Antibiotic use and consumption have been increasing globally, and projections indicate that this trend is likely to continue. A recent study estimated that, under the scenario of no policy interventions, worldwide antibiotic consumption in 2030 could be as much as 200% higher than the level in 2015 (Klein et al. 2018). Anthropogenic, or human-caused, antibiotic residues in the environment can result in a number of serious environmental problems. Thiamphenicol is one example of an antibiotic that has been shown to have adverse environmental impacts. Research has indicated that thiamphenicol can interfere with nitrate reduction processes in soil, which can result in increased levels of nitrous oxide (N_2O) being released into the atmosphere (Yin et al. 2016). The widespread use of antibiotics can put significant selective pressure on microorganisms, leading to the enrichment of ARGs (Wang et al. 2019; Migliorini et al. 2019). Antibiotic resistance is a complex phenomenon that can occur through various mechanisms, namely, enzymatic destruction, efflux pumps, cellular protection and both target defence and antibiotic deactivation (Zhang et al. 2019; Chen et al. 2019; Wilson et al. 2020). Antibiotic resistance can significantly impair the efficacy of antibiotics against pathogens, making it difficult or impossible to treat infections effectively (Pärnänen et al.

2019). Horizontal gene transfer (HGT) is a key mechanism by which antibiotic resistance genes (ARGs) can spread and become more prevalent in the environment. Mobile genetic elements (MGEs) which include transposons, integrons and plasmids are self-contained pieces of DNA that can move from one bacterium to another, often across species boundaries. These MGEs can carry multiple ARGs, providing bacteria with a "toolkit" of resistance mechanisms (Wang et al. 2018; Pallares-Vega et al. 2019; Zhao et al. 2019a). ARGs and antibiotics have frequently been found in rivers (Rodriguez-Mozaz et al. 2015; Singh et al. 2019; Das et al. 2020), lakes (Tang et al. 2015; Yang et al. 2018a), groundwater (Tong et al. 2020; Zainab et al. 2020) and coastal and estuarine environments (Griffin et al. 2019; Zheng et al. 2021). The presence of pollutants, including antibiotics and antibiotic resistance genes (ARGs), in these environments can have negative impacts on the health of estuarine and coastal ecosystems (Ward et al. 2020). The presence of ARGs and antibiotics in coastal and estuarine environments can have far-reaching consequences for biogeochemical cycling, ecological security and human health (Leonard et al. 2015; Zhao et al. 2019b).

Antimicrobial resistance in bacteria is currently the most prevalent type of resistance in microbes and a significant public health issue. The overuse and misuse of antibiotics have led to the evolution of bacteria that are resistant to multiple antibiotics, making it difficult to treat bacterial infections.

12.2 Taxonomic Profiling

Understanding the taxonomic classifications of resistome components is crucial for locating the bacteria that produce a resistome. Taxonomic assignment analysis of resistome elements can provide information about the composition and relative abundance of the microbial community in a sample (Ruppé et al. 2018; Rice et al. 2020). There are two main approaches for identifying bacterial community composition from metagenomic data. The first approach is based on direct analysis of raw sequencing reads and does not require contig assembly. The second approach involves the assembly of contigs from the raw sequencing reads, followed by taxonomic assignment of the contigs (de Abreu et al. 2021). Taxonomic classification of metagenomic data without contig assembly can be a faster and more computationally efficient approach compared to contig assembly (Rodríguez-Brazzarola et al. 2018). The length and quality of sequences are crucial considerations during taxonomy classification analysis. Short reads or low-quality reads may have a higher error rate and may not provide enough information to accurately identify the bacterial taxa present in a sample (Breitwieser et al. 2019; Ye et al. 2019). The length of contigs generated by contig assembly is an advantage for taxonomic classification, as longer contigs provide more information to accurately identify the bacterial taxa present in a sample. Taxonomic classification and contig-based taxonomic classification rely heavily on the use of reference databases (Rodríguez-Brazzarola et al. 2018). Contig assembly can sometimes enable the reconstruction of partial genomes of previously unknown or uncultured bacterial

organisms. In contig assembly, short reads from different bacterial taxa can be erroneously assembled together into a single contig, resulting in a chimeric contig. The quality of the assembled contigs and the accuracy of the taxonomic assignments made based on the assembled contigs can strongly influence the interpretation of the microbial community composition and function (Behera et al. 2020a, b, 2021a, b, 2022; Rout et al. 2022).

Furthermore, genome assembly can enable the identification of possible HGT regions, which are genomic regions that have been acquired by a bacterium from another organism through HGT mechanisms such as transduction, transformation or conjugation. In the context of antibiotic resistance genes, the size of gene sequences can have a substantial effect on gene annotation transfer and the investigation of biological mechanisms linked with resistance (ARGs). Taxonomic assignment by contig assembly can be a useful tool for identifying and understanding resistance mechanisms, particularly in the context of studying the structural relationships between microbiota and the resistome. The type of sample being worked with can influence the quality and quantity of DNA/RNA obtained, which can in turn impact the success of the assembly process. The sequences in the datasets are of good quality and long enough to be aligned directly to a reference database. The taxonomic classification can be performed using alignment-based methods, which are computationally less intensive than de novo assembly approaches (Rodríguez-Brazzarola et al. 2018). High-throughput sequencing can be used to study the taxonomic assignments of resistome elements in various environments, including water reservoirs to identifying antibiotic resistance genes (ARGs) in host-pathogen relationships such as hospitals (Chng et al. 2020), water reservoirs (Yu et al. 2020; Ekwanzala et al. 2020), soil (Chen et al. 2017), human faeces (Karkman et al. 2019), livestock wastewater and faeces (Jia et al. 2017), air (Yang et al. 2018b; Li et al. 2021) and biogeochemical and biogeographical processes (Kuang et al. 2016; Liu et al. 2018).

12.3 Functional Study and ARGs Database

The investigation of taxonomic signatures can assist us in gaining a deeper comprehension of the connections that exist among the various members of a microbial diversity. Functional metagenomics is an approach that purposes to identify functions in a microbial community by discovering new enzymes, biosynthetic gene clusters and antibiotic resistance genes (ARGs). Functional annotation typically involves several steps, including gene prediction, annotation transfer and functional assignment (Dong and Strous 2019). Metagenomics is a powerful tool for studying microbial communities and has the potential to provide important insights into microbial ecology, evolution and biotechnology (Zhang et al. 2011). There are numerous databases and methods available for identifying a microbial community's taxonomic diversity and undertaking functional assessments. These include databases of reference genomes, protein sequences and metabolic pathways, as well as bioinformatic tools for taxonomic classification, functional annotation and

pathway analysis. Functional analysis of metagenomic data provides a wealth of information that can be used for various sub-analyses, depending on the sequencing depth and research question. These sub-analyses can include protein-protein interaction, pathway, functional category, gene ontology, protein family and subsystem analysis, among others. Each of these analyses provides different levels of detail and can be used to gain insights into the functional potential and metabolic activities of the microbial community. There are open-source software/applications, like Mothur (Schloss et al. 2009), MEGAN (Huson et al. 2007) and QIIME (Caporaso et al. 2010) for taxonomy and functional analysis. BLAST^{+} is an important tool in genomic research and is widely used to annotate new genome sequences and to investigate the evolutionary relationships between different species (Altschul et al. 1997). DIAMOND is a powerful tool for annotating genomic data, particularly for the analysis of large datasets such as metagenomic samples. Its speed and accuracy make it an attractive option for researchers working with large amounts of sequence data (Buchfink et al. 2014). USEARCH is a useful tool for bioinformatic analysis, particularly for sequence searches and clustering (Edgar and Bateman 2010). RAPSearch2 is a powerful tool for sequence searching and is well suited for large-scale analyses (Zhao et al. 2012).

The development of new methods and tools for comprehensive metagenomic analyses is an ongoing process, and it is important for researchers to stay up-to-date with the latest advancements in the field in order to conduct the most effective and informative analyses. Resistome databases are crucial resources for understanding antimicrobial resistance and are constantly evolving as new information becomes available (Danko et al. 2021). The use of database for sequence analysis can introduce database bias that can affect the accuracy and relevance of the results, particularly for metagenomic analyses. The genomic surveillance of AMR is crucial for understanding the distribution and spread of resistance genes, as well as for identifying new resistance mechanisms (de Abreu et al. 2021). To support this effort, numerous annotation softwares and databases have been established to facilitate the analysis of antibiotic resistance gene (ARG) content in bacterial genomes or next-generation sequencing (NGS) metagenomic samples. These tools and databases provide valuable resources for researchers and practitioners to identify, annotate and compare ARGs in different bacterial genomes and metagenomes. Some of the most widely used annotation tools and well-known AMR databases are tabulated in Table 12.1.

The Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2020) is unique in that it combines sequence data with bioinformatic tools to aid in the detection and analysis of AMR genes. For example, the database includes curated detection models that can be used to identify AMR genes in sequenced bacterial genomes, as well as tools for visualising and analysing the genomic context of these genes. In addition to resistance genes, CARD also includes information on resistance mutations, which are genetic changes that can confer resistance to antibiotics. Like the resistance genes, the resistance mutations are organised by bacterial species. CARD focuses on delivering high-quality reference material and molecular

Table 12.1 Several bioinformatic databases available for studying antibiotic resistance genes (ARGs)

Database	Web link	References
CARD	https://card.mcmaster.ca/	Alcock et al. (2020)
ARDB	https://ardb.cbcb.umd.edu/	Liu and Pop (2009)
ResFinder	https://cge.cbs.dtu.dk/services/ResFinder/	Florensa et al. (2022)
SARG	https://smile.hku.hk/SARGs#	Yin et al. (2018)
ARGminer	https://bench.cs.vt.edu/argminer/#/home	Arango-Argoty et al. (2020)
NDARO	https://www.ncbi.nlm.nih.gov/pathogens/refgene/	Feldgarden et al. (2021)
MEGAres	https://megares.meglab.org/	Doster et al. (2020)
AMR++	https://github.com/Microbial-Ecology-Group/AMRplusplus	Bonin et al. (2023)
ARG-ANNOT	http://www.mediterranee-infection.com/article.php?laref=282&titre=arg-annot	Gupta et al. (2014)
ARG-database	https://smile.hku.hk/SARGs	Yang et al. (2016)

sequences that are arranged using the Antibiotic Resistance Ontology (ARO), a regulated vocabulary.

ARDB (Liu and Pop 2009) also known as the Antibiotic Resistance Genes Database tracks ARGs. It was first released in 2005 and is maintained by the Antibiotic Resistance Genes Reference Center at the University of Alberta, Canada. It includes information on a wide range of antibiotic resistance genes, including those found in both Gram-positive and Gram- negative bacteria. The database provides detailed annotations for each gene, including information on its function, location and associated resistance mechanisms.

Resfinder (Florensa et al. 2022) is a useful tool for the characterisation and identification of ARGs, and its use can help researchers better understand the distribution and prevalence of resistance genes in bacterial populations and metagenomic datasets. Resfinder also provides information about the sequence similarity and identity of the identified resistance genes, allowing researchers to compare the resistance genes to known reference sequences and assess the potential impact of any genetic variations or mutations. This can be important for understanding the mechanisms of resistance and for predicting the potential effectiveness of different antimicrobial therapies.

ARG-ANNOT (Gupta et al. 2014) is a web-based tool for both complete genomes and draft genomes and can be applied to both Gram-negative and Gram-positive bacteria. The tool provides detailed annotations for each detected AR gene, including information on its function, resistance mechanism and associated resistance phenotype. It also provides information on the genomic context of each gene, including its location on the chromosome and any associated mobile genetic elements.

ARG-database (Yang et al. 2016) ARGs-OAP (Antibiotic Resistance Genes Online Analysis Pipeline) is a bioinformatic tool designed to facilitate the analysis and finding of antibiotic resistance genes (ARGs) in metagenomic data. It includes a large and diverse database of reference sequences for comparison and can be applied to both short-read and long-read sequencing data. By providing a comprehensive and up-to-date database of reference sequences and detailed annotations for each gene, it helps to facilitate research on the molecular mechanisms of antibiotic resistance and on the development of new strategies for combating it.

SARG (Yin et al. 2018) database was designed to help researchers identify ARGs in metagenomic samples, which are complex mixtures of DNA from different microorganisms. SARG v2.0 builds on this concept by incorporating a much larger number of reference sequences for ARGs, which allows for more comprehensive coverage of the resistance gene landscape in different environments. ARGs-OAP v2.0 is designed to help researchers analyse and annotate high-throughput sequencing data for the presence of antibiotic resistance genes (ARGs). The database includes a large number of reference sequences for ARGs, which can be used to identify similar sequences in raw sequencing data using a similarity search strategy.

ARGminer (Arango-Argoty et al. 2020) able to capture a broader range of ARGs and provide more comprehensive information about each gene, including its sequence, function and resistance profile. It looks like the sequences from the different databases that were used to build ARGminer were processed to get rid of duplicates and mark them also with highest similarity from each of the databases. Overall, the use of an ensemble database like ARGminer can be a powerful tool for understanding the genetics and mechanisms of antibiotic resistance, which is a critical public health concern.

NDARO (Feldgarden et al. 2021) is the central hub for scholars to access data related to antimicrobial resistance (AMR) in infective organisms. The purpose of real-time AMR surveillance is that it keeps an up-to-date database of AMR genes and a combination of genetic and antibiotic susceptibility data. The Reference Gene Catalog, which is maintained by the NDARO, is a curated collection of antimicrobial resistance (AMR) genes from various bacterial pathogens. NDARO has expanded its focus beyond just antimicrobial resistance (AMR) genes to include other genetic elements that are important for understanding the biology of clinically important pathogens.

MEGAres (Doster et al. 2020) is an updated version of the MEGARes database, which is a comprehensive resource for studying antimicrobial resistance genes. It contains sequence data for thousands of hand-curated antimicrobial resistance genes, but it also includes additional features that are designed to aid in the analysis of metagenomic sequencing data. A crucial part of preventing AMR is identifying its genetic causes in the fight against this global public health threat. By using MEGARes to analyse metagenomic data, researchers can identify the presence and abundance of AMR genes in various environments and track their distribution and evolution over time.

AMR^{++} (Bonin et al. 2023) is a powerful and flexible bioinformatic pipeline that is designed to aid in the analysis of antimicrobial resistance genes. In the context of

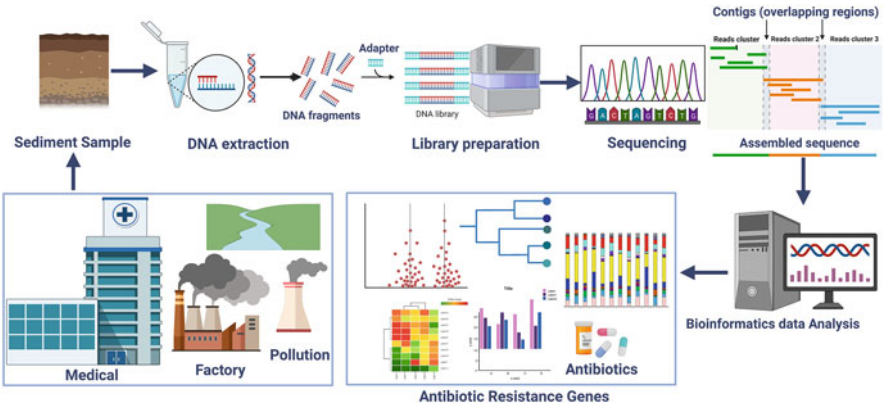

Fig. 12.1 Antimicrobial resistance genes showing the sources, and drivers of antimicrobial resistance in the aquatic environment

metagenomics, the DNA of a given sample or set of samples is sequenced using high-throughput sequencing technologies. The resulting data can be quite complex, with millions or even billions of short DNA sequences that need to be sorted and analysed. By providing a comprehensive view of the resistome in a given bacterial population, the pipeline can help researchers to better understand the mechanisms and evolution of antibiotic resistance and develop more effective strategies for combatting this important public health. The sources and drivers of AMR in the aquatic environment is crucial for developing effective strategies to mitigate its impact. These strategies may involve improved wastewater treatment processes, responsible antimicrobial use in agriculture and aquaculture, and enhanced surveillance and monitoring programs. Additionally, promoting public awareness and education regarding the proper disposal of pharmaceuticals and reducing unnecessary antimicrobial use are important steps towards combating AMR in the aquatic environment (Fig.12.1).

12.4 Antimicrobial Resistance in Bacteria

Resistome is a term used to describe the total genetic potential of an ecosystem to resist antimicrobial agents (Martínez et al. 2014; Crofts et al. 2017). The resistome of an ecosystem is shaped by a variety of factors, including the presence of anthropogenic stressors, such as the release of antibiotics and other toxic compounds into the environment, and the presence of natural stressors, such as heavy metal ions and other toxic substances (Kraemer et al. 2019). Microorganisms have evolved the ability to detect, interact with and digest tiny compounds that control the antibiotics (Berendonk et al. 2015). The development of novel antibiotics that can avoid resistance can be influenced by knowledge about the evolution of resistance (McGarvey et al. 2012). The genomic era has led to new insights into the biology

of bacteria, which could potentially lead to the discovery of new antibiotics, but the process of drug development is complex and time-consuming (Li et al. 2015). Soil microorganisms produce natural antimicrobial compounds, and these have been a rich source of antibiotics used in clinical medicine (Wrighton 2018; Crits-Christoph et al. 2018). Metagenomic mining has revealed that resistance genes have existed in microbial populations long before the modern "antibiotic era" (Yadav and Kapley 2021). The diversity and distribution of ARGs in the environment can help to inform the development of new antibiotic resistance methods, such as using natural chemicals from microbial communities to inhibit the propagation of resistance genes (Berendonk et al. 2015). In this study, antibiotic resistance in environmental bacteria can provide important insights into the natural history of resistance and the mechanisms that underlie the development and spread of resistance in clinical settings (Aminov 2009). The fast growth of antibiotic-resistant infections has revealed our limited understanding of the environmental mechanisms occurring in microbial communities (Waseem et al. 2017). Multi-drug-resistant bacteria (MDR) have evolved the ability to metabolise antimicrobials and can transfer these properties to other bacterial species through horizontal gene transfer (Holt et al. 2015).

12.5 Hotspots for the Spread of Antibiotic Resistance

Antibiotic use in agriculture, veterinary medicine and human medicine can lead to the growth and spread of resistant microorganisms in the environment. Antibiotic-resistant bacteria have been found in various environmental sources, including soil, water and wildlife; there is no direct evidence that these bacteria have existed four million years ago, in caves (Bhullar et al. 2012). ARG bacteria have also been discovered in the gastrointestinal tracts of persons living in distant places who have never been exposed to antibiotics as well as in samples of permafrost that are thousands of years old (Kunhikannan et al. 2021). The use of antibiotics in agriculture and animal husbandry can lead to the selection and spread of ARGs in the gut microbiota of animals (Wichmann et al. 2014; Berendsen et al. 2015). Antibiotic-resistant bacteria can live in close proximity to each other in the soil, which can facilitate the transfer of antibiotic resistance genes through horizontal gene transfer (Christensen et al. 1998). Horizontal gene transfer is a major factor promoting the growth of ARGs in the environment, and it highlights the importance of responsible use of antibiotics to minimise the emergence and spread of antibiotic-resistant bacteria. The use of antibiotics in animals can contribute to the development of antibiotic resistance in humans. When antibiotics are used in animal agriculture, bacteria can become resistant to these drugs and may spread to humans through food or other environmental pathways. This can lead to the rise and spread of ARG infections in humans, which can be difficult to treat with traditional antibiotics. Therefore, reducing the use of antibiotics in animal agriculture and promoting responsible use of antibiotics in human medicine are important strategies for minimising the emergence and spread of antibiotic-resistant infections in humans

(Mann et al. 2021). Workers who handle and process meat or work in agriculture may be exposed to bacteria that are resistant to antibiotics, and this can increase the risk of developing antibiotic-resistant infections (Manyi-Loh et al. 2018). It is critical to investigate the various environmental hotspots that contribute to the spread of antibiotic resistance in both pathogenic and non-pathogenic bacteria. Hotspots for antibiotic-resistant bacteria can be found in various environmental sources, with pharmaceutical manufacturing sites, wastewater systems and aquaculture, food and animal production and hospitals (Berendonk et al. 2015).

It is important to find the major drivers that contribute to the development and spread of antimicrobial resistance. Some of the major drivers include the following:

- Overuse and misuse of antibiotics: Antibiotic-resistant bacteria can arise and spread due to the overuse and improper use of antibiotics in both animals and human beings.
- Poor infection prevention and control practices: Poor infection control and prevention procedures in healthcare settings can facilitate the spread of antibiotic-resistant bacteria among patients.
- Inadequate sanitation and hygiene: Inadequate sanitation and hygiene can lead to the spread of antibiotic-resistant bacteria in the environment, particularly in water and soil.
- Agricultural and animal husbandry practices: The use of antibiotics in agriculture and animal husbandry can lead to the development and spread of ARGs in animals and the environment.
- Global travel and trade: The global movement of people, animals and goods can facilitate the spread of antibiotic-resistant bacteria across borders.
- Insufficient funding for research and development: The lack of investment in research and development of new antibiotics and alternative treatments for infectious diseases can limit our ability to effectively treat antibiotic-resistant infections.

Identifying and addressing these drivers is critical to addressing the problem of antimicrobial resistance and preserving the effectiveness of antibiotics for future generations.

12.6 Conclusion

Metagenomics is an effective method for finding and investigating antibiotic resistance pathways utilising both sequence-based and function-based methods. It allows for the comprehensive analysis of complex microbial communities, providing insights into the diversity and distribution of ARGs. Antimicrobial resistance studies are commonly related to other components of the study being conducted, such as investigations of mutations, metabolic pathways, gene expression and infections. These other factors can affect the development and spread of antibiotic resistance, and understanding their interplay with resistance mechanisms is important for

developing effective strategies to combat antimicrobial resistance. These studies involve large, complex datasets and require advanced computational and bioinformatic tools for their analysis. Proper data pre-processing, quality control and statistical analysis are essential to ensure the accuracy and reproducibility of results.

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