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Review of Antibiotic Resistance Genes (ARGs) in Salmon Aquaculture and Empirical Data on Spatial and Seasonal Trends in the Bay of Fundy

Grace M. Murphy and Shawn M.C. Robinson

Fisheries and Oceans Canada
St. Andrews Biological Station
125 Marine Science Drive
St. Andrews, NB E5B 0E4

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Foreword

This series documents the scientific basis for the evaluation of aquatic resources and ecosystems in Canada. As such, it addresses the issues of the day in the time frames required and the documents it contains are not intended as definitive statements on the subjects addressed but rather as progress reports on ongoing investigations.

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ABSTRACT

The purpose of this paper is to review the background information available for antibiotic microbial resistance (AMR) in the aquaculture sector and to provide empirical data on the presence of antibiotic resistant genes (ARGs) associated with Atlantic salmon farms in the Bay of Fundy.

The continual decline of wild fish stocks worldwide and increasing demand for fish from increasing human populations and rising per capita consumption has resulted in the increasing production of the aquaculture industry to meet the global demand. However, the higher culture densities of intensive fish farms in comparison to wild conspecifics provides a prime environment for the spread of bacterial diseases, which pose a significant fish health and financial threat to the aquaculture industry. These diseases are generally controlled with antibiotics, but rising resistance to these drugs by bacteria that could be transferred to human pathogens in some parts of the world is becoming more common, raising concerns about the cost to human health, national economies and highlighting the need for research into alternative treatments. Resistance develops through the propagation of ARGs by natural selection of certain pathogens, and the sharing of ARGs between bacteria by horizontal transfer. Along with pathogens in the fish gut, environmental bacteria are also exposed to antibiotics in the water and sediments in the vicinity of fish farms, creating a further risk of the spread of ARGs to human pathogens from this vector. There have been cases of antibiotic resistance in human pathogens linked to the use of antibiotics in aquaculture and agriculture, and so many countries have imposed legislation over the use of antibiotics, with various levels of success. Finally, there have been attempts into using alternative treatments to control the spread of infectious disease in aquaculture, including bacteriophage therapy, quorum sensing inhibitors, vaccines, probiotics, immunostimulants, and herbal therapy.

Empirical data from the Bay of Fundy show that Atlantic salmon aquaculture farms are hotspots for microbial activity and that bacterial populations differ in their community structure depending on their proximity to the fish farms. The classes of bacterial populations closest to the farm generally tend to be anaerobic and sulphur reducers. We sampled for the ARGs related to the drugs florfenicol, tetracycline and sulfonamide. There was a general trend in increasing relative ARG abundance close to the farm, but the patterns were not the same for all ARGs. There also seemed to be a decrease in the concentration of some ARGs over time ranging from 3 to 12 months.

The information gathered from the literature review and the conclusions of the empirical study looking for AMR in relation to salmon farming showed that the process of modifying/enhancing ARGs at salmon aquaculture farms is present. This is consistent with other studies, both terrestrial and aquatic, that show AMR in bacterial populations respond to anthropogenic activities that involve the use of antibiotics. These data are some of the first in Canada to look at AMR in relation to aquaculture. While they give some insight into levels of ARGs and the environment, the scale (spatial and temporal) at which this is happening and the implications for the probable transmission to humans through the food supply is yet unknown. Further research is required on this topic in order to better define scales of AMR in comparison with other known reservoirs (e.g., wastewater treatment plants, agriculture activities), the degree of spatial dispersion involved of ARGs, linkages with wild populations of organisms that are part of the human food chain and the probability of transmission of ARGs to pathogens affecting human or animal health. Once a better understanding is gained on these aspects, a proper risk assessment can be done on aquaculture activities to answer questions such as: appropriate treatment regimes, probable impacts on the environment, implications of site selection and overall risk to human health.

REVIEW: MICROBIAL ANTIBIOTIC RESISTANCE IN AQUACULTURE

INTRODUCTION

This review is written in three parts. The first part deals with a brief review of the status of the global seafood industry and its role in providing food for the human populations. While this may seem esoteric in a review on antibiotic resistance, it essentially forms the basis for the demand curve of seafood and is strongly correlated with the number of interactions that occur between farmed species and other wild species in the surrounding ecosystems at all size scales, including bacteria. The second part deals with a review of the literature on various aspects of the development of antibiotic resistance and other potential approaches for dealing with bacterial diseases in the future. The third part is a presentation of some empirical data that were gathered for this particular project to begin assessment of the presence/absence of antibiotic resistant genes in association with salmon farms in the Bay of Fundy. These data are valuable in order to begin evaluation of the current ARG status on Canadian aquaculture farms and to compare with the data from aquaculture farms in other temperate parts of the world.

Fish have been a major staple in human diets for millennia, either directly as a meal item or indirectly as a diet ingredient in the food for other animals that humans grow for nutrition. However, fish stocks worldwide have been rapidly declining in the past few decades. The percentage of stocks considered maximally fished was recorded at 59.9% worldwide in 2016, compared to 7% that are still under-fished (FAO 2018b) (Figure 1). At the same time, as the human population increases and globalization makes it easier for products to be imported from all over the planet, the demand for fish has increased, particularly in developing countries (FAO 2018b). China continues to bring in large harvests, but overall, fisheries worldwide have been stagnating or slowly declining after peaking, over two decades, since 1996 (Figure 2). Therefore, to meet the demand, industrial and government attention has turned to aquaculture to feed the growing demand for fish for human consumption. Aquaculture, or the farming of aquatic organisms, is one of the fastest-growing industries on the planet, employing 19.3 million people in 2016, up from 12.6 million in 2000 (FAO 2018b). Aquaculture now accounts for nearly half of fish produced for food and non-food uses and continues to rise, although most of that new production is from Asia. All manner of aquatic organisms are being farmed, with carps and similar fish being the most common, but seaweeds and invertebrates such as crustaceans, clams and oysters are also important (Figure 3). The mass of animals and plants farmed for food in 2016 reached a total of 110.1 million tonnes with an estimated value of 302 billion CAD (FAO 2018b). In the same year, 37 countries were producing more fish and seafood through farming than fishing, while another 22 countries produced between 30% and one half of their fish and seafood through aquaculture. The continual growth of the aquaculture industry (5.8% between 2000 and 2016) means that the industry must contend with the logistics associated with mass-producing large amounts of fish, similar to their cousin-industry agriculture. High densities of cultured organisms provide an opportunity for a multitude of microorganisms (some of them pathogenic) to interact with and successfully complete their life cycles.

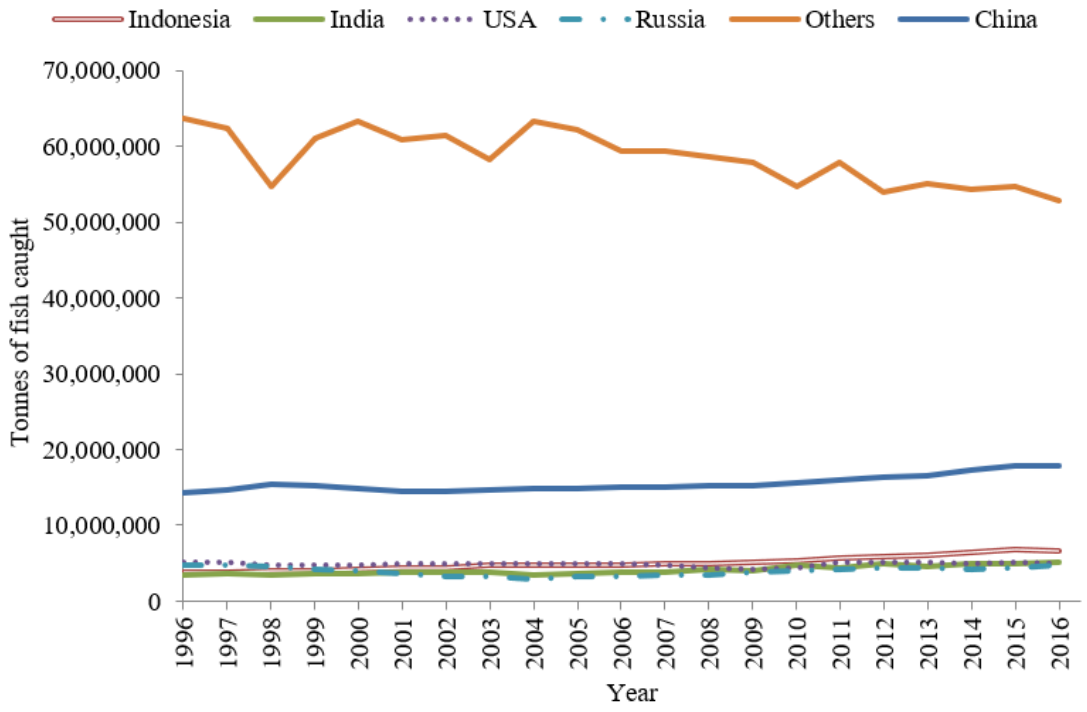

Figure 1. Production in capture fisheries overall between 1996 and 2015 by country (tonnes). Anchoveta are excluded from the fish data, as productivity depends on events such as El Niño and other environmental factors (FAO 2018a).

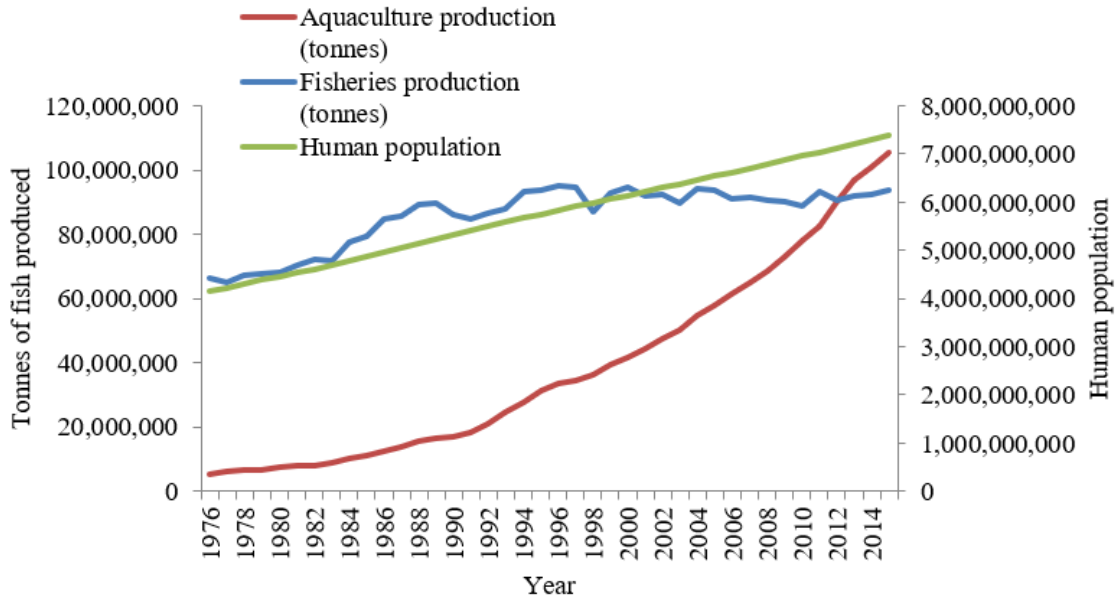

Figure 2. Productivity in capture fisheries and in aquaculture overall between 1976 and 2015 (tonnes) compared with number of people in the world. Anchovies and similar species are excluded from the fish data, as productivity depends on events such as El Niño and other environmental factors (FAO 2016, UN 2017, FAO 2018a, b).

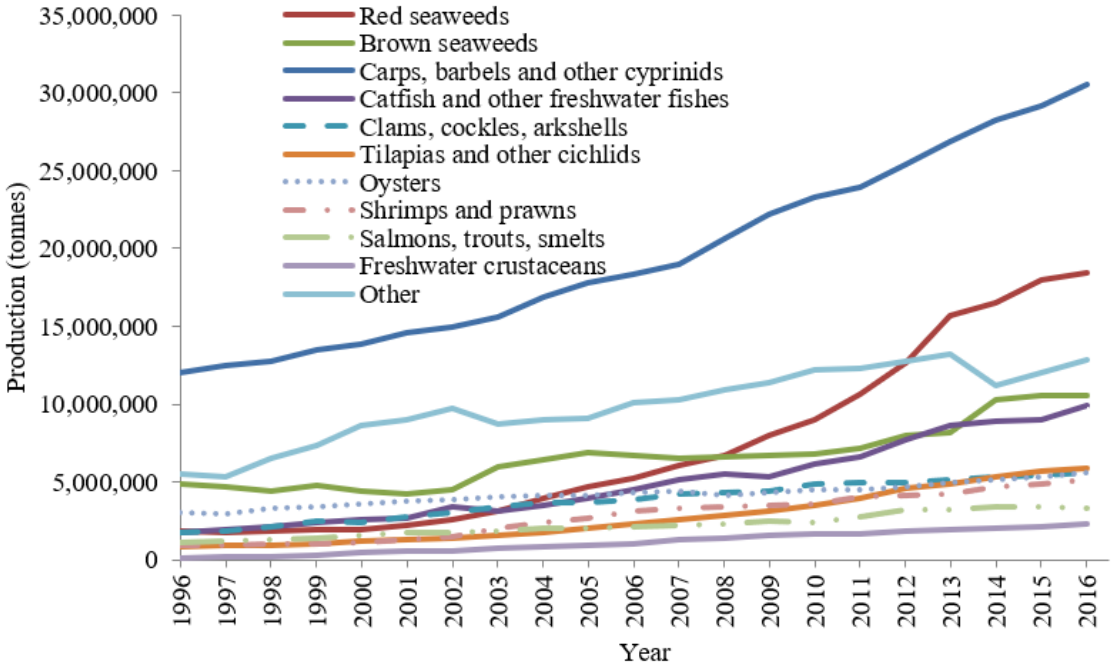

Figure 3. Increased productivity in aquaculture by ISSCAAP group, (International Standard Statistical Classification of Aquatic Animals and Plants), 1996-2016 (tonnes) (FAO 2018c).

Since its inception, the aquaculture industry has had to contend with infectious disease outbreaks in fish stocks as a practical and monetary obstacle. Diseases, which can be caused by bacteria, parasites, viruses, and fungi, cause loss of fish along with being costly and time-consuming to treat (Dadar et al. 2017). Traditionally, due to the cost-effective availability of broad-spectrum antibiotics such as tetracyclines, amphenicols and sulfonamides, antibiotic treatments have been used to control outbreaks of bacterial disease in aquaculture (Watts et al. 2017). This was not a permanent solution as was once hoped, as bacteria have been able to develop resistance against many of the drugs used against them (Watts et al. 2017). The spread of antibiotic resistance comes from the propagation of antibiotic resistance genes (ARGs), or genes that code for a protein or function that will grant an organism resistance to an antibiotic or class of antibiotics. These genes produced by natural selection are natural defenses of bacteria against antibiotics produced by other bacteria or against toxic substances such as heavy metals or xenobiotics found in the environment. These genes may also grant immunity to a human-produced antibiotic (Kummerer 2009). ARGs are spread by vertical transfer, when bacteria possessing the gene are “fitter” and thus more likely to survive and divide into more similar bacteria (Alonso et al. 2001), or by horizontal transfer, where mobile genetic elements including plasmids allow bacteria to share ARGs in a mass pool called a resistome (Watts et al. 2017). As resistance spreads, many bacteria have become resistant to multiple antibiotics, and so simply switching to another drug is no longer an option (Watts et al. 2017). ARGs may also act as indicators of environmental pollutants, where the antibiotics can affect the biodiversity of bacterial communities in the water and sediments in the vicinity of fish farms (Watts et al. 2017).

The biggest concern of antibiotic resistant genes in our food, or the environment in which it is grown, is the potential transfer of ARGs from naturally occurring bacteria to pathogens that either affect our crops (e.g., livestock, plants, fish, shellfish etc.) or human health. In Canada,

the Council of Canadian Academies (CCA) states that "*AMR is both a One Health and One World problem. There is no single sector at fault, no part of the world immune, and no one solution to solve the challenges brought on by resistant microbes. There is also a time lag between the cause and effect of AMR: AMU [antimicrobial use] in one place today may lead to resistant infections in another place tomorrow. The negative impacts of AMR are already experienced in Canada and around the world; inaction today ensures that, as resistance grows, these impacts will only worsen with time*". The CCA shows estimates that 1 in 19 deaths are attributed to AMR and that it costs Canada $1.4 billion a year in healthcare costs (Council of Canadian Academies 2019). Antibiotic use and the resulting AMR in terrestrial and aquatic food production systems is the subject of increasing concern internationally by all industrial sectors (veterinarians, industry, healthcare managers) and has been identified as a major environmental and health care risk (e.g., Kraemer et al. 2019; Lulijwa et al. 2020). The need for coordinated action on addressing this issue, both domestically and internationally, is urgently required.

The possibility of this introduction from the aquaculture industry has raised a significant amount of international concern, which has resulted in a number of reviews on the subject (e.g., Cabello 2006, Heuer et al. 2009, Cabello et al. 2013, Cabello et al. 2016, Watts et al. 2017, Topp et al. 2018, Lulijwa et al. 2020). The transfer of ARGs from a farm location to humans could happen along three different pathways: 1) consumption of improper/undercooked contaminated products, 2) close or direct contact with animals or 3) through the environment (Tiwari et al. 2013). Although there are relatively few studies specifically examining the transfer of ARGs from aquaculture situations to humans, there are some data. A study in Chile found a plasmid-mediated quinolone resistance gene from a marine bacterium was being passed to *E. coli* (Aedo et al. 2014). Similarly, a study in China investigated the quinolone resistance development. They found that in addition to hospitals, aquaculture was a possible source of *aac(60)-Ib-cr* and *qnrB2* in aquatic environments (Wen et al. 2016). Enterobacteriaceae, monitored in water quality controls, were important hosts of these two genes and the ubiquitous bacteria, *Aeromonas* spp., served as vectors for *qnr*S2 with the help of *Inc*Q-type plasmids. A 3rd study investigated the resistance development to quinolones and fluoroquinolones in Asia (Poirel et al. 2012). They found that most of the *PMQR* encoding genes originated from bacterial species that were naturally present in the aquatic environment and suggested that they represented the main source of the problem.

There has been a much larger effort invested in the subject of farm to human transfer of antibiotic resistance in the field of agriculture. An example of this was a study that looked at the usage, the resistance that developed, the evidence for transmission and an overall summary for three antibiotics streptothricins, glycopeptides, and colistin (Webb et al. 2017). They found convincing evidence that antimicrobial resistance can be transferred to humans.

A schematic diagram that highlights the linkages between farming and the potential pathways of antibiotic resistance genes in *E. coli* in the biosphere to pathogenic bacteria of humans shows a number of conduits (Fig. 4a) (Hawkey and Jones 2009).

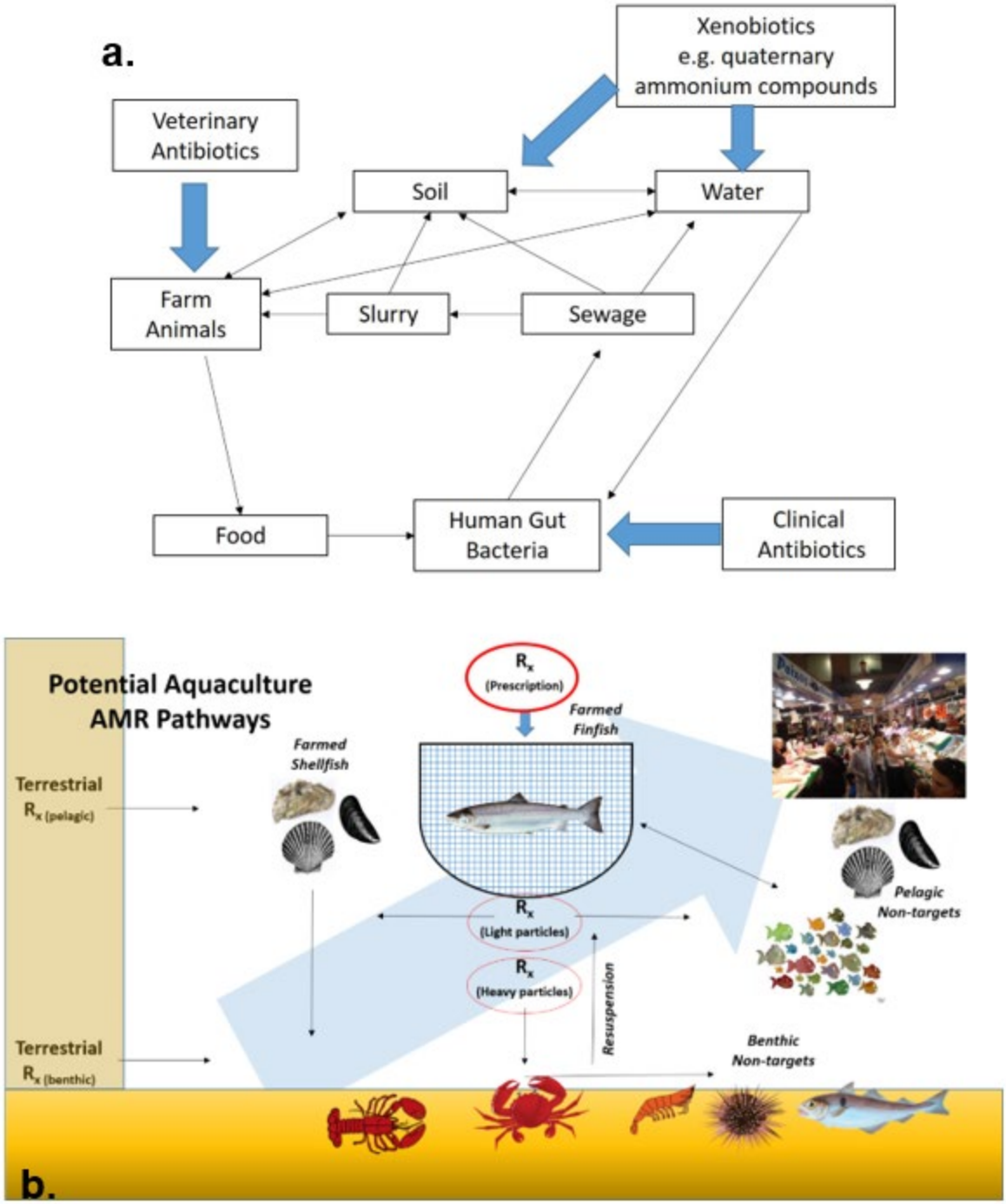

Figure 4 a.) Flow of antibiotic resistance genes in E. coli in the biosphere. Thick arrows show major selective pressures on antibiotic resistance genes, thin arrows show the significant directions of gene flow (redrawn and modified slightly from Hawkey and Jones 2009). b.) Pathways of effects of the potential flow of antibiotics and ARGs in the marine ecosystem ultimately leading to human food (upper right).

Marine aquaculture activities can create a number of other pathways of effects as there are a number of multiple trophic interactions occurring that all lead towards human consumption (Fig. 4b). Antibiotics can be introduced from the farm itself due to prescriptive control of disease or

they may come in from terrestrial sources through wastewater treatment plants that discharge into the natural environment. These antibiotics and potentially resulting ARGs can then get transferred to wild species that associate with the inputs and then make it into human diets.

Thus, the potential exists for aquaculture farms in Canada to contribute to the overall growth of AMR. We are using the same basic culture technologies being employed in other countries and using the same suite of antibiotics, for the most part. Ultimately, the degree to which ARGs are amplified and spread to the resistome is basically related to the rates and probabilities associated with the biology of the bacteria, the environment they live in and the frequency of genetic exchanges that occur. The calculation of these risks will most reasonably done by modelling (e.g., Baker et al. 2016, Rico et al. 2017).

As a result, alternatives to antibiotics in aquaculture are needed to deal with the upcoming issue. It is estimated that by 2050, Canada's healthcare costs as a result of increasing AMR could grow up to $8,billion per year (Council of Canadian Academies 2019). There has already been some success with using vaccines to prevent the spread of bacterial diseases, especially in Norway where the use of antibiotics has been greatly reduced over the past 20 years (Midtlyng et al. 2011). There have also been promising attempts to use bacteriophage therapy to treat bacterial diseases (Wang et al. 2017b). Bacteriophages, or viruses specific to bacteria, show potential as a consumer-safe and eco-friendly alternative to antibiotics due to their specificity to the bacteria they target (Wang et al. 2017b). The use of cultured bacterial species, either as a probiotic to boost the ability of the host to fight off infections, or because they outcompete pathogen-causing bacteria in the host gut, has also been proven effective in fighting fish diseases (Sihag and Sharma 2012). Immunostimulation has been used as a way to combat the stress placed on fish immune systems by farming conditions (Vaseeharan and Thaya 2013). Immunostimulants can often be made of common plants, and many of them have additional benefits as growth promoters (Nya and Austin 2009b). Similarly, some herbal extracts have been shown to contain antimicrobial substances (Dorucu et al. 2009), and the sediment *red clay* has been shown to aggregate to bacterial cells so that they can be removed from water (Jung et al. 2016). These measures are not usually meant as a replacement to antibiotics, but are meant to supplement existing treatments in order to provide the best possible integrated treatment plans as well as to combat the spread of ARGs.

UNDERSTANDING BACTERIAL DISEASES IN AQUACULTURE

Bacteria are small prokaryotic organisms that thrive in complex communities in almost every environment on earth, including inside and outside of living organisms—every animal and plant is the target of bacterial diseases (Drexter 2010). Bacteria are simpler than eukaryotic organisms, with a single circular chromosome. Replication is achieved by binary fission, where the cell splits into two identical offspring cells. Pathogenic (or disease-causing) bacteria enter the body of a larger organism such as a human or fish through the wounds in the skin, mucous membranes, or openings such as eyes, noses, gills or genitals (Drexter 2010). They can also be carried by parasites, consumed in food and water and transmitted by skin-to-skin contact or sexual activity (Drexter 2010).

Bacterial diseases have different modes of action for invading and infecting a host (Drexter 2010). The symptoms of several common fish pathogens that will be discussed in this article can be found below in Table 1. Symptoms begin to appear as bacteria are multiplying in the body and begin to damage cells by secreting harmful compounds or competing with host cells for space and nutrients. Other bacteria may suppress the body's immune defenses to allow themselves to propagate more quickly, or they may secrete toxins that will trigger such a massive immune response that the host itself is harmed. The goal of any bacterial infection is to multiply as widely as possible and spread to as many new hosts as possible (Drexter 2010).

Bacteria are responsible for a majority (54.9%) of disease outbreaks in aquaculture (Dadar et al. 2017). Outbreaks are a major economic burden to the industry due to fish mortalities (Serrano 2005) and the costs associated with treatment (Dadar et al. 2017).

The practices associated with aquaculture provide a prime breeding ground for bacterial diseases due to the relatively high densities of fish. A *sea cage* (a structure also referred to as a *net pen* or a *sea pen*) is a large enclosure placed directly into the ocean in a coastal region and stocked with juvenile fish (Burrells et al. 2001). There is free exchange of water with the ocean and substances such as fish feed, medications and faeces are able to pass through the net pen into the surrounding environment (DePaola et al. 1995). Fish in a sea cage are crowded and stressed, besides not able to engage in their natural migratory behaviours (Burrells et al. 2001). Movement of fish from cage to cage generally is accomplished by a well boat, or a boat where fish and water are sucked in before being moved to another location (Hjeltnes et al. 2017). Fish are also handled by staff (Burrells et al. 2001). The stress placed on fish by these conditions has a negative effect on their immune systems (Burrells et al. 2001). Given the massive exports of the products of aquaculture around the world, there is a risk of the spread of bacterial disease from location to location through farmed fish, especially when fish are not showing symptoms and appear healthy (Dadar et al. 2017) which is common (Table 1 describes several common fish pathogens that may exist in fish without showing symptoms).

Increasing resistance to antibiotics used to treat these diseases has been recorded in recent years. As a result, there have been extensive studies, and motions to implement, alternative treatment and prevention methods (Dadar et al. 2017). These are expanded upon below in the *Alternative Approaches to Bacterial Control* section, and include bacteriophage therapy, vaccination, and immunostimulation. There have also been laws and regulations implemented by various countries to try to limit the use of antibiotics in order to prevent the spread of antibiotic resistance, and these are summarized in Table 3 in the *Status quo* section below.

Table 1. Common pathogens found in farmed fish, their symptoms and the treatment traditionally used to handle outbreaks.

Disease name	Type of Pathogen	Effects	Treatment	Source
Infectious salmon anemia	Viral	Lethargy, fin rot, skin lesions (occasionally deep enough to damage muscle tissue), severe anemia, necrosis (tissue death) in the liver and other organ damage	No treatment has been developed, but a vaccine is available.	(Falk et al. 1997, Bouchard et al. 2001) (Brudeseth et al. 2013)
Infectious pancreatic necrosis	Viral	Loss of appetite, hyperventilation, abnormal swimming, abdominal swelling, damage	No treatment has been developed, but a vaccine is available	(Roberts and Pearson 2005, Brudeseth et al. 2013)

Disease name	Type of Pathogen	Effects	Treatment	Source
		to intestinal mucus, liver swelling, pancreatic necrosis		
Enteric red mouth disease/yersiniosis (*Yersinia ruckeri)*	Bacterial	Loss of appetite, lethargy, reddening of the mouth area, hemorrhaging (bleeding) under the skin, in the eyes, at the base of fins, around the anus, and in internal organs, accumulation of fluid in intestines and eventual necrosis of intestinal mucus lining	Treatment relies on antibiotics. Effective drugs include: amoxicillin, oxolonic acid, oxytetracycline,sulfadiazine in combination with omethoprim or trimethoprim, and florfenicol.	(Furones et al. 1993, Newaj-Fyzul et al. 2007, Kumar et al. 2015)
Winter ulcer (*Moritella viscosa), Tenacibaculum sp.)*	Bacterial	Skin lesions, occasionally deep enough to damage muscle tissue, muscle degeneration	Florfenicol	(Lunder et al. 1995)
Piscirickettsiosis (*Piscirikettsia salmonis*)	Bacterial	Skin lesions and nodules, lethargy, loss of appetite, abnormal swimming, anemia, abdominal swelling, hemorrhaging at base of fins, around the anus and internal organs, and swelling of internal organs. Mortality may occur without	Florfenicol, oxolinic acid, flumequine, oxytetracycline.	(Rozas and Enriquez 2014)

Disease name	Type of Pathogen	Effects	Treatment	Source
		symptoms in some cases.		
Vibriosis/cold water vibriosis (various species of *Vibrio)*	Bacterial	Lesions, anemia, hemorrhaging at the gills, fins and intestines, inflammation of intestines, muscle necrosis, suppressed immune response	Oxytetracycline, sulfonamides in combination with omethoprim or trimethoprim,	(Bullock 1977, Serrano 2005)
Amoebic gill disease (*Neoparamoeba perurans*)	Caused by amoeba	Lesions and excretion of mucus at the gills, cysts, anemia	Vaccines are still in development.	(Ruane and Jones 2013)
Classical and atypical furunculosis (*Aeromonas salmonicida*)	Bacterial	Lesions, blood-filled pustules, hemorrhaging. Mortality may occur without symptoms in some cases.	Sulfonamides in combination with omethoprim or trimethoprim.	(Gudmundsdottir and Gudmundsdottir 1997, Serrano 2005)
Bacterial haemorrhagic septicaemia (*Aeromonas hydrophila, Aeromonas sobria, Pseudonomas*)	Bacterial	Abnormal swimming, pale gills, bloating and skin lesions. Death can also be observed suddenly and without symptoms.	Oxytetracycline	(Serrano 2005, Kayansamruaj et al. 2017)
Strep infection (*Streptococcus iniae*)	Bacterial	Lethargy, abscesses, stiffening of the dorsal region, abnormal swimming, and central nervous system damage often leading to rapid death.	Vaccines have been developed and implemented in many cases.	(Baiano and Barnes 2009) (Weinstein et al. 1997)

DEVELOPMENT OF RESISTANCE

There are two main ways that bacteria can share resistance determinants with other bacteria without dividing. The transfer of genetic material from one bacterium to another is referred to as *horizontal/lateral transfer*, while genetic material passing to daughter cells after division is referred to as *vertical transfer*.

Some species of bacteria are able to divide fast enough to double their numbers multiple times an hour, which means that natural selection works on them much more quickly in comparison to other organisms. For this reason it was originally thought that beneficial gene mutations granting bacteria additional fitness and increasing their chances of successful replication and division was the main vector for the spread of antibiotic resistance (Alonso et al. 2001). This describes the process of *vertical transfer*, where genes are passed on generationally as bacteria divide. Later research showed that an unexpectedly important role was played by *horizontal transfer* (Alonso et al. 2001), or the sharing of genes that improve fitness in different environments (Watts et al. 2017). Horizontal transfer occurs through *mobile genetic elements*, which in cases where genes are being moved from one cell to another includes plasmids and transposons (Bennett 2008). Fish farms are a prime environment for this kind of activity (Watts et al. 2017). While the pool of shared genetic material has always been large, studies have shown that before wider use of antibiotics, resistance genes were much less common than they are today (Alonso et al. 2001). As a result, it has been concluded that selective pressure exerted on bacterial communities results in the addition of resistance genes to the shared genetic pool (Alonso et al. 2001). The pool of antibiotic resistance genes being exchanged by a bacterial community is referred to as the *resistome* (Watts et al. 2017).

Selective pressure

Antibiotic resistance is not a new phenomenon, as bacteria have always had to find ways to deal with harmful compounds in the environment in order to survive (Watts et al. 2017). Bacteria over millions of years have developed resistance to heavy metals and other compounds that will be encountered in their habitats, as well as to natural antibiotics produced by other bacteria in the sediment (Kummerer 2009). Exposure to sub-lethal levels of antibiotics results in bacteria carrying resistance genes surviving and reproducing at higher levels compared to bacteria without these genes. These factors lead to antibiotic resistance being a very common problem on fish farms, particularly where antibiotics are used regularly in a prophylactic manner. In one study, up to 90% of bacteria tested were found to be resistant to at least one antibiotic (Watts et al. 2017).

Selective pressure is exerted constantly on the bacterial communities in fish farms whenever antibiotics are in use (Watts et al. 2017). In situations where fish in a large net pen are being treated with antibiotics, the medication is usually mixed with feed and dropped directly into the water(Romero et al. 2012). Thus bacteria living in the gut of the fish are exposed to the treatment, and when feed goes uneaten or un-metabolized antibiotics pass in fish faeces, bacteria in the water or in the sediments beneath the farm are exposed to a non-lethal dose of the compound(Romero et al. 2012). This results in selective pressure favoring bacteria that have antibiotic resistance genes and an eventual increase in the types of bacteria that have resistance to the medication (Romero et al. 2012). This common process is an example of acquired resistance for an area, where the bacteria becomes resistant to a compound where it was previously susceptible (Romero et al. 2012). Acquired resistance is the most concerning form of antibiotic resistance and the biggest threat to public health as it interferes with our ability to treat pathogens that, before antibiotics, were considered much more serious (Romero et al. 2012).

Many cell structures conferring antibiotic resistance allow bacteria to resist damage from metals in the environment—such as efflux pumps, which are proteins in the membranes of bacteria that allow them to pump harmful compounds out of the cell (Watts et al. 2017). While certain metals are important micronutrients for bacteria, they may become toxic depending on concentration and environmental conditions (Seiler and Berendonk 2012). Heavy metals such as mercury, cadmium, copper and zinc aid in the selection for antibiotic resistance genes when released into the environment in combination with antibiotics and allowed to reach a critical concentration (Seiler and Berendonk 2012). Heavy metals are found in additives to feed and to fertilizers as well as in anti-fouling agents (previously used on the nets of open-water fish pens to prevent biological growth on the structure) and in pesticides, resulting in a bacterial community exposed simultaneously to antibiotics and to heavy metals(Seiler and Berendonk 2012). The coupling of bacterial resistance mechanisms against heavy metals and antibiotics results in increased selection, or co-selection, for antibiotic resistance genes(Seiler and Berendonk 2012).

Horizontal transfer- sharing genetic material

A *mobile genetic element* refers to the method by which cells pass genetic material among themselves (Watts et al. 2017). This can be a structure that allows DNA to move from cell to cell, or between different locations inside the cell.

Conjugation refers to the passing of genetic material between two organisms that have physical contact. This is also called bacterial mating. The mobile genetic elements in this case are known as *plasmids,* small circular pieces of DNA that are able to facilitate their own transfer from one cell to another and *transposons*, or a gene with the ability to jump between chromosomes and plasmids (Bennett 2008). A conjugative plasmid can then pass through the donor cell membrane into the recipient cell, before the transposon is transcribed and possibly incorporated into the chromosome (Bennett 2008).

Transformation is the process by which a cell acquires DNA that is floating freely in the environment (Bennett 2008). Similarly to conjugation, a mobile genetic element is taken up by the cell, and the genes it contains can then be transcribed and translated (Bennett 2008). DNA released by lytic bacteriophages (See below in the *Bacteriophages* section) may be picked up by another cell, and there are concerns that this could include antibiotic resistance genes, either present in the cell beforehand or inserted via the viral genome (Madhusudana Rao and Lalitha 2015).

Transduction refers to a case where a bacteriophage inserts genetic material into a cell, which the cell then incorporates into its genome. Bacteriophages are plentiful in nature, especially in aquatic environments, where transduction is very common (Kutter and Sulakvelidze 2004).

Role of plasmids

Plasmids are small, usually circular pieces of DNA that replicate in cells, independently from that organism's chromosomes (Bennett 2008). They do not contain any essential genes for survival or replication (Bennett 2008). The genes carried by plasmids may improve an organism's fitness in certain situations, which makes it evolutionarily advantageous for a bacterium to pick plasmids up in the process known as *transformation* (Bennett 2008). A plasmid carrying genes that confer resistance to antibiotics or to other lethal substances is called a *resistance plasmid* (Bennett 2008). Resistance plasmids are believed to be responsible for most cases of resistance to common antibiotics that have been recorded to date (Bennett 2008).

Another mobile genetic element important to discussions around plasmid-mediated antibiotic resistance is the transposable element or *transposon*, which allows a gene to be transferred

from one DNA molecule to another (Bennett 2008). In cases where a plasmid has been taken up by a cell, a transposon may cause an antibiotic resistance gene to be transferred from the plasmid to the bacterium's chromosome (Bennett 2008). The resistance gene is then incorporated into the bacterial genome and will be reproduced as the cell divides (Bennett 2008).

Role of bacteriophages

Phages, short for *bacteriophages*, are viruses that are nearly omnipresent in the environment and specifically target bacteria (Madhusudana Rao and Lalitha 2015). Phages exploit bacteria because phages have all the genetic material, but none of the cellular machinery required to propagate offspring, and as a result need to hijack the cellular machinery of a bacterium in order to reproduce their genomes and synthesize proteins for new phages (Madhusudana Rao and Lalitha 2015). In *lytic* or *virulent* bacteriophages, this quickly results in the death of the host as the cell membrane and peptidoglycan layer are compromised in order to release the propagated phages into the environment (Oliveira et al. 2012). For this reason, lytic bacteriophages have been extensively studied as potential alternative to antibiotics in aquaculture, with great success (Oliveira et al. 2012). More information on lytic bacteriophages and their therapeutic potential can be found below in the *Alternatives to Antibiotic Use—Bacteriophages* section.

Concerns about temperate or lysogenic phages usually center on the transfer of foreign genes into bacteria (Madhusudana Rao and Lalitha 2015). Unlike virulent phages, they do not lyse the host to release new viruses into the environment (Oliveira et al. 2012). In unsuitable environmental conditions, the virus simply inserts its genetic material into the host's genome (or remains in the cell as a plasmid) and allows it to be reproduced over and over as the host cell replicates its own DNA and divides (Oliveira et al. 2012). In this situation, the propagated viral genome is referred to as a *prophage*, which can enter the lytic cycle when conditions are right (Madhusudana Rao and Lalitha 2015) or can be secreted from the cell without killing it (Oliveira et al. 2012). For this reason, lysogenic phages are useless as a treatment, but can be responsible for the transfer of genetic elements conferring virulence or antibiotic resistance on bacteria (Madhusudana Rao and Lalitha 2015). A prophage being packaged can include elements of a host genome along with its own, and then insert these into a new host bacterium, causing the recipient to integrate these genes and begin to reproduce them (Madhusudana Rao and Lalitha 2015). As a result, the recipient bacteria can acquire resistance to multiple antibiotic compounds, or acquire dangerous virulence factors, such as the ability to produce lethal toxins (Madhusudana Rao and Lalitha 2015). This acquisition of virulence factors can lead to a bacterium gaining the ability to infect new organisms in a process known as *phage conversion* (Madhusudana Rao and Lalitha 2015).

Role of integrons

Integrons, which exist in some DNA molecules, are another method that bacteria use to collect non-essential but potentially useful genes (Bennett 2008) These elements contain gene cassettes, small, circular pieces of DNA that do not replicate and typically contain one gene at a time (Bennett 2008). Gene cassettes are moved about inside an integron, or from one integron to another, and similar to transposons this can facilitate the insertion of a foreign gene into a bacterial genome (Bennett 2008). While transposons tend to insert genes into the chromosome at random, or with slight preference for certain sites, integrons encode an enzyme that causes the insertion of the gene at a specific site (Bennett 2008).

Common strategies of resistance

Antibiotics have either a bactericidal or bacteriostatic mechanism of action (Burridge et al. 2010). Bactericidal antibiotics directly kill the targeted organism or make it vulnerable to being

killed by the host immune response, often by targeting the synthesis of membranes or cell walls, protein synthesis, DNA replication or important enzymes (Romero et al. 2012). Bacteriostatic antibiotics prevent the growth and division of the pathogen, allowing it to die off naturally or be killed by the host immune response (Romero et al. 2012). This is achieved by interfering with the production of cell products or DNA, or by blocking aspects of bacterial metabolism (Romero et al. 2012). The mechanism of action of an antibiotic is important to determining how a pathogen defends itself against it. The mechanisms of actions of some common types of antibiotics and strategies used by resistant bacteria are summarized in the table below.

Table 2. Common antibiotics used in aquaculture, along with their commercial names, mechanisms of action and common strategies of resistance against them.

Name of antibiotic	Commercial names	Mechanism of action	Strategy of resistance	Ref
Quinolones (oxolonic acid, flumequin)	Oxolinsyre vet, Litoflox, Bandrol, Flox-feed, Flumepren	Targets DNA gyrase and topoisomerase IV, both involved in DNA replication	Resistance stems from point mutation affecting the topoisomerase gene.	(Burridge et al. 2010) (Romero et al. 2012, Norwegian Veterinary Institute 2016a, Lozano et al. 2018)
Tetracycline (oxytetracycline)	Tetroxy Aquatic, Oxymarine, Pennox, Oxysentin, Terramycin, Bactitab, Renamycin, Terrivet, Zanil	Targets protein synthesis by reversibly binding to the ribosome of a prokaryotic cell	Varied: mutations in the ribosome, inactivation of the drug, active efflux of the drug from the cell, or protecting the ribosome using a cytoplasmic protein.	(Romero et al. 2012, Sharker et al. 2014, Kumar and Roy 2017, Lozano et al. 2018)
Amphenicols (florfenicol, chloramfenicol)	Aquaflor, Floraqpharmavet, Duflosan, Aquafen	Targets the 50S subunit of a bacterial ribosome and inhibits protein elongation.	Includes proteins such as RNA methyltransferases and hydrolases as well as protein transporters to remove the drug from the cell	(Burridge et al. 2010) (Romero et al. 2012, Norwegian Veterinary Institute 2016a, Kumar and Roy 2017, Watts et al. 2017)
Sulfonamides (Sulfadimethoxine ormetoprim,	Romet-30, Sulfamerazine, Sulfatrim, Cotrim-vet	Inhibits folic acid metabolism, usually given in	Alteration of the DHPS enzyme reduces functionality	(Burridge et al. 2010) (Skold 2000,

Name of antibiotic	Commercial names	Mechanism of action	Strategy of resistance	Ref
trimethoprim, sulfadiazine, sulfamethoxazole)		combination with trimethoprim (tribrissen) for inhibition at two different levels. This is achieved by targeting the enzyme dihydropteroate synthase (DHPS).	but also increases resistance to sulphonamides.	Sharker et al. 2014, Kumar and Roy 2017, Lozano et al. 2018)
Amoxicillin	Acimox, Renamox, Ranamox, Amox-feed	A b-lactam antibiotic that disrupts the synthesis of peptidoglycan, thus weakening a Gram positive pathogen's cell wall or a Gram negative pathogen's peptidoglycan layer.	Synthesis of B-lactamases to cleave the B-lactam ring and thus render the drug ineffective.	(Burridge et al. 2010, Rasul et al. 2017, Wang et al. 2017b, Lozano et al. 2018)
Erythromycin	Vetromic, Eritofeed	Interferes with mRNA-bound 70S ribosomes and degrades the 50s subunit.	Ribosome modification to alter the target site and prevent antibiotic binding.	(Vester and Garrett 1987) (Weisblum 1995, Cao et al. 2011)

Multi-drug resistant pathogens- MDRPs

A phenomenon of concern recorded in recent years is the appearance of *multi-drug resistant pathogens*, or bacteria that are resistant to multiple antibiotics (Watts et al. 2017). Up to 20% of bacteria found in some fish farms are resistant to at least five antibiotics (Watts et al. 2017). In many cases, these antibiotics are also critical for use in human medicine (Heuer et al. 2009). This can be a serious obstacle to the control of human disease outbreaks if resistance genes are passed to human pathogens (Heuer et al. 2009).

Multi-drug resistance has been found in fish pathogens as well. Strains of *Aeromonas* (Table 1) have been found to be resistant to sulfonamides, streptomycin, spectinomycin, trimethoprim and/or tetracycline all at once (Serrano 2005). Between 2007 and 2015, the Ministry of Agriculture's Animal Health Centre in British Columbia collected isolates of bacterial pathogens from samples submitted from fish farmers and tested them for resistance to the antibiotics usually used to treat them (Centre for Coastal Health 2016). Two of the diseases included in this study were *Yersinia ruckeri* and *Aeromonas salmonicida,* both isolated from farmed Atlantic salmon. Antibiotic resistance in *Y. ruckeri* was found to be very rare. Meanwhile, in cases where

resistance in *A. salmonicida* was recorded, resistance to multiple drugs was found in the majority of instances.

STATUS QUO

Therapeutic use of antibiotics refers to cases where antibiotics are given to sick fish for the purpose of directly treating a specific pathogen infecting them (Romero et al. 2012). The development of antibiotic resistance genes against these drugs, and the spread of these genes to human pathogens, is concerning as many of the same drugs are also used in human medicine (Heuer et al. 2009). In Canada, there is a small suite of antibiotics available to the fish farming industry through the veterinarians: oxytetracycline, florfenicol, erythromycin, Ormetoprim and Trimethoprim. From the period 2016 to 2018, oxytetracycline represented 78%, florfenicol 20% and the others represented approximately 2% of the use by weight (Dataset).

Preventative or metaphylactic use of antibiotics refers to the giving of antibiotics to healthy animals in order to prevent an outbreak before it starts (Romero et al. 2012). This may be done at the same time sick fish are treated (Romero et al. 2012). The purpose of this is to avoid the monetary loss resulting from fish mortalities in an outbreak and the additional cost of a therapeutic treatment. However, there is an increased risk of development of antibiotic resistance since bacteria are constantly being exposed to low levels of antibiotics (Romero et al. 2012). None of this is happening in Canada at the present time.

Prophylactic use of antibiotics refers to the giving of antibiotics to healthy animals in order to promote increased feeding efficiency and growth (Serrano 2005). This works by eliminating microbes, harmful or not, living in the intestinal tract which utilize nutrients in the host's feed for their own use (Serrano 2005). Without these organisms, the host is better able to absorb the nutrients it consumes, leading to faster growth and weight gain (Serrano 2005). While there is a financial incentive for doing this, the extended presence of antibiotics in small doses creates a prime environment for resistance to develop (Kummerer 2009). While developed countries in the Western world have generally banned or heavily regulated the use of antibiotics for prophylactic and prevention purposes, this practice is still common in Asia (Heuer et al. 2009).

The usage of antibiotics varies by country (Kummerer 2009). As much as 90% of the world's aquaculture production comes from Asia, where regulations restricting the use of antibiotics are less common, and use is often underreported (Heuer et al. 2009). For this reason the true volume of antibiotics used to treat farmed fish is not known (Kummerer 2009). Out of the major fish producers in the Western world, Chile reports the highest antibiotic use. Unlike in Norway, Canada or the UK, antibiotic use has actually shown an upwards trend in Chile over the past 10 years (Lozano et al. 2018) (Fig. 5). There is also less transparency in Chile regarding the effects of the administered antibiotics on humans and animals (Lozano et al. 2018). These data show that Chile was using approximately 487 Kg (SD=121) of antibiotic per tonne of salmon produced. In comparison, in 2016-2019, Canada was using on average 0.13 kg (SD=0.01) (Dataset) and in 2016, Norway was using 0.000017 Kg of antibiotic per tonne of salmon produced (Midtlyng et al. 2011).

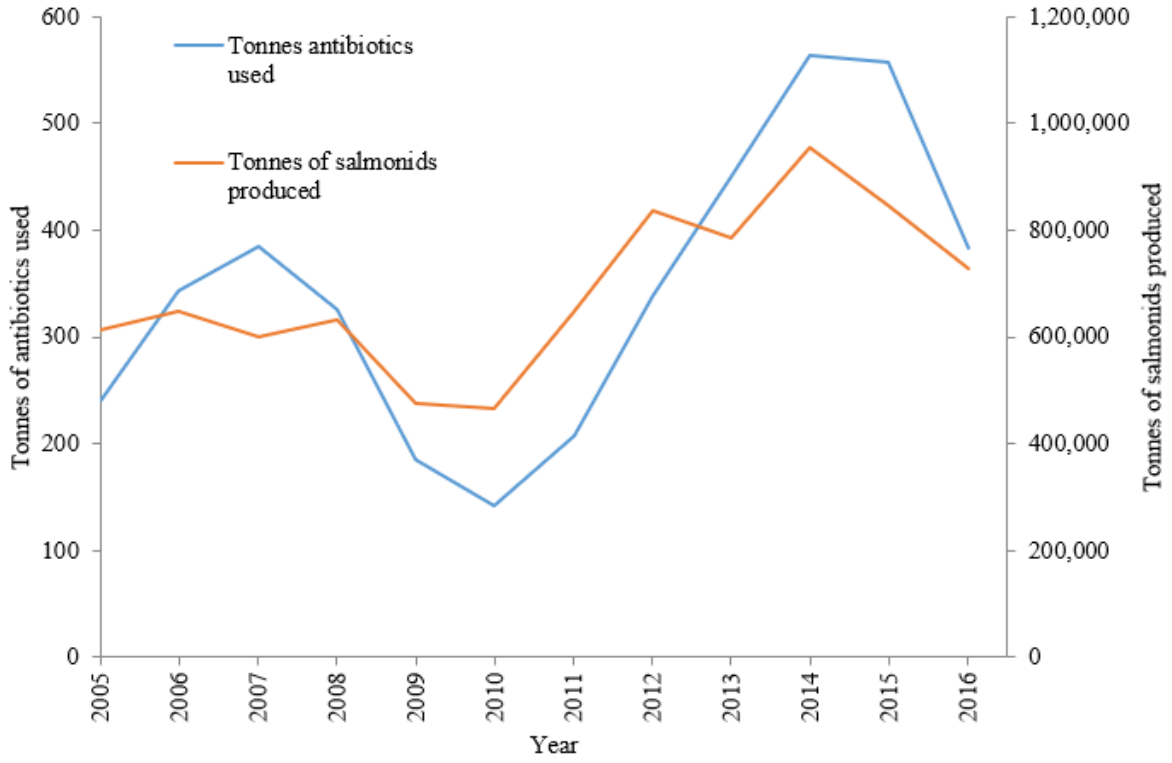

Figure 5. Antibiotic use in Chile by year, compared with the production of salmonids (tonnes) (Lozano et al. 2018).

While antibiotic use in Chile is clearly following trends in the biomass of fish produced (Figure 5), antibiotic use in Norway has actually sharply decreased (Fig. 6), despite continual growth in the aquaculture sector (FAO 2018c) (Midtlyng et al. 2011). Norway has managed to decrease its antibiotic use with extensive vaccination programs along with spatial planning of fish farms to prevent the spread of disease and other technical solutions (Midtlyng et al. 2011). More information can be found about Norway's success below in the *Vaccines* section.

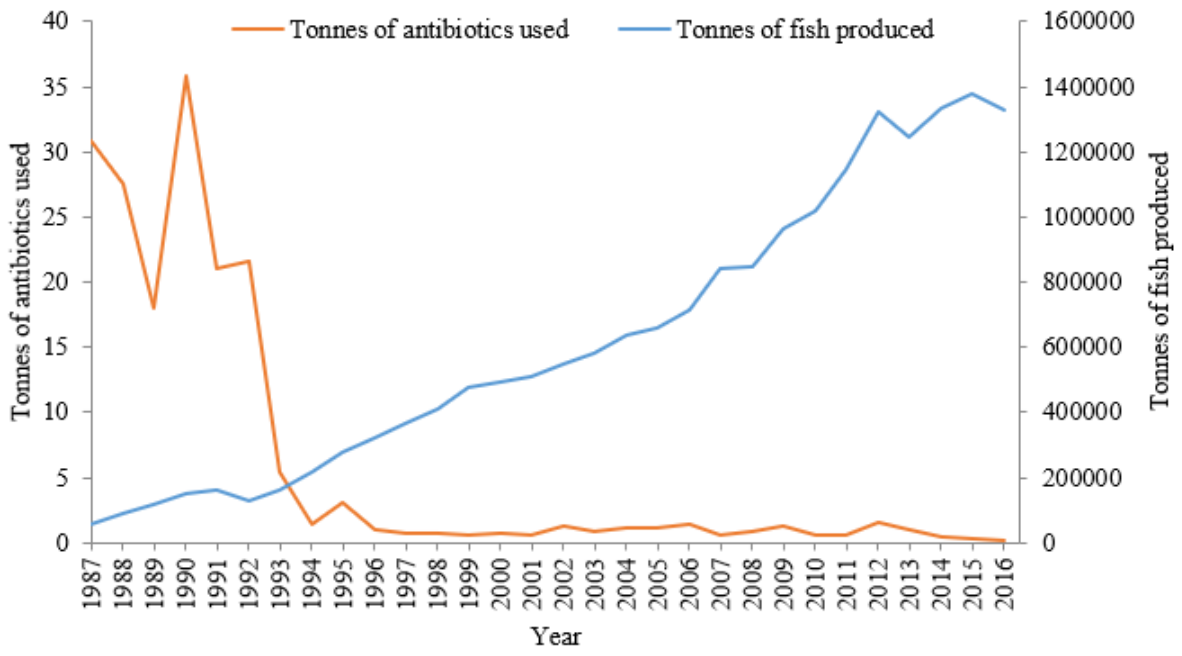

Figure 6. Antibiotic use in Norway by year, compared with the production of farmed fish (tonnes) (Hjeltnes et al. 2017, FAO 2018c).

Table 3: Legislation concerning the use of antibiotics in aquaculture, by country.

Country	Regulations	Ref
United States	Oxytetracycline, a mix of sulfadimethoxine and ormetoprim and florfenicol are licensed for use on catfish and salmonids. Usage is restricted to the specified species for the treatment of enteric redmouth and furunculosis respectively. Aquaculture makes up only a small percentage of antibiotics sold for use in animals in the United States.	(Serrano 2005, Kummerer 2009, Kumar et al. 2015)
Canada	Antibiotics must be administered under the supervision of a licensed veterinarian. The use of oxytetracycline, sulfadimethoxine mixed with ormetoprim, and florfenicol are all licensed for use in farmed fish.	
Norway	Antibiotics must be administered under the supervision of a licensed veterinarian. Antibiotics approved for use on farmed fish include florfenicol, oxolinsyre and oxytetracycline.	(Hjeltnes et al. 2017)
China	Antibiotic use is underreported and weakly regulated. Antibiotics approved for use in aquaculture in China include sulfonamides, nystatine, terramycin, aureomycin, penicillin, streptomycin, penicillin, streptomycin, doxycycline, erythromycin, and oxolinic acid.	(Serrano 2005)

Country	Regulations	Ref
Chile	Antibiotic use is higher in Chile than in Norway or other major salmon producers. Regulations are less strict, with 12 generic and 25 branded antibiotics approved for use, without specifications regarding to which species they may be administered. However antibiotics can only be applied for the direct treatment of a specific disease, instead of as a preventative or prophylactic measure. Treatment is required to be supervised by a veterinarian.	(Lozano et al. 2018)
United Kingdom	Oxytetracycline, oxolinic acid, amoxicillin and a combination of trimethoprim and sulfadiazine are authorized for use.	(Kayansamruaj et al. 2017)

ARGS FOUND IN AQUACULTURE ENVIRONMENTS

Methods of entry and reservoirs

There are concerns that antibiotics remaining in soil and water in the vicinity of fish farms could contribute to the development of antibiotic resistance in human pathogens, particularly in developing countries due to widespread, uncontrolled use. The reasons for this include that developing countries are typically located in hotter climates, meaning that pathogens are acclimatized to higher temperatures that would allow them to live in the human gut (Serrano 2005) and the warmer temperatures may also decrease reproductive times allowing for faster population growth rates.

The sediments of the ocean floor are home to a thriving bacterial community possessing a massive pool of shared genes (Watts et al. 2017). Selective pressure from antibiotics administered to farmed fish causes this resistome to contain more antibiotic resistance genes, changing the makeup of bacterial communities and promoting the spread of antibiotic resistance (Watts et al. 2017). It has been determined that most antibiotics have a half-life of two to three weeks in soil, which is extended at temperatures below 20^{o}C (Serrano 2005). Oxytetracycline, one of the most widely used antibiotics in aquaculture, can have a widely ranging half-life from nine to 419 days depending on the substrate and the degree of anoxia (Björklund et al. 1990; Capone et al. 1996). Dilution is thought to be the main mechanism for the reduction in concentration, so resuspension events of the sediment on the sea bottom likely play a large role in determining local concentrations.

Antibiotics are also a threat to bacteria living in surface water. Nitrifying bacteria endemic to fresh water, that play an important ecological role, were harmed by antibiotics used in aquaculture in a simulated environment in laboratory studies (Kummerer 2009).

Antibiotics and ARGs can enter sediments on the ocean floor beneath farms through uneaten fish feed and through fish excrement (Muziasari et al. 2014). When farmed fish receive antibiotics through feed, the dose may be higher compared to that which is given to livestock, as feed consumption is less efficient in fish, especially when they are sick and suffering a loss of appetite since there is less control over individual animals (Watts et al. 2017). When antibiotics are consumed by fish and not completely metabolized, the antibiotic that exits the body in faeces is still an active compound (Kummerer 2009). In some studies, up to 80% of antibiotics given to fish can enter the environment over the course of treatment (Watts et al. 2017). The antibiotics may then remain in the sediment (Kummerer 2009), accumulate in the tissues of wild fish, which then not only cause them to travel far from their original sources, but also exposes

naive bacteria which then may develop resistance (Zhao et al. 2015). These fish may also be caught for human consumption, which could theoretically transfer ARGs or resistant bacteria into the human gut, potentially causing disease or transfer of the ARGs to human pathogenic bacteria (Zhao et al. 2015).

Oxytetracycline is one of the most common antibiotics used in aquaculture due to its low cost and wide range of effectiveness (Romero et al. 2012). However, oxytetracycline is very difficult to metabolize, and as a result a large amount of the medication must be given in order to reach the desired dose (Romero et al. 2012). Up to 70-80% of the oxytetracycline given in fish feed is excreted into the water, where most of it biodegrades. However, some persists in the water and in the sediments below the farm (particularly when currents are weak) (Romero et al. 2012). A Chinese study in 2017 demonstrated the presence of bacterial genes conferring resistance to oxytetracycline (and to sulfonamides) in water and sediments associated with fish farms in the Guangzhou region of China (Su et al. 2017). The authors suggest that incomplete treatment of wastewater from terrestrial sources can significantly affect fish farms may result in the dissemination of ARGs and antibiotics into the environment (Su et al. 2017). In another study, on a Norwegian fish farm, oxytetracycline continued to be detected in the water and sediments beneath a fish farm 89 to 142 days after treatment (Samuelsen et al. 1992). As above, prolonged exposure to non-lethal concentrations of an antibiotic lead to selection for ARGs in bacteria. This genetic information may eventually make its way into the human food supply or be laterally transferred to human pathogens.

Another possible route of introducing ARGs to fish farms would be through some of the more “natural” health practices that use biological entities as a solution. An example of this would be the use of large numbers of cleaner fish, such as a wrasse (Labridae) or lumpfish (Cyclopteridae) , as natural predators that would graze sea lice from the skin of cohabiting salmon in a sea cage. Millions of cleaner fish a year are produced in hatcheries and then stocked with salmon. These cleaner fish are susceptible to many of the same pathogens that affect salmon and consequently, are treated with the same antibiotics giving rise to the concern of increasing AMR (Erkinharju et al. 2020). Vaccines being developed for cleaner fish are lagging behind efforts on vaccines for salmon.

Effect on bacterial communities

The presence of antibiotics and antibiotic resistant genes in the environments surrounding fish farms alters the biodiversity of the bacterial communities endemic to those areas (Watts et al. 2017). This occurs because resistant strains of bacteria can out-compete non-resistant strains (Watts et al. 2017).

A mathematical model proposed by Rico et al. (2017) used data previously compiled on the resistance thresholds of environmental bacteria to model the risks of the development of resistance when antibiotics are released into the environment. Similar to Su et al. (2017), the water and sediments from which the data originated came from aquaculture environments in southeast Asia, where antibiotics are used regularly and with much less regulation than in North America and Europe (Rico et al. 2017). In nearly all of the calculated scenarios, at least some of the antibiotics released into the environment exceeded the threshold required for the propagation of ARGs (Rico et al. 2017).

Samuelsen et al. (1992) sought to outline the resistance properties and abundance of the bacteria in the sediment below the farm over a year and a half post-treatment with oxytetracycline (Samuelsen et al. 1992). There were an extremely high number of resistant bacteria (up to 90%) in the sediment directly after treatment, which then dropped and levelled

off before gradually increasing towards the end of the study, with the highest percentage being approximately 50% under one of the three cages on the final day (Samuelsen et al. 1992).

A 1995 study by Depaola, Peeler and Rodrick (1995) monitored oxytetracycline resistance in the bacterial communities in the water in a catfish farm, and in the internal microbiota of the fish, during a treatment with oxytetracycline given in medicated feed. The authors found that resistance to oxytetracycline in the bacteria associated with medicated fish rose steadily from approximately 10% to 40% during the fall treatment period before slowly returning to normal during the post treatment period (DePaola et al. 1995). During the spring experiment, resistance fluctuated from 10% before treatment to nearly 90% during the middle of the treatment period, before dropping to about 30% during the post treatment period (DePaola et al. 1995).

Consequences for human health

There have already been discernable consequences for public health stemming from the use of antibiotics in aquaculture and the resulting propagation of ARGs. Varying levels of antibiotics, some at levels harmful to human health, have been found in the products of Chinese fish farms (Mo et al. 2017). The consumption of these products may result in the accidental medication of the consumer, which can disturb the biodiversity and balance of the individual's natural flora, leading to a weakened ability to fight off disease and increased risk of infections (Heuer et al. 2009). Children, the elderly and other individuals with lowered immune responses are particularly at risk (Serrano 2005). There are also risks of development of antibiotic resistance in the human's gut flora, and issues with diagnosing allergies to medications due to inaccurate history of antibiotic usage (Mo et al. 2017). In developed countries there is usually a required time period, between seven and 40 days, between when a fish can be treated with antibiotics and when it can be slaughtered for food in order to prevent accidental medication of the of the consumer by residues in the animal's tissues (Serrano 2005).

As described above, environmental bacteria are capable of sharing antibiotic resistance genes with one another. These antibiotic resistance genes may make their way into the human microbiome (Heuer et al. 2009). There are a few ways this could occur. Resistant bacteria may be found in farmed fish that is harvested for human consumption (Weinstein et al. 1997), may pass to workers at fish farms that handle fish, be consumed in drinking water (unlikely in developed countries) (Heuer et al. 2009), or may be passed from farmed fish into wild fish that are later caught for human consumption (Zhao et al. 2015). Regardless of the route of transfer, the use of antibiotics in fish farming has been linked to the development of antibiotic resistance in human pathogens (Heuer et al. 2009). Many antibiotics used to treat farmed fish are also used to treat humans, which makes the propagation of resistance genes against these medications in the bacterial gene pool additionally dangerous (Heuer et al. 2009). In a similar vein, some fish pathogens developing resistance belong to the same genus as (or are otherwise similar enough to) human pathogens to increase the likelihood of transferable genes between them (Heuer et al. 2009). Human pathogens can also be present in aquatic environments and develop ARGs as the direct result of selective pressure (Heuer et al. 2009). These pathogens include species from the genera *Salmonella* and *Shigella*, as well as opportunistic pathogens such as *Escherichia coli, E. tarda* and *Streptococcus iniae* (Heuer et al. 2009). Propagation of antibiotic resistance in human pathogens leads to more disease outbreaks and an increased difficulty in treating them where they occur (Heuer et al. 2009). Studies from Japan that were specifically studying tetracycline and the possibility of transfer of ARGs to farmed fish concluded that *tet* genes from fish farm bacteria had the same origins as those from clinical resistant strains (Furushita et al. 2003).

ALTERNATIVE SOLUTIONS

Removal of antibiotics from the environment

Constructed wetlands

A constructed wetland utilizes the natural processes of sediments, plants and microbes to remove contaminants from wastewater (Bôto et al. 2016). A microcosm study in the laboratory was used to test the ability of constructed wastelands to remove antibiotics and bacteria containing ARGs from the wastewater of a fish farm (Bôto et al. 2016). The antibiotics in question were enrofloxacin and oxytetracycline, both widely used in aquaculture (Bôto et al. 2016). The study was a great success, showing 99% removal of antibiotic particles and 95% removal of the targeted bacteria (Bôto et al. 2016). However, while constructed wastelands are viewed as a green solution to ARG contamination in fish farm wastewater, little is known about the environmental impact of the strategy, as well as about the risks of the release of toxic compounds if the plant barrier is damaged (Bôto et al. 2016).

Photolysis

Some antibiotics have sensitivity to light, and their decomposition may be sped up by exposure (Kummerer 2009). Photo-degradation of antibiotics takes place mainly in surface water, and efficacy depends on the intensity and frequency of the light source as well as the turbidity of the water (Kummerer 2009). Attempts to remove antibiotics from water by exposing them to light should be approached with caution, as incomplete decomposition may render compounds unstable and additionally toxic (Kummerer 2009). Oxytetracycline is particularly susceptible to photo-degradation (Kummerer 2009). Fluoroquinolines can be degraded by UV light (Kummerer 2009).

Hydrolysis

Hydrolysis refers to a chemical reaction where water is consumed in order to cleave a molecule in two. Certain antibiotics undergo this process under the right conditions, depending usually on temperature and pH (Kummerer 2009). B-lactams are very easily hydrolysed (Kummerer 2009). Bacteria resistant to this class of antibiotics make use of the hydrolysis process to open the B-lactam ring, which leads to deactivation of the compound's activity (Kummerer 2009). The introduction of such bacteria to an aquaculture environment so that they may deactivate residual antibiotics does not seem to be an option for reducing the spread of antibiotic resistant genes—just the opposite, as these bacteria could potentially pass their resistance genes to others in the vicinity.

NEED FOR NEW APPROACHES

Aeromonas salmonicida is a pathogen that causes huge economic loss and mortality of fish in aquaculture in many northern regions (Romero et al. 2012). It is especially known for its ability to quickly gain resistance to a new antibiotic (Romero et al. 2012). Originally the treatment for A. salmonicida was sulfonamide, but over 75% of isolates tested are now resistant (Romero et al. 2012). This is only one case of antibiotics becoming ineffective due to rising resistance. Given the health concerns surrounding the fast-growing resistance of pathogens to common antibiotics, it is essential to find new ways of either surpassing this resistance or of treating bacteria without antibiotics. Multiple solutions will be required, as the causes of the issue are complex and many bacteria are already resistant to multiple drugs (Finch and Hunter 2006). Simply switching to another antibiotic in the case of resistance is no longer a viable option due to the risk of increasing the development of MDRPs (Finch and Hunter 2006).

There are few efforts in major pharmaceutical companies to research new antibiotics due to the low financial incentive compared to “lifestyle” drugs (Finch and Hunter 2006). There is also a lower demand for broad-spectrum antibiotics compared to drugs that treat only a few specific infections, which is also not cost-effective for drug companies (Finch and Hunter 2006). Despite increases in funding, there has also been a decrease in the discovery of antimicrobial compounds (Finch and Hunter 2006).

Before funding waned in favour of research into broad-spectrum antibiotics, vaccines were considered the most promising option for defending against infectious bacteria (Finch and Hunter 2006). Where vaccine research is funded in the modern day, it is usually for diseases for which no antibiotic treatment is available (Finch and Hunter 2006). More funding should be directed into research for vaccines for infections that are currently treated with antibiotics (Finch and Hunter 2006).

Ultimately what happens in Canada in regard to replacement strategies for antibiotics in fish culture will reflect what is also happening internationally, since the fish farming industry is closely linked. Many of the salmon farming companies in Canada are multi-nationals with headquarters located in Norway and the large pharmaceutical companies are also international in scope with national connections to all the countries that are currently growing salmon. Solutions that are developed in one country tend to spread quickly to others, often through commercial sales (e.g., vaccines, cleaner fish technology, probiotics etc.). Norway, for example, is the dominant player in the salmon aquaculture industry and as a result is investing extensively in new approaches to control diseases and parasites. As a result of less investment in this sector by the Canadian government, Canada generally tends to take more of an opportunistic approach and license and sometimes modify the technologies that have been developed by others, although there are some exceptions. This has happened with cage design, feeding systems, vaccines, parasite control, nutrition etc. Any of the of alternative approaches will likely be linked to other international efforts currently underway as the licensing and regulation of the technology will have to be demonstrated to work under Canadian conditions and would have to be consistent with the relative level of risk that is currently accepted by Canadian resource managers.

Bacteriophage therapy

Bacteriophages, viruses that specifically target bacteria, are one of the most promising options for controlling bacterial diseases in farmed fish. Bacteriophages, as viruses, consist fundamentally of a genome (DNA or RNA) encased inside a protective coat known as the *capsid,* which is made of proteins and/or lipoproteins (Kutter and Sulakvelidze 2004). They lack the cellular machinery required to reproduce their genetic information or synthesize proteins for their protective capsids, and so must hijack a host organism’s machinery to do so (Kutter and Sulakvelidze 2004). In lytic/virulent phages, this results in the death of the host (Kutter and Sulakvelidze 2004). Bacteriophages are the single most abundant group of organisms on Earth and virulent phages play a vital role as predators in bacterial ecosystems (Kutter and Sulakvelidze 2004).

Bacteriophages can be classed into two groups—*lytic/virulent* and *temperate/lysogenic* (Oliveira et al. 2012). A lytic or virulent phage is one that requires a *lytic cycle* in order to reproduce. At the beginning of the lytic cycle the virus invades the host cell, releases its genome, and interrupts host metabolism, DNA replication and protein synthesis (Kutter and Sulakvelidze 2004). The virus may also contain proteins that destroy the host genome and enzymes or tamper with the membrane (Kutter and Sulakvelidze 2004). Once the virus has been replicated, the host cell lyses, or bursts, and releases new phages into the environment to continue the cycle (Kutter and Sulakvelidze 2004). There also exist temperate or lysogenic phages, which

have the option to live in a host cell indefinitely in a *lysogenic cycle*, where the viral genome is integrated into the host genome or remains in the cell in the form of a plasmid (Oliveira et al. 2012). These dormant viral genomes, known as *prophages*, are quietly reproduced as the host cell replicates itself, and may under the right conditions be activated and enter the lytic cycle (Kutter and Sulakvelidze 2004). Bacteriophages being studied for use in medicine are generally virulent bacteriophages (Oliveira et al. 2012).

The most common bacteriophages are *tailed phages*, which have a "tail" of fibrous proteins attached to their protein coats for use in infecting the host cell. This process is known as *adsorption* (Kutter and Sulakvelidze 2004). The virus recognizes and binds to a specific molecule on the host cellular surface—virtually any kind of membrane molecule can be an unwitting receptor for a phage (Kutter and Sulakvelidze 2004). After phage tail fibres bind to these receptors, enzymes in the tail tip degrade the bacterium's protective peptidoglycan layer and the phage genome is injected into the cell (Kutter and Sulakvelidze 2004). The virus contains *phage promoters* which recognize the host RNA polymerase and compel it to transcribe viral *early genes*, which code for proteins that will arrest host DNA replication and inactivate host defensive mechanisms such as proteases (Kutter and Sulakvelidze 2004). As mentioned above these proteins may have destructive properties to host nucleic acid, proteins or membranes (Kutter and Sulakvelidze 2004). The cell is then reprogrammed to synthesize genetic material and proteins to build new phages. When conditions are right, viral proteins known as *lysins* and *holins* will compromise the host membrane and peptidoglycan layer, killing the host allowing new phages to escape into the environment (Kutter and Sulakvelidze 2004).

Each species of bacteriophage specializes on a specific strain or strains of a host species (Kutter and Sulakvelidze 2004). This suggests that phage cocktails can be developed to target only a specific organism without seriously targeting host natural flora, which gives them potential as a means of treating infectious diseases in human and veterinary medicine. This specificity also means that the first step in developing a phage treatment is to positively identify the bacterium acting as the causative agent of disease (Oliveira et al. 2012). Subsequent steps include isolating phages in the bacteria's surroundings, many purification cycles, genetic characterization of the phages, establishing virulence against the target bacteria and testing its efficacy both in the laboratory (on plates and in tanks) and in the field (Oliveira et al. 2012).

Phage applications in aquaculture

Bacteriophage treatments for farmed fish have been studied extensively over the past 20 years, and the first were implemented against *Lactococcus garvieae* in Japan in 1999 (Wang et al. 2017b). A few experiments on different farmed organisms and pathogens are summarized below, giving some insight into the advantages and disadvantages surrounding the issue.

Case Study 1

Vibrio is a family of common fish pathogens that causes high levels of mortality when infections get out of control (Table 1). Different species of *Vibrio* are pathogenic against a wide variety of aquatic animals, from oysters to salmon (Wang et al. 2017b). *V. harveyi* is a species of *Vibrio* found to infect many species of farmed invertebrates including oysters, shrimp and lobster. Bacteriophage treatments have been tested and found to be effective against *V. harveyi,* with several different phages found to have virulence against dozens of strains. In a recent study, a sample of *Vibrio harveyi* was used to amplify bacteriophages isolated from diseased abalones (Wang et al. 2017b). Two lytic bacteriophages were identified and named vB_VhaS-a and vB_VhaS-tm (Wang et al. 2017b). Both phages share similarities with previously identified *Vibrio* phages (Wang et al. 2017b). Eight strains of *V. harveyi* were cultivated with the isolated and amplified phages in order to determine which of them could be targets for the virus (Wang et al. 2017b). vB_VhaS-tm was found to have virulence against all eight *V. harveyi* strains tested,

while vB_VhaS-a was virulent against six out of eight strains tested (Wang et al. 2017b). When abalones infected with *V. harveyi* were treated with a cocktail containing vB_VhaS-tm, the survival rate was 70%, compared to 0% of the group infected with *V. harveyi* and not treated (Wang et al. 2017b). The phage and broth control groups had no effect on survival, suggesting that the phage cocktail was the cause of the increased survival in the diseased fish (Wang et al. 2017b).

Case Study 2

V. harveyi has also been successfully treated with another broad-range bacteriophage (effective against 50 tested *V. harveyi* strains) in larval shrimp in a laboratory environment. This treatment lead to a survival rate of 80% in comparison with the control group survival of 25% (Vinod et al. 2006). These promising results led to the treatment being tested in a shrimp hatchery, where 35000 shrimp larvae were treated for a naturally occurring *V. harveyi* outbreak (Vinod et al. 2006). Two other groups were also tested in the study (Vinod et al. 2006). One group was given the standard antibiotic treatment, and another group received no treatment (Vinod et al. 2006). The experiment took place over 17 days, and results can be viewed below (Fig. 7). The phage treatment had a significantly higher rate of survival than the antibiotic group and the control group (Vinod et al. 2006).

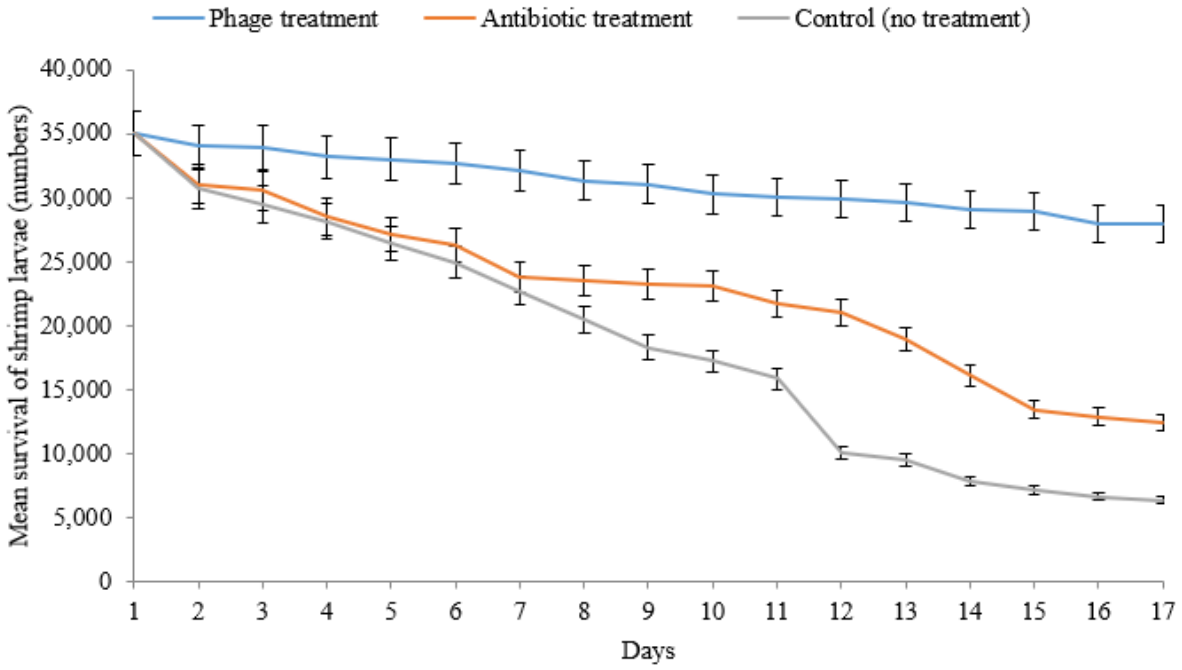

Figure 7. Survival of larvae during treatment of a V. harveyi outbreak in a shrimp hatchery, based on whether they were given the bacteriophage treatment, the antibiotic treatment, or no treatment (Vinod et al. 2006).

Case Study 3

V. harveyi is not the only pathogen against which bacteriophage treatments have been successful. In Indian freshwater aquaculture, a B-lactam antibiotic called carbapenem is usually used to treat infections of the common fish pathogen *Pseudomonas aeruginosa* (Table 1), as it is immune to most B-lactamases (Khairnar et al. 2013) (B-lactam antibiotics require a molecular feature called the *B-lactam ring* to be effective. *B-lactamases* are bacterial enzymes that cleave this ring, granting resistance against the antibiotic). However, recent *P. aeruginosa* outbreaks

have developed genes coding for a *metallo-B-lactamase* (MBL), which *is* effective against carbapenem, and as a result the usual treatment has been rendered ineffective (Khairnar et al. 2013). Alternative antibiotic treatments exist, but are very expensive or have severe negative health consequences for fish (Khairnar et al. 2013). The MBL gene is also plasmid-mediated and spreading rapidly among bacterial strains (Khairnar et al. 2013). As a result, an alternative to the standard treatment is desperately needed. As bacteriophages targeting a bacteria can be found in large numbers wherever their hosts gather (Kutter and Sulakvelidze 2004), wastewater from these fish farms were used in order to isolate phages specific to *P. aeruginosa* (Khairnar et al. 2013). Twenty infected catfish were infected with antibiotic-resistant *P. aeruginosa* and the phage cocktail was tested in a lab environment (Khairnar et al. 2013). Ten of these had the phage cocktail applied to their skin lesions, while ten had a diluent applied as a controls (Khairnar et al. 2013). It was found that this phage therapy did lead to reduced skin lesions in fish suffering from a *P. aeruginosa* infection compared to the control group (Khairnar et al. 2013).

Case Study 4

The fish pathogen *Streptococcus parauberis* is a Gram-positive, non-motile coccus usually treated with erythromycin, amoxicillin or florfenicol (see Table 1), but resistant strains (including multi-drug resistant strains) are becoming more and more common (Kwon et al. 2017). In the hopes of isolating a bacteriophage specific to this pathogen, a confirmed *S. parauberis* isolate was cultivated with filtrate of homogenized tissue samples from diseased fish. Similarly to the example described above, the purpose of this was to find viruses gathering in a host-rich environment. After repeated cultivation, bacteriophage purification and DNA analysis the novel bacteriophage Str-PAP-1 was identified (Kwon et al. 2017). To test its potential as a treatment, 55 *S. parauberis* strains were isolated from an infected fish farm for use in the lab, along with isolates of 5 other common fish pathogens for comparison purposes (one isolate each of *Lactococcus garvieae, Streptococcus inae, Vibrio anguillarum, Vibrio ichthyoenteri,* and *Edwardsiella ictaluri* were used in the study) (Kwon et al. 2017). Of the 55 *S. parauberis* isolates tested in the study, the growth of 35 was inhibited by Str-PAP-1 (Kwon et al. 2017). The virus did not show virulence against any of the other five bacteria (Kwon et al. 2017). When fish were given Str-PAP-1-medicated feed, a lower abundance of *S. parauberis* was detected in their organs at the end of the study, and infection and mortality from the disease was reduced (Kwon et al. 2017). Unexpectedly, fish growth was also enhanced when feed was medicated with Str-PAP-1 (Kwon et al. 2017), suggesting it as an alternative to prophylactic antibiotics. There were hopes that Str-PAP-1 would also have effectiveness against the other fish pathogens tested in the study, was found to only be virulent against *S. parauberis* (Kwon et al. 2017).

As shown by these promising results, there is a strong case for bacteriophage treatment, with several advantages over antibiotics. Additional resources are currently required to research bacteriophages, but once treatments are developed they could potentially be cheaper than antibiotic treatments (Oliveira et al. 2012), especially since phages quickly multiply when provided with hosts (Madhusudana Rao and Lalitha 2015). The environmental impact of phage treatments is believed to be lower, as phages are already abundant in the environment (Madhusudana Rao and Lalitha 2015). The specificity of phages to their targets is likely their greatest advantage over antibiotics, as their effects are restricted to certain organisms (Oliveira et al. 2012)—this stands in contrast to the action of broad-spectrum antibiotics, which can seriously damage the microbiome of an animal or environment (Madhusudana Rao and Lalitha 2015). Also contrary to antibiotics, this specificity also makes them safe to consume if they are present in food (Madhusudana Rao and Lalitha 2015).

Drawbacks

However, there remain several disadvantages to be considered when developing bacteriophages for use in aquaculture. Inefficient feeding of sick animals due to reduced appetite makes it less feasible to administer phage therapy through feed (Oliveira et al. 2012). Injections are a more direct route, but are not practical in cases of large fish farms with hundreds of thousands or even millions of animals to treat (Oliveira et al. 2012). Phages are strain-specific rather than species-specific, which limits their efficacy and requires the identification and isolation of bacteriophages against numerous bacterial variants (Oliveira et al. 2012) in order to constantly update the phage libraries. While some phages are effective against up to 90% of strains of a species of bacteria, others are only effective against a few (Oliveira et al. 2012). This disadvantage can be negated by delivering several phages at once in a cocktail, but the identification of a bacteriophage virulent to each strain is a time consuming process that requires expertise and equipment (Oliveira et al. 2012). Thirdly, bacteriophages that behave in a virulent fashion in the lab may become lysogenic in the harsher conditions of the field, requiring extensive *in vivo* tests before a treatment could be considered for wider implementation (Oliveira et al. 2012). Fourthly, there are concerns that a lysogenic phage could transfer antibiotic resistance genes to a bacterium, or that a lytic phage could cause the release of these genes in the environment to be taken up by pathogens via transformation (Madhusudana Rao and Lalitha 2015).

Finally, similar to antibiotics, bacteria are able to gain resistance to bacteriophages by developing genes that code for phage-neutralizing antibodies or by mutating to lose the surface molecules the phages use as receptors (Oliveira et al. 2012). However, lytic phages kill their hosts before new viruses escape into the environment, making it less of a concern that a resistant bacterium will multiply (Oliveira et al. 2012). Giving higher doses of a phage also negates the effects of phage-neutralizing antibodies (Oliveira et al. 2012), and giving multiple phages in cocktails accounts for strains that are resistant to a particular phage (Madhusudana Rao and Lalitha 2015). Phage resistance in bacteria can also be tackled by combining phage treatment with antibiotic treatment (Madhusudana Rao and Lalitha 2015). Therefore, this should be considered to be an ongoing battle for cultured organism health and the maintenance of physiological stasis.

Quorum Sensing Inhibitors

Quorum sensing (QS) is a phenomenon discovered in bacteria which represents a method of intercellular communication between individuals of a species and possibly other species in the biofilm community. This communication system enables bacteria to undertake processes that are costly and non-effective when cell densities are low, but become much more effective at the community level when cell densities are high such as during periods for virulence factor synthesis, biofilm formation, and protease and siderophore production. A QS system consists of QS signal molecules and regulatory protein components that can control physiological behaviors and virulence gene expression of bacterial pathogens (Remy et al. 2019, Zhao et al. 2019). The premise is that if this QS system can be inhibited, then the exponential rise of pathogen numbers in the microbiome of the organism will not happen. Populations of bacteria are naturally controlled in nature by other bacteria and this feature has led to the exploration for inhibitors that may be put to use in human industries and healthcare. A review of the subject shows that there are several marine species that produce quorum sensing inhibitors, either small molecules or inhibitor enzymes (Zhao et al. 2019). The authors suggest that this approach would be potentially valuable for applications in food preservation, aquaculture, human healthcare, ecological protection, etc., by controlling QS-mediated food spoilage and inhibiting QS-mediated biofilm formation and virulence of pathogens.

Quorum sensing inhibition has been shown to occur in the digestive systems of fish. A Chinese study with goldfish showed that a quorum quenching (quorum sensing inhibition) enzyme decreased the percentage of pathogenic bacteria in the gut (Zhou et al. 2016). The authors suggested that quorum quenching probiotics may be useful as a non-antibiotic feed additive that could control bacterial diseases in aquaculture. Other studies that have examined quorum sensing inhibition have shown that it can be effective in human treatments for the pathogen *Burkholderia* (Koch et al. 2014) and also in plants (Helman and Chernin 2015).

In summary, quorum sensing inhibition seems to be a field that is developing rapidly and that may have a future role to play in strategies to combat bacterial infections in both a healthcare perspective as well as for industrial processes.

Vaccines

Vaccination is the process of exposing an organism to *antigens* (defined below) associated with a pathogen against it in order to trigger an immune response. The method of exposure varies by the type of vaccine, but the common thread in all types is triggering the immune system to produce antibodies and memory cells against a pathogen in order to enable it to effectively fight the pathogen off in the case of a future infection (HHS 2017).

The processes of the vertebrate immune system are explained in more detail below in the *Immunostimulants* section, but a few concepts are required for a basic understanding of how vaccines work. When the body detects a foreign invader, it sends cells known as *macrophages* to engulf and destroy it (NIAIDS 2012). The macrophage will then expose, or present, an *antigen* of that organism (molecule present on the surface of the pathogen that distinguishes it from other organisms) on its own surface, which will be recognized by other immune cells called *B* and *T* cells. The T cells either modulate the activity of other immune cells or kill cells that have been infected by the organism carrying the antigen, and the B cells will begin producing *antibodies* against the antigen. Antibodies recognize the antigen on the surface of the pathogen and attach to it, eventually covering the invading organism and preventing it from functioning or attacking host cells. Antibodies also signal to immune cells to come and destroy the invader. After the pathogen is gone, some B and T cells involved in fighting it will be converted to *memory cells*, which can quickly divide and resume their functions if the pathogen is encountered again.

The purpose of a vaccine is to simulate an infection to allow the recipient to produce antibodies and memory cells in advance of an encounter with the pathogen. Types of vaccines include live-attenuated vaccines (where the pathogen is administered alive and disabled), inactivated vaccines (where the pathogen is administered dead), subunit vaccines (where parts of the pathogen that the body will recognize as foreign are administered, such as surface proteins), and toxoid vaccines (where a harmful product secreted by the pathogen is administered) (HHS 2017). Vaccines can also be monovalent, divalent or multi-component, depending on how many pathogens are included.

As fish are very simple vertebrates with immune systems that rely heavily on the action of macrophages, the development of vaccines for them must take this into consideration (Dadar et al. 2017). Other concerns include how a vaccine will be administered to huge populations of fish in sea cages—in-feed, immersion and injection routes have all been explored (Dadar et al. 2017). In most cases, the vaccines used in aquaculture contain dead/inactivated pathogens, which are produced by exposing large numbers of the organism to a lethal substance such as formalin that will kill the microbes without destroying their identifying molecules/antigens (Dadar et al. 2017). Inactivated vaccines are also safer than live/attenuated vaccines in many cases (HHS 2017), so long as care is taken when the pathogens are replicated and killed (Dadar et al.

2017). Common fish pathogens (which are elaborated upon in Table 1) that are the subjects of inactivated vaccines include several species of *Vibrio* including *Vibrio salmonicida,* as well as *Aeromonas salmonicida* and *Yersinia ruckeri.* One disadvantage of inactivated vaccines is that they must be delivered in an injection, which is very time consuming when it comes to large numbers of fish (Dadar et al. 2017). They also generally require multiple doses to be effective, which exacerbates this issue (HHS 2017). Attenuated vaccines also show potential in aquaculture. They generally give a higher level of protection when compared to inactivated vaccines, and do not have to be injected (Dadar et al. 2017). However, they require extensive testing before implementation to ensure that the weakened pathogen is not able to cause a full-scale infection (Dadar et al. 2017). Finally, vaccines can be *homologous* or *heterologous*. In a homologous vaccine, the pathogen being introduced is the target, while in a heterologous vaccine one organism is introduced to grant immunity to another (Kapczynski et al. 2017).

Vaccines have been explored as a method for preventing disease in aquaculture since 1938, when carp were injected with inactivated *Aeromonas punctata,* resulting in immunity to the disease (Dadar et al. 2017). With the technology available at the time, however, this route was considered too time-consuming for wider application (Dadar et al. 2017). Vaccines did not begin to be licensed for use in aquaculture until the 1970s, and have been widely implemented since 1990s (Dadar et al. 2017). There has been massive success in reducing the use of antibiotics via vaccines in Norway (see below). There have also been great reductions in fish morbidity and mortality where vaccines have been implemented (Dadar et al. 2017).

The Norwegian aquaculture sector has been using vaccines to reduce the amount of antibiotics used to treat farmed fish since 1987, with great success (Midtlyng et al. 2011). By 2016, only 212 kg of active substance was sold in Norway for the antibiotic treatment of farmed fish (Fig. 8). These results come as a result of collaboration between government and fish farmers to vaccinate fish against the common pathogen classical furunculosis and focused efforts towards quality vaccine development by the pharmaceutical industry, combined with non-vaccine initiatives including mandatory yearly fallowing and spatial planning of fish farming sites to minimize the spread of disease (Midtlyng et al. 2011).

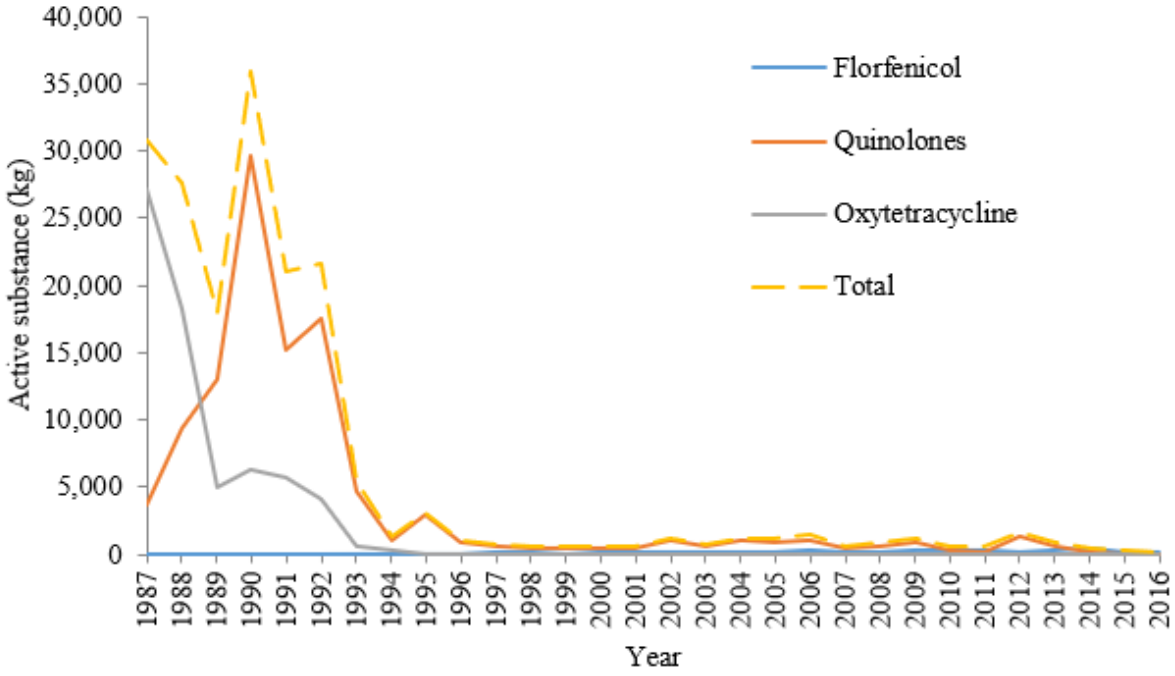

Figure 8. Antimicrobials sold for use in Norwegian aquaculture by category, 1987-2016 (kg active substance) (Olsen 2005; Public Health Institute 2015).

While Norway has had the most success regarding vaccinating farmed fish in order to reduce antimicrobial usage, efforts are also ongoing around the world. Vaccines are available in 40 countries for 17 species of farmed fish and have been developed against 22 bacterial and six viral pathogens (Brudeseth et al. 2013). Nonetheless it remains an uncommon practice in countries such as China, which has a high level of unregulated, unreported antimicrobial use in its aquaculture sector (Brudeseth et al. 2013). Canada, Chile and the United Kingdom have all implemented large-scale programs for vaccinating fish against common pathogens (Brudeseth et al. 2013). In order to show a general example for the procedures used around the world, vaccination procedures for Atlantic salmon are summarized in the table below.

Table 4. Vaccination efforts for Atlantic salmon by country (Brudeseth et al. 2013).

Country	Most common diseases	Vaccination procedures
Norway	Classical furunculosis, infectious salmon anemia, vibriosis, cold water vibriosis and winter ulcer	Six component water/oil based vaccine for the aforementioned six diseases injected into salmon smolts at the freshwater site. About 30% of smolts also receive a monovalent vaccine against infectious pancreas necrosis.
Chile	Furunculosis, vibriosis, piscirickettsiosis, IPN and ISA	Five-component water/oil-based vaccine against the diseases mentioned in the previous column, with occasional oral vaccination against piscirickettsiosis or monovalent immersion vaccine against IPN at farmers' discretion.

Country	Most common diseases	Vaccination procedures
Canada	Classical furunculosis, infectious salmon anemia, infectious hemorrhagic necrosis vibriosis and cold-water vibriosis, enteric redmouth	East coast: water/oil based vaccination against furunculosis, ISA and three kinds of vibriosis. West coast: same as above, with an additional inter-muscular plasmid vaccine against IHN. There is also an immersion vaccine against enteric redmouth (ERM).
USA	Classical furunculosis, enteric red mouth, infectious salmon anemia, vibriosis and cold-water vibriosis	Similar to Canadian vaccination practices on both coasts, but no vaccine against IHN is licensed in the United States. Salmon fry receive an additional vaccine against ERM and furunculosis.
United Kingdom	Infectious pancreatic necrosis, classical furunculosis	Water/oil based divalent vaccination against furunculosis and IPN, as well as monovalent vaccine against pancreatic disease
Faroe Islands	Classical furunculosis, Infectious salmon anemia, infectious hemorrhagic necrosis, vibriosis, cold-water vibriosis, winter ulcer	National contingency plan to vaccinate farmed fish against ISA. Over 90% of smolts vaccinated with a 7 component vaccine against common bacterial and viral diseases.
Australia/New Zealand	Amoebic gill disease, enteric redmouth	Vaccines are still in development and use is not currently licensed. However, New Zealand is using a vaccine for enteric redmouth.

The efficacy of vaccines in reducing fish loss to bacterial diseases has been documented worldwide (especially in the Norwegian sector) but also in laboratory environments. A few cases are summarized here, relating to the process of development of vaccines against *Streptococcus iniae*, a marine pathogen responsible for heavy losses in farmed fish, especially tilapia (Klesius et al. 2000). As summarized in Table 1, the pathogen attacks the central nervous system of the fish, causing up to 50% mortality and massive economic loss in some cases (Low et al. 1999, Baiano and Barnes 2009). Due to rising resistance in recent years antibiotic treatment is becoming less and less effective, highlighting the need for an alternative method of preventing and/or treating the disease (Klesius et al. 2000).

There is an additional sense of urgency in the case of this disease, as *S. iniae* is also an opportunistic human pathogen—while it primarily targets fish it will also infect humans given the opportunity (Low et al. 1999). In one case, the bacterium was isolated from human patients suffering from meningitis and cellulitus (Bachrach et al. 2001). The transfer from fish to human hosts has been shown to occur when a person with bare hands prepares fresh, raw fish for cooking (Weinstein et al. 1997).

Case study 1

Tilapias in a laboratory environment were injected with the vaccine to be tested. Two vaccines for *S. inae*, one homologous and one heterologous, were administered to fish in a tank

environment both by intraperitoneal and intramuscular routes. The vaccinated fish were found to have a statistically significantly lower mortality than the unvaccinated fish in all trials, with mortality ranging from 4% to 63% compared with the 79% accumulative mortality of the unvaccinated fish. The most effective vaccination procedure was the heterologous vaccine given intraperitoneally (Klesius et al. 2000).

Case study 2

S. iniae continues to be a problem in aquaculture in regions including Israel and North America (Bachrach et al. 2001). A study examines rainbow trout from Israeli fish farms, which were all vaccinated against *S. iniae* from 1995-1997 resulting in a 50% reduction of mortality to approximately 5% (Bachrach et al. 2001). However, 1997 reported a new outbreak and increase in mortality in Israeli fish farms, with the sick fish exhibiting symptoms more serious than those in previously reported *S. iniae* outbreaks (Bachrach et al. 2001). Samples were taken from the fish, isolated and underwent DNA analysis, biochemical testing and morphological observation (Bachrach et al. 2001). It was concluded that the organism responsible for this outbreak was indeed *S. iniae*, albeit a new strain against which the 1995 vaccine was not effective (Bachrach et al. 2001). As the vaccine was unable to completely eradicate the pathogen from large, densely packed fish stocks, a second, formerly less prominent serotype was able to take over, rendering the vaccine ineffective (Bachrach et al. 2001). This incident suggests that fish that are being vaccinated should be monitored for new strains of previously common pathogens, in order to anticipate and hopefully develop vaccines or treatments to prevent outbreaks such as the one described above (Bachrach et al. 2001).

Case study 3

A more recent study, in 2017, details the testing of a new vaccine (henceforth referred to as pEno) against *S. inae* in Nile tilapia (Kayansamruaj et al. 2017). This vaccine was intended to prevent outbreaks such as the one described in the Bachrach study above. DNA vaccines are lauded for their ability to trigger both a cellular and humoral immune response, in order to hopefully defend against a range of bacterial strains (Kayansamruaj et al. 2017). A-enolase, which is a surface protein highly conserved across a broad range of streptococcal species, was chosen as a vaccine target for this reason (Kayansamruaj et al. 2017). The vaccine contained sequences of DNA associated with this feature (Kayansamruaj et al. 2017). The group of fish vaccinated with pEno enjoyed a 72.5% survival rate, compared to 40% and 25% in the mock vaccination and control groups (Kayansamruaj et al. 2017).

In summary, vaccines have already had success and continue to show promise for reducing antimicrobial usage in aquaculture worldwide, and as a result reducing the exposure of bacteria in the environment to compounds that would lead to the propagation of ARGs. More research should go into the development of vaccines for farmed fish, especially those targeted towards features that are conserved across multiple bacterial strains in order to prevent unexpected outbreaks from less common serotypes.

Bacterial biocontrol and probiotics

In order to survive in a harsh environment with a diverse community of microorganisms, many species of bacteria have developed the ability to produce metabolites that kill or inactivate other bacteria. This feature stems from natural selection and exists to help bacteria overcome potential predators or competitors for nutrients. As a result, there exist species of bacteria that can be used to kill or weaken other species. Attempting to harness this ability for use in controlling pathogens is called *bacterial biocontrol.*

Bacteriocins are one example of these bacterially produced products. They are proteins that attach to receptors on the cell wall of a sensitive bacterium and are absorbed inside the cell (similarly to a bacteriophage), where they then damage the recipient's cellular machinery: some punch holes in the inner membrane, destroy genetic material, or inhibit metabolic processes (Madhusudana Rao and Lalitha 2015). The use of pure bacteriocins is either not a feasible option for the control of bacterial diseases in aquaculture, or requires extensive future research before such a treatment becomes a possibility (Madhusudana Rao and Lalitha 2015). They have a very narrow range of specificity, usually restricted to species very similar to the producer, and the cultivation of bacteria to collect these proteins is not financially practical (Madhusudana Rao and Lalitha 2015). However, the production of bacteriocins is one indicator of a species' potential as a *probiotic* (Madhusudana Rao and Lalitha 2015).

Probiotics are beneficial microbes that are directly introduced to a host (Sihag and Sharma 2012). This includes those introduced in order to inhibit the growth of pathogens in that organism's system (Sihag and Sharma 2012). They have been successfully used to treat human gastrointestinal disease in the past (Sihag and Sharma 2012) and as alternatives to antibiotics in aquaculture (Newaj-Fyzul and Austin 2015). Bacteria and fungi can both be used as probiotics (Sihag and Sharma 2012), and a wide range of organisms have been tested in aquaculture. They have several possible strategies to interfering with the growth and function of harmful microorganisms, including the production of metabolites harmful to pathogenic bacteria such as antibiotics, lysozymes, proteases and the aforementioned bacteriocins (Sihag and Sharma 2012). They may also compete with pathogens for nutrients such as iron, for available energy, or for adhesion sites in the gut of the host (Sihag and Sharma 2012). Probiotics may also fortify the natural processes of an organism, by promoting thickening of the epidermis, increasing digestive function, reducing stress or aiding metamorphosis (Newaj-Fyzul and Austin 2015). Finally, there is evidence for probiotics enhancing the host immune response, cleaning water by converting organic matter (such as discarded feed) to CO_2, producing vitamins or other helpful compounds, and inhibiting the growth of algae or other fouling agents (Sihag and Sharma 2012). The action of some probiotic agents are summarized in the table below.

Advantages to the use of probiotics include that they may be produced locally and cost-effectively-- given adequate and appropriate nutrients, microbes will quickly multiply (Newaj-Fyzul and Austin 2015). Other benefits include growth enhancement and disease resistance (presenting an alternative to prophylactic antibiotic treatment) and an overall reduction in the need for chemical treatments (Newaj-Fyzul and Austin 2015). However, there is a lack of solid evidence that probiotics are actually harmless to the host, as research in this area has been sparse (Newaj-Fyzul and Austin 2015). There are financial concerns around how long probiotic bacteria can be stored in feed before beginning to die and lose their effectiveness (Newaj-Fyzul and Austin 2015). There are also concerns regarding the possibility of probiotic bacteria acquiring virulence or antibiotic resistance genes from pathogenic bacteria in the gut of the host (van Reenen and Dicks 2011). In one case laboratory tests identified antibiotic resistance genes in several organisms used as probiotics in aquaculture, with particularly high rates of resistance in the *Enterococcus, Lactobacillus, Weissella* and *Pediococcus* genera (van Reenen and Dicks 2011).

Table 5. Probiotics, their targets and their benefits for the host organism.

Probiotic	Type of bacterium	Target organisms	Method of action	Ref.
Bacillus amyloliquefaciens	Gram-positive, rod shaped. Extremely similar to *Bacillus subtilis*	*Aeromonas hydrophila,*	Produces extracellular products to inhibit the growth of the target.	(Cao et al. 2011)
Pseudoalteromonas S2V2	Gram-negative, rod-shaped.	*Vibrio* sp.	Produces compounds with antibacterial activity including H_2S, gelatinases, oxidases and catalases. Also produces an unknown, non-proteinaceous antibiotic against *Vibrio.*	(Isnansetyo et al. 2009)
Bacillus coagulans	Lactic acid-producing, gram positive, rod-shaped.	*Vibrio vulnificus, Enterococcus faecalis, Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Klebsiella oxytoca,* and *Pseudomonas aeruginosa*	Modulates immune response, enhances expression of beneficial genes, and produces antibacterial compounds including lactic acid.	(Pan et al. 2012)
Bacillus subtilis	Gram-positive, rod shaped. Extremely similar to *Bacillus amyloliquefaciens*	*Aeromonas* sp.	Improved respiratory burst and lysosomal activity. Enhanced innate immunity. Production of antimicrobial compounds including peroxidase and a1-protease.	(Newaj-Fyzul et al. 2007)
Brevibacillus brevis	Gram-positive, rod shaped.	*Vibrio sp.*	Improves digestion,	(Mahdhi 2012)

Probiotic	Type of bacterium	Target organisms	Method of action	Ref.
			competes with pathogen, reduces stress response	
Enterococcus gallinarum	Lactic acid-producing Gram-positive coccus	*Vibrio anguillarum*	Production of lactic acid, acetic acid and ethanol, which have antimicrobial properties.	(Sorroza et al. 2013)
Lactococcus lactis	Lactic acid-producing Gram-positive coccus	*Streptococcus iniae, Streptococcus parauberis, Enterococcus viikkiensis* and *Lactococcus garviae*	Modulates immune response for increased lysosomal activities and production of antibodies.	(Kim et al. 2013)
Pediococcus pentosaceus	Lactic acid-producing Gram-positive coccus	*Vibrio anguillarum*	Increases production of red and white blood cells. Enhanced phagocytic activity in the head and kidney regions. Production of lactic acid.	(Huang et al. 2014)
Pseudomonas M162 and M174	Gram-negative, rod shaped	*Flavobacterium psychrophilum*	Compete with *F. psychrophilum* for iron when resource is scarce, increases respiratory burst activity.	(Korkea-aho et al. 2011, Korkea-aho et al. 2012)

Immunostimulants

Every living organism has mechanisms to defend it against invasion from foreign agents. In vertebrates, this takes the form of innate/humoral and specific/acquired immunity. Innate immunity is the first line of defense, and refers to any immune action that is not adapted to fight off a specific pathogen (Hoseinifar et al. 2015). This includes the action of cells known as monocytes/phagocytes (which will locate and eat invading pathogens), neutrophils and natural killer cells (Hoseinifar et al. 2015). This aspect of the immune system is almost immediately triggered after the body detects the presence of *pathogen-associated molecular patterns*, or molecules that the body will recognize as foreign because they do not typically occur in eukaryotic organisms (Hoseinifar et al. 2015).

The unnatural conditions in which farmed fish are kept—in static, densely populated sea cages, along with handling by staff (Burrells et al. 2001)—result in stress to the animals and a lowered immune response (Vaseeharan and Thaya 2013). This causes the fish to have a heightened vulnerability to infections and a lowered ability to defend against them (Vaseeharan and Thaya 2013). There have been motions toward combatting disease in farmed fish by giving immunostimulants to counter this. An *immunostimulant* is any substance that induces or increases an immune response in the recipient (Divyagnaneswari et al. 2007). The heavy reliance of fish on their innate immune system (Divyagnaneswari et al. 2007) means that immune stimulation could aid in the animal's defense to a wide range of pathogens. Immunostimulants have also been shown to aid in fish growth and feeding (Nya and Austin 2009b), and have been implemented as an alternative to prophylactic antibiotics (Logambal et al. 2000).

There have also been studies into the use of medicinal plants for their antimicrobial properties. Many common plants, including garlic and cinnamon, have been shown to inhibit the growth of bacteria due to properties of the extracellular substances they produce.

There have also been studies of immunostimulants being given in combination with a vaccine in order to enhance and prolong the recipient's immune response (Logambal et al. 2000). It has been found that in most cases, response is maximized when the immunostimulant is given shortly before the vaccination, suggesting that the immunostimulant prepares the organism to respond to a vaccine (Logambal et al. 2000).

Another advantage to the use of medicinal plants is that they are biodegradable and may as a result not linger in the environment to the extent that antibiotics do (Logambal et al. 2000). They are also safer for consumption in the event that they make their way into fish sold for food, as many of them are also used in cooking (Dorucu et al. 2009). The extensive cultivation of these plants make them less expensive to acquire than many antibiotic drugs (Dorucu et al. 2009).

Table 6. Immunostimulants used in aquaculture and their methods of action.

Immunostimulant	Characteristics	Method of action	Ref.
Garlic *(Allivum sativum)*	Type of onion.	Increases fish resistance to *A. hydrophila.* Enhances the multiplication of erythrocytes and leukocytes as well as phagocytic, respiratory burst, anti-protease and lysozyme activities. The plant is also observed to inhibit the growth of bacteria.	(Nya and Austin 2009b)
Ginger (*Zingiber officiale)*	Root of flowering plant.	Shows potential as a prophylactic due to positive effects on fish growth. Also functions as an appetizer to improve feeding efficiency, which results in enhanced growth. Contains antimicrobial compounds gingerol and	(Nya and Austin 2009a, Punitha et al. 2008)

Immunostimulant	Characteristics	Method of action	Ref.
		camphene. Innate immune responses are enhanced.	
Black cumin (*Nigella sativa*)	Seeds of flowering herb.	Specific immunity is stimulated, resulting in increased production of immunoglobulins and serum proteins. Has a weaker effect than many of the other immunostimulants discussed in this paper, but shows potential as a prophylactic feed additive.	(Dorucu et al. 2009)
Dietary nucleotides	Building blocks of DNA or RNA.	Increase resistance to bacterial disease including *V. anguillarum* and *P. salmonis.*	(Burrells et al. 2001)
Cinnamon (*Cinnamosma fragrans*)	Inner bark of tree	Contains *cinnamaldehyde*, a chemical compound with antimicrobial activity against *S. inae.*	(Randrianarivelo et al. 2010, Rattanachaikunsopon and Phumkhachorn 2010)
Vitamin D_3	Fat-soluble steroid also known as cholecalciferol	Increases activity of phagocytic cells and other aspects of the innate immune system, with very little to no effect on the specific immune system.	(Cerezuela et al. 2009)
Vitamin C	Ascorbic acid	Few benefits seen to diets with increased vitamin C, but consequences recorded when depleted amounts are given. Fish with depleted levels of vitamin C suffer increased mortality when infected with *Aeromonas salmonicida,* suggesting that feed could be supplemented with Vitamin C if there are concerns that fish are not receiving adequate levels.	(Hardie et al. 1991)
Bermuda grass (*Cynodon dactylon)*	Warm-season grass.	Shows potential as a prophylactic due to positive effects on fish growth. Increases production of albulin, globulin, cholesterol, glucose, triglycerides and several important proteins. Phagocytic	(Punitha et al. 2008, Citarasu et al. 2006)

Immunostimulant	Characteristics	Method of action	Ref.
		and lysozyme activity enhanced. Contains antimicrobial substances cynodin, hydrocyanic acid and triticin. Slightly reduces mortality in fish when challenged with *Vibrio vulnificus.*	
Indian bael (*Aegle marmelos)*	Fruit-bearing tree.	Shows potential as a prophylactic due to positive effects on fish growth. Increases production of albulin, globulin, cholesterol, glucose, triglycerides and several important proteins. Phagocytic and lysozyme activity enhanced. Slightly reduces mortality in fish when challenged with *Vibrio vulnificus*	(Citarasu et al. 2006)
Winter cherry (*Withania somnifera)*	Fruit-bearing plant related to deadly nightshade.	Shows potential as a prophylactic due to positive effects on fish growth. Increases production of albulin, globulin, cholesterol, glucose, triglycerides and several important proteins. Phagocytic and lysozyme activity enhanced. Slightly reduces mortality in fish when challenged with *Vibrio vulnificus*	(Citarasu et al. 2006)
Thoodhuvalai *(Solanum trilobatum)*	Flowering herb.	Both water and hexane soluble fractions found to enhance nonspecific immunity via production of reactive oxygen species, production of reactive nitrogen species and activity of serum lysozyme. Water-soluble fractions are significantly more effective. Both fractions increase fish resistance to *A. hydrophila.*	(Divyagnaneswari et al. 2007)

Immunostimulant	Characteristics	Method of action	Ref.
Holy basil (*Ocimum sanctum)*	Flowering herb.	Leaf extract contains water soluble phenolic compounds and other substances that are believed to stimulate primary and secondary antibody response in the weeks after administration. The length of the primary response is increased, and the lag period between primary and secondary antibody response is reduced in comparison to a control group. Resistance to *A. hydrophila* is enhanced.	(Logambal et al. 2000)
Indian long pepper *(Piper longum)*	Seeds of flowering plant	Positively effects fish appetite, improving feeding efficiency. Produces the compounds piperine, piplartine piperlongumime, sylvatine, guineesine, piperlongumime and filfiline, which have antimicrobial properties. Improves survival when recipients are challenged with *V. harveyi.*	(Punitha et al. 2008)
Gale-of-the-wind (*Phyllanthus niruri*)	Tropical plant	Produces antimicrobial compounds phyllanthin, phyllochrysine, phyltetralin, and quercitrin. Also improves liver function. Improves survival when recipients are challenged with *V. harveyi.*	(Punitha et al. 2008)
Coatbuttons (*Tridax procumbens*)	Flowering plant	Produces antimicrobial compound *b*-Sitosterol. Improves survival when recipients are challenged with *V. harveyi.*	(Punitha et al. 2008)

There is a wealth of evidence for the use of simple garlic, *Allivum sativum*, as an immunostimulant in aquaculture. Its molecular products have been shown to stimulate the immune responses of both fish and humans, and have also been shown to inhibit the growth of bacteria and viruses (Nya and Austin 2009b). In one example, a 2009 laboratory study tested the effects of a range of amounts of garlic in combatting an infection of *Aeromonas hydrophila* in rainbow trout (Nya and Austin 2009b). When 0.5 g of garlic or more was given per 100 g of feed, mortality in the tested fish was massively reduced (Fig. 9).

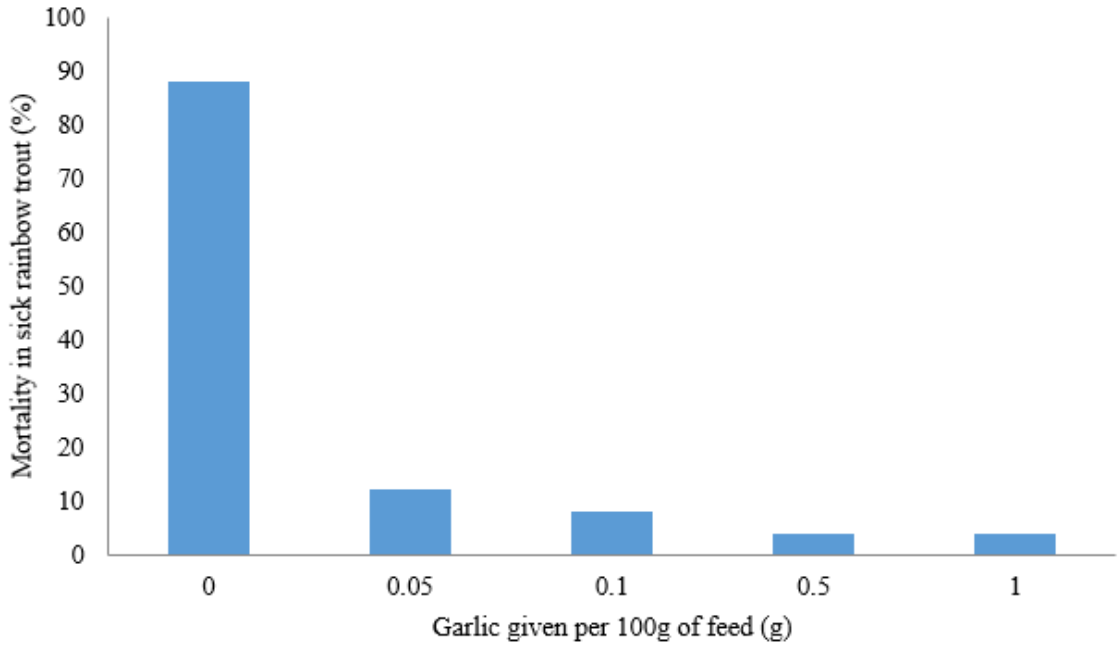

Figure 9. Percentage of mortalities recorded in rainbow trout infected with A. hydrophila, relative to the amount of garlic given in their feed.

Prebiotics

Fish, like mammals, have a diverse community of bacteria living in their intestinal tract. Gut bacteria provide important functions to the host, such as breaking down compounds the host is unable to digest and outcompeting pathogens, but also may have negative effects by causing a drain on host resources. As described above in the *Probiotics* and *Immunostimulants* sections, modifying an animal's diet has effects on the microbiota living in its gut and as a result on the health of the animal itself (Ringø et al. 2016). The composition of the microbial population in the gut is also effected by the sex and age of the animal as well as a variety of environmental factors including water salinity and water quality (Ringø et al. 2016).

As in other animals, gut microbiota in fish help break down nutrients that the animal is unable to digest on its own. This often takes the form of fermentation of certain kinds of carbohydrates, which is an anaerobic (not requiring oxygen) process that takes place along the intestinal tract. Fermentation leads to the production of beneficial acids that modulate the gut environment or that may be absorbed and processed for energy (Guerreiro et al. 2017). When substrates for the fermentation process are available, the bacteria that perform these reactions are favoured and able to multiply more quickly than competing bacteria (Guerreiro et al. 2017). If these bacteria have positive effects on the health of the host (*Bacterial biocontrol and probiotics*), it is desirable to supplement fish feed with these substrates in order to encourage their growth.

A prebiotic is any compound that encourages the growth of beneficial gut bacteria. Prebiotics are often used in conjunction with probiotics and other feed additives in order to obtain the ideal mix of bacteria in the animal's gut (Egerton et al. 2018), and have been in use in terrestrial animal agriculture for nearly 50 years (D'Abramo 2018). Prebiotics act as a substrate for the action of beneficial bacteria in the fish gut, allowing that bacterium to multiply more rapidly as a result of ample resources (Guerreiro et al. 2017). They cannot be digested by the fish, and must instead be digested by beneficial bacteria in order to encourage their multiplication (Guerreiro et al. 2017). The propagation of these bacteria result in the production of beneficial compounds,

secretion of digestive enzymes into the gut and an increase in digestive activity (Guerreiro et al. 2017). Prebiotics have potential as an alternative to metaphylactic and prophylactic antibiotics due to their well-studied positive effects on fish growth and digestion of feed, as well as by acting as immunostimulants and increasing disease resistance (Guerreiro et al. 2017, D'Abramo 2018). While results are somewhat mixed, trials have shown that prebiotics can reduce mortalities in fish as well as promote quicker weight gain and general wellbeing (Ringø et al. 2016). *Oligosaccharides* or carbohydrates containing 3-10 units of sugars such as glucose or fructose, are the most common prebiotics used in aquaculture (Table 7).

Prebiotics are also a solution to some of the problem with giving probiotics to farmed animals. As probiotics are live bacteria they are able to interact with and exchange genetic information with the bacterial community in the fish gut and on the fish farm, which can result in unexpected consequences and the spreading of virulence factors or ARGs (Guerreiro et al. 2017). Probiotic feeds also have a short shelf life. Prebiotic feeds are much longer-lasting and do not run the risk of introducing new bacteria into fish, as they only modulate the bacteria that the fish are already carrying (Guerreiro et al. 2017).

Table 7. Classes of prebiotics tested in aquaculture and their effects.

Class of prebiotics	Characteristics	Method of action	Ref.
Fructo-oligosaccharides (FOS)	Non-digestible oligosaccharides made of short to medium chains of glucose and fructose	The giving of dietary FOS encourages the growth of the bacteria that the host requires to ferment it. These bacteria may confer additional health benefits and FOS supplementation has as a result been shown to improve fish growth and health.	(Ringø et al. 2016)
Short-chain fructo-oligosaccharides (scFOS)	Similar to FOS, but with shorter glucose/fructose chains.	Encourages the growth of beneficial gut bacteria resulting in improved digestion/feed conversion and fish growth.	(Ringø et al. 2016)
Yeast	Fungus commonly used for fermentation reactions.	Has a positive effect on host microbes which results in stimulation of the immune system.	(Ringø et al. 2016)
Mannan oligosaccharides	Oligosaccharides bearing a mannose residue	Interacts with bacterial pathogens to prevent binding to the host gut wall. Pathogens use carbohydrate-binding proteins to adhere to oligosaccharides in the membranes of gut cells. Mannan-oligosaccharides can act as substitutes for these receptors, preventing colonization of the host by the pathogen. Subsequently the	(Ringø et al. 2016)

Class of prebiotics	Characteristics	Method of action	Ref.
		growth of bacteriocin-producing beneficial species is increased.	
Arabinoxylan-oligosaccharides	Short-medium chain carbohydrates with arabinoxylan residues. Arabinoxylan is a form of cellulose found in the cell walls plants such as wheat.	Has been shown to increase the growth of lactic acid producing bacteria (see *Probiotics*) in the gut.	(Ringø et al. 2016)

Red clay

Clay is a group of sediments formed when feldspar minerals undergo weathering from rain (Sciencing 2017). It contains a group of minerals including SiO_2 and traces of various metal oxides. Grains are less than 4 micrometres, but are known for their water-attracting properties and may expand up to 100% in a wet environment. The particles also stick together very well, and are known to attract and retain metal ions in solution. Clay deposits containing high levels of iron and aluminum oxides are referred to as red clay.

A 2016 Chinese study evaluated the usefulness of red clay as an antimicrobial against three fish pathogens: *Aeromonas salmonicida*, *Vibrio alginolyticus*, and *Streptococcus equinus*. While the clay was successful in reducing the growth of *A. salmonicia* and *V. alginolyticus* once concentrations reached 5-10%, it actually acted as a probiotic for *S. equinus*, enhancing the bacterium's proliferation and protein synthesis (Jung et al. 2016).

To investigate the reasons for these effects as well as the changes in the cell structure during the treatment, all three species were studied under a phase contrast microscope and a scanning electron microscope (Jung et al. 2016). Clay nanoparticles aggregated to *A. salmonicida* and *V. alginolyticus*, and elongation was also observed in *A. salmonicida* even at very low concentrations (Jung et al. 2016). Nanoparticles did not attach to *S. equinus* and no morphological changes were observed (Jung et al. 2016). Electron microscopy revealed damage to the membranes of *A. salmonicida* and *V. alginolyticus*, while later assays confirmed that these bacteria were under increased oxidative stress (Jung et al. 2016). The immunity of *S. equinus* was attributed to its increased membrane fluidity, which appears to be an adaptation to prevent aggregation with harmful substances in the water (Jung et al. 2016). Once aggregated with red clay, the other cells could be removed from surface water (Jung et al. 2016).

Natural antibiotics

Metals, in their pure form, have been known for millennia as natural antibiotics and have been used to control bacteria (particularly copper which was relatively easy to obtain) by ancient Egyptian societies and was being actively incorporated into medical treatments in the 1800's in Europe (Grass et al. 2011). There is renewed interest in the use of metals to reduce the use of antibiotics in industrial activities such as hospitals and food processing facilities, but this is

primarily for surfaces (Yasuyuki et al. 2010, Grass et al. 2011). The metals usually work through the disruption of the cell membrane of the bacteria. In the environment, the buildup of metals and their various forms in sediments is generally a reflection of the industrial activity taking place (Dean et al. 2007). For example, the cupric oxide used as an anti-foulant coating on the nets of salmon cages reduces the settlement of bivalves and hydroids through its relative toxicity to larvae and allowing more water to flow through the net cages. This coating is designed to constantly ablate exposing fresh material to settling larvae, but the old material generally deposits to the bottom increasing heavy metal concentrations in the sediments. This practice has been mostly replaced now with automated high-pressure water washing of the untreated nets on a regular cycle. Similarly, zinc is added to the diets of fish for nutritional physiological reasons, but a certain proportion of the element is not retained by the fish due to its biological availability and deposits to the sediments underneath the cages.

However, despite the antibacterial nature of the metals in their pure forms, there is substantial information from a multitude of environmental studies that clearly show a strong correlation between the development of antibiotic resistance and increasing levels of heavy metals in sediments (e.g., Yu et al. 2011, Zhao et al. 2017, Han et al. 2020, Lu et al. 2020). This correlation seems to arise because of the environmental conditions, such as anoxia, drive the selection for bacteria that can withstand these environments by modification of their physiological processes. These processes, are genetically driven and controlled by the genes of the organism, some of which can be located on the plasmids where the genes for antimicrobial resistance are often found as well.

Therefore, heavy metals are probably not a panacea to reducing the use of antimicrobials and the aquaculture industry. They may play a role in certain aspects of it such as processing and product handling, but it is probably worthwhile trying to keep them out of the sediments and maintaining concentrations at background levels, since they are a normal part of the ecosystem.

PAPER: EMPIRICAL DATA ON ARGS IN AQUACULTURE

A SURVEY OF MICROBIAL POPULATIONS AND ANTIMICROBIAL RESISTANCE GENES (ARGS) IN ASSOCIATION WITH SALMON FARMS IN THE BAY OF FUNDY

Shawn M.C. Robinson[1], Lorraine Hamilton[2], Benjamin Forward[3], Jonathan Day[1]

[1]St. Andrews Biological Station, Fisheries and Oceans Canada, St. Andrews, New Brunswick

[2]Bedford Institute Of Oceanography, Fisheries and Oceans Canada, Dartmouth, Nova Scotia

[3]Research Productivity Council, Fredericton, New Brunswick

INTRODUCTION

In 2018, a DFO-funded program was initiated under the Canadian Environmental Protection Act-Disposal at Sea (CEPA-DAS) regulations to investigate the impact the chemicals and antibiotics being used in the salmon farm industry was having on the local environment. One of the projects that came from this program was targeted on measuring the prevalence of antimicrobial resistance genes (ARGs) in the surrounding bacterial populations of salmon farms, similar to other studies that were done in other parts of the world on salmon farming (Tamminen et al. 2011a, Buschmann et al. 2012b). It was premised that the ARGs might have a longer residence time than the actual antibiotics and therefore may give a better picture of the overall risk of antibiotic exposure to the surrounding environment. As a result, a survey was done in the summer and late fall of 2018 with the goal to look at the microbiological activity around farms, the bacterial population diversity on a spatial and temporal basis and the relative percentage of ARGs in the associated bacteria.

MATERIALS AND METHODS

Study sites

The salmon farm study sites for this project were chosen based on fish health treatment records over the last five years in conjunction with the fish health professionals at the New Brunswick Department of Agriculture, Aquaculture and Fisheries (DAAF) and those at Cooke Aquaculture Inc.(CAI). For this initial survey of ARGs, we chose a site that had a history of high antibiotic use, medium use and low use. The healthcare professionals provided a list of 5 fish farms in each of the 3 categories, and we (SABS) chose one from each category based on the logistics of working at the site and any historic data that we may have had from previous studies. The sample sites chosen were Charlie Cove (high), Navy Island (medium) and Davidson's Head (low) (Fig. 10). The Charlie Cove site was in a different Bay Management Area and therefore was in fallowing year. There were also slightly different hydrographic characteristics for each site, but all are long-term salmon farms that are representative of the farms in the Bay of Fundy.

Reference sites were chosen for comparison with the farm sites based on the criteria that they had to be at least 200 m away from the farm and provided some background context for the farm samples (Fig. 10). This included two sewage treatment plants in the region, one in St. Stephen and the other in St. Andrews.

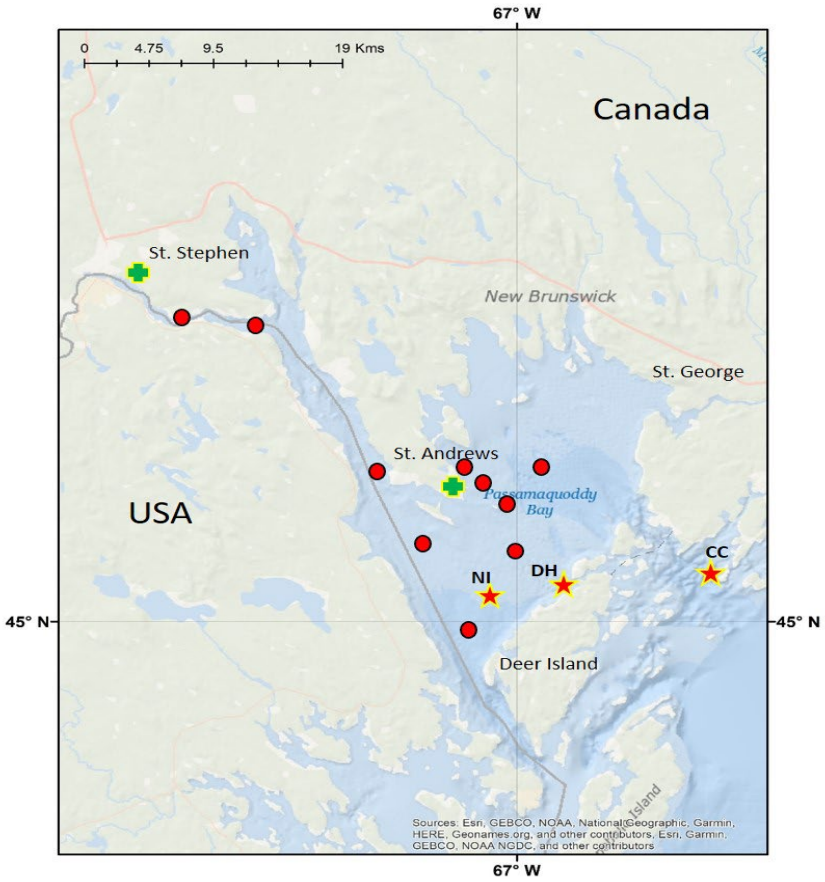

Figure 10. Location of the fish farms (stars), reference sample stations (red dots) and the sewage treatment facilities (green crosses) sampled in this study. NI=Navy Island, DH=Davidson's Head, CC=Charlie Cove.

Sample design

Samples at the farm and reference stations were taken from the surface of the benthos, and midwater support structures at 5 m (the grid) that hold the cages in place on the farm. The samples were analysed for ATP, eDNA and for ARGs. Two time periods were chosen for sampling: August 2018 when water temperatures and production were at their highest and December when water temperatures were colder and production less. Benthic samples radiating away from a farm with two transects at each farm were done in the summer. For the winter period, samples were only taken from one farm (Davidson’s Head) and more reference samples were taken.

Sampling

Benthic samples were taken with a small remote operated vehicle (ROV) equipped with a custom-designed syringe-sampler that could hold five syringes and be remotely triggered. The ROV was deployed to the bottom at a sampling station where the pilot would settle it gently on the bottom. Using the thrusters, the pilot would gently shuffle the ROV left and right where some teeth on the bottom of the sampler would re-suspend the surface sediment in a small cloud.

One of the 50 mL syringes would be triggered and a sample of this cloud would be taken. Then the pilot would fly the ROV off the bottom 1-2 metres away in a random direction where the process would be repeated. Five replicate samples were taken at each station. The ROV was then retrieved to the vessel where the syringes were removed and replaced with new sterile ones. The syringes with the samples were immediately emptied into sterile 50 mL falcon tubes, 1 mL was extracted with an automatic pipette input into an extraction solution for ATP (Ultralyse) and the tube was placed on ice for transport back to the laboratory. Then the vessel would move to the next station (farm or reference) where another sample would be taken using the same protocols.

The mid-water sampling was done by SCUBA diver on either the grid from the farm or from a reference buoy at least 200 m away from the farm (e.g., navigation buoy). For the farm samples, potential sampling stations were calculated from the north, south, east and west sides of each cage (e.g., if there were 10 cages on a farm, that would give us 40 potential sampling locations). We then randomly chose five stations to sample. To take the samples, two divers would swim along the grid at 5 m to a sampling station. Using a plastic bag over his hand, a diver would run his hand over approximately 50 cm of the rope and re-suspend the sediment and attached epifauna. Two replicate samples would be taken at each station and then the divers would return the samples back to the vessel where they were processed as described above for the benthic samples.

Sample processing

Once the samples were back at the lab they were processed on the same day, with the exception of the ATP which was stable for several days. A 50 mL sample was taken from the ice, re-homogenized and 5 mL were filtered through a pre-weighed 25 mm GFC filter. This filter was then placed in a 80°C drying oven for 24 hours where was then re-weighed to calculate mg/mL of sediment in the 50 mL falcon tube. The remaining sample in the 50 mL falcon tube was pipetted out onto a 0.2 µm sterile filter in 5 mL aliquots until the filter was dark brown or the remaining sample was used up. The filter was then folded using sterile forceps, placed into a sterile 15 mL falcon tube and 1 mL of 100% ethyl alcohol was added. The samples were stored in the fridge until transport to the Research Productivity Council lab in Fredericton, New Brunswick, where the DNA was extracted for further processing.

ATP analysis

The ATP analysis was done using the analytical protocols set out by LuminUltra® Technologies Ltd. . Briefly, 1 mL of the original sample taken by the ROV was pipetted into a prepared UltraLyse tube (mentioned above in sampling). This was shaken and then allowed to stand undisturbed for up to 24 hours. Depending on the amount of material in the original sample, a colloidal layer generally settled to the bottom of the tube. A 1 mL sample was taken using an automatic pipette from the UltraLyse tube, not disturbing the colloidal layer, and pipetted into a prepared UltraLute tube where it was inverted three times to mix the contents and allowed to stand for 4 minutes. Using an automatic pipette, 0.1 mL of the solution was placed into a sample cuvet and 0.1 mL of Luminase was added. The mixture was swirled 5 times and placed into the luminometer for reading. The luminometer and the Luminase were calibrated at the start and end of each processing run.

eDNA analysis

DNA was extracted from samples and the 16S rRNA gene was amplified according to the workflow suggested by Illumina, but incorporating modifications as described elsewhere (Caporaso et al. 2011, Caporaso et al. 2012). The V3 or V4 (or both) regions of the bacterial

16S rRNA gene was sequenced as this region has been shown to be very reliable for community clustering (Liu et al. 2007, Hamady and Knight 2009). Sequencing was performed on an Illumina MiSeq instrument using 2 x 300 bp paired end reads and generate from 2,000 to 45,000 reads per sample.

ARG analysis

All DNA extracts were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific) and read on a Synergy H1 plate reader (BioTek). The DNA samples were diluted 1:2 to ensure sufficient volume for all qPCR assays. All samples were checked for qPCR inhibition using the TaqMan Exogenous Internal Positive Control (IPC) Reagents (ThermoFisher Scientific). Quantitative PCR reactions were setup per the manufacturer's recommendations using 3 µL of the diluted DNA extracts and Luna Universal Probe qPCR Master Mix (New England BioLabs). All qPCR reactions were performed on a QuantStudio 7 Real-Time PCR system (ThermoFisher Scientific). There was no evidence of PCR inhibition.

Amplification was assessed for the reference locus 16S and nine antibiotic resistance genes (see Table 8). The 16S qPCR reactions consisted of 3 µL of the diluted DNA extracts, 1X Luna Universal qPCR Master Mix (New England BioLabs), 0.5U Antarctic thermolabile UDG (New England BioLabs) and 0.025 µM each of the 16SF and 16SR primer (see Table 8). The qPCR cycles consisted of 25°C for 10 minutes, 95°C for 1 minute and 45 cycles of 94°C for 15 seconds and 60°C for 30 seconds followed by melt curve analysis.

For the nine antibiotic resistance genes evaluated, the qPCR reactions consisted of diluted DNA extracts, 1X Luna Universal qPCR Master Mix (New England BioLabs), and 0.025 µM each of the forward and reverse primers (see Table 8). For tetA, tetK, tetM and sul2, the qPCR cycles consisted of 95°C for 1 minute and 45 cycles of 94°C for 15 seconds and AT°C (see Table 8) for 30 seconds followed by melt curve analysis. For loci tetB, int1 and sul1, the qPCR cycles consisted of 95°C for 1 minute and 45 cycles of 94°C for 15 seconds and AT°C (see Table 8) for 20 seconds and 72°C for 20 seconds followed by melt curve analysis.

Amplification for IPC, 16S and all antibiotic resistance genes were done in triplicate. All qPCR plates included a seven point (10^6 to 10^0) standard curve (in triplicate) created from the serial dilution of gBlocks (Integrated DNA Technologies) with the target loci. The melt curve results were evaluated using QuantStudio Rea-Time PCR Software v1.3 (ThermoFisher Scientific) for all antibiotic resistance genes were visually inspected and the result (number of copies) was only included when at least two of the three replicates showed melt curves consistent with the gBlock controls. The average and standard deviation of the two or three qPCR reactions meeting this criteria was calculated. Where there was only one or no replicates showing a melt curve peak consistent with the gBlock controls a result of no amplification was reported.

Table 8. Antibiotic resistance loci, primer information and qPCR annealing temperature.

Locus	Antibiotic	Citation for qPCR primers	qPCR annealing temperature (°C)	Cycle type
16S	Not applicable (Reference)	Jang et al. (2018)	60	Fast
tetA	Oxytetracycline	Tamminen et al. (2011b)	64	Fast

Locus	Antibiotic	Citation for qPCR primers	qPCR annealing temperature (°C)	Cycle type
tetB	Oxytetracycline	Jang et al. (2018)	53	Standard
tetK	Oxytetracycline	Buschmann et al. (2012a)	60	Standard
tetM	Oxytetracycline	Tamminen et al. (2011b)	60	Standard
floR	Florfenicol	Su et al. (2017)	60	Standard
int1	Not applicable (Class 1 integrase)	Jang et al. (2018)	58	Standard
sul1	Sulfadiazine and Sulfadimethoxine	Jang et al. (2018)	56	Fast
sul2	Sulfadiazine and Sulfadimethoxine	Jang et al. (2018)	68	Standard

Reporting of the ARG data was done through a standardized measurement where the total number of copies of a particular gene was divided by the total number of copies of the 16S ribosomal gene. This provided a number which was proportional to the total population size of bacteria processed in the sample.

RESULTS

ATP results

The data from the ATP samples showed a strong spatial pattern in relation to proximity to the aquaculture farm. Despite a problem with some of the loss of replicate samples for the summer sample in the benthic transect (due to human error), pooling the samples from the different farms at each of the distances still showed a strong increase in the ATP concentration per gram dry weight at the farm edge dropping very quickly towards background levels by 20 m (Fig. 11). Mean densities ranged from approximately 25,000 pg ATP/g DW at the farm to approximately 4,000 from 20 m out. Variability (standard error) was higher at the 0 meter station, but was relatively homogeneous at all the other distances. The late fall samples from Davidson's Head showed the same general trend as the summer with significantly higher concentrations of ATP in the benthic samples at the farm compared to the 200 m reference site. The late November sample means from the farm (approximately 80,000 pg ATP/g DW) were almost three times higher than the August farm samples (25,000 pg ATP/g DW). The midwater samples showed a different pattern as the reference site showed almost twice as much ATP compared to the farm site (Fig. 12a). There was also a difference between farms for grid samples with Charlie Cove being the lowest (Fig. 12b). The November samples at the Davidson's head site show that there was no significant difference between the farm and the reference site with regard to the ATP concentrations (23,700 vs 25,000 pg ATP/g DW, respectively).

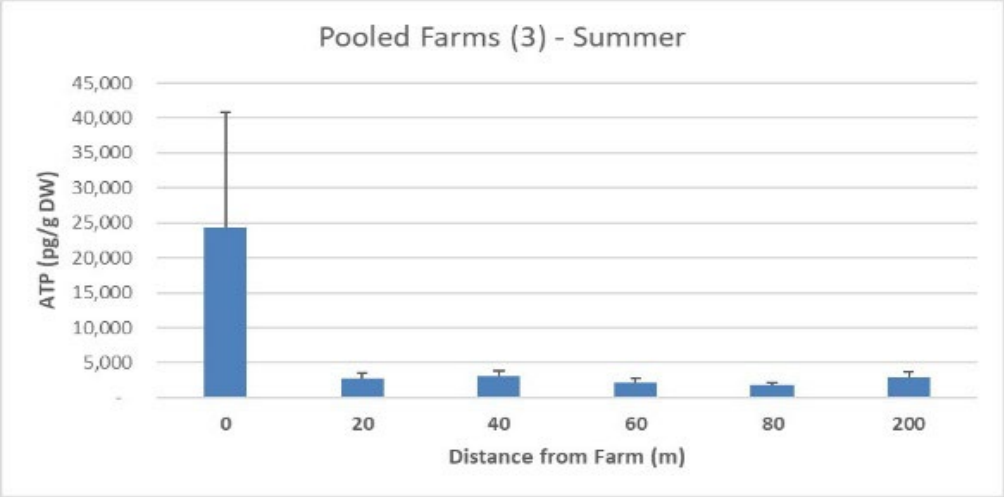

Figure 11. ATP concentrations (pg ATP/g dry weight) with distance away from the farm edge. Three sites are pooled. Error bars are one standard error.

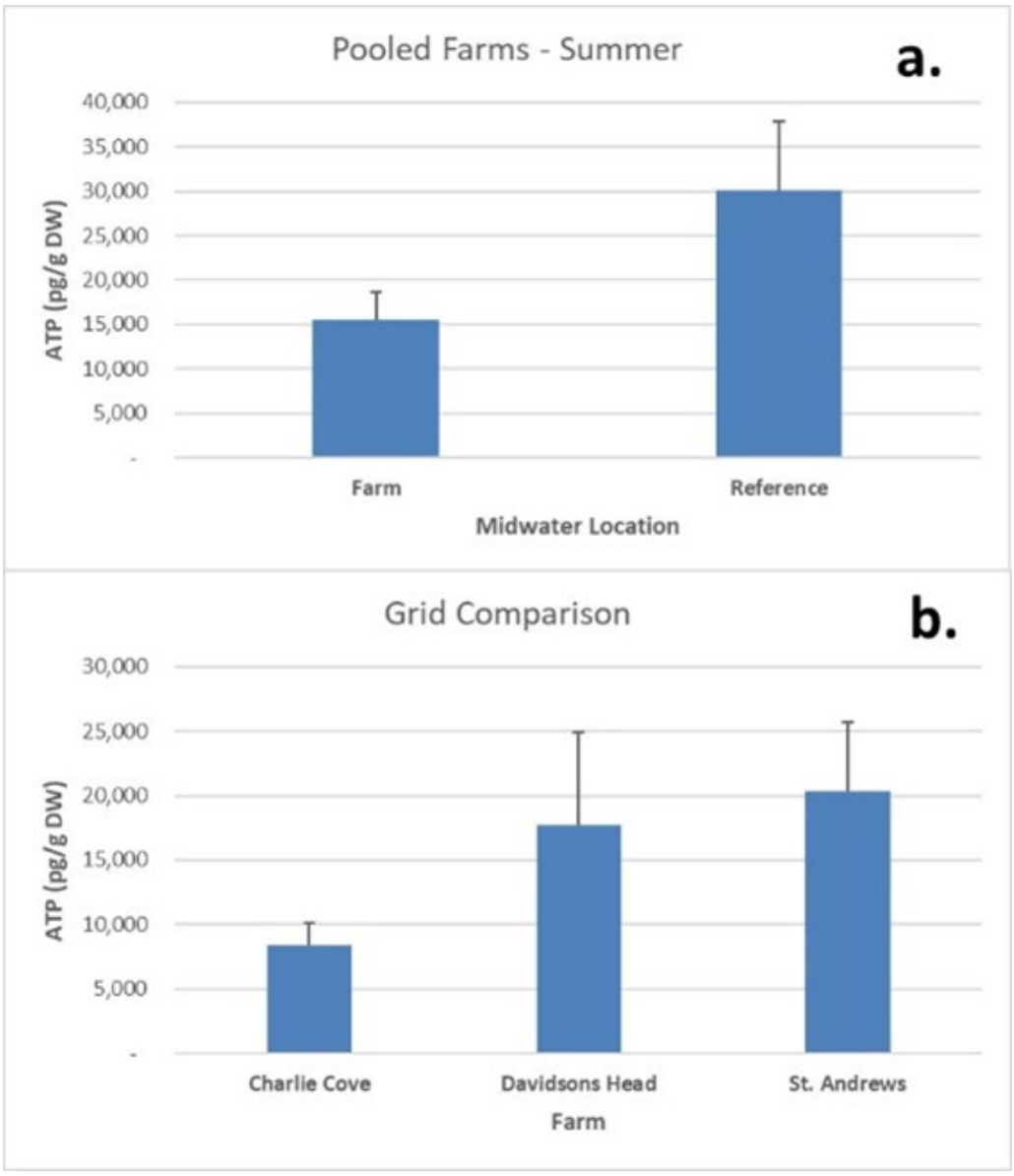

Figure 12. a.) Mean midwater ATP concentrations at pooled farms versus reference sites in August 2018. b.) Comparison of ATP concentrations on the grid at the 3 farms in August 2018. All error bars are 1 standard error.

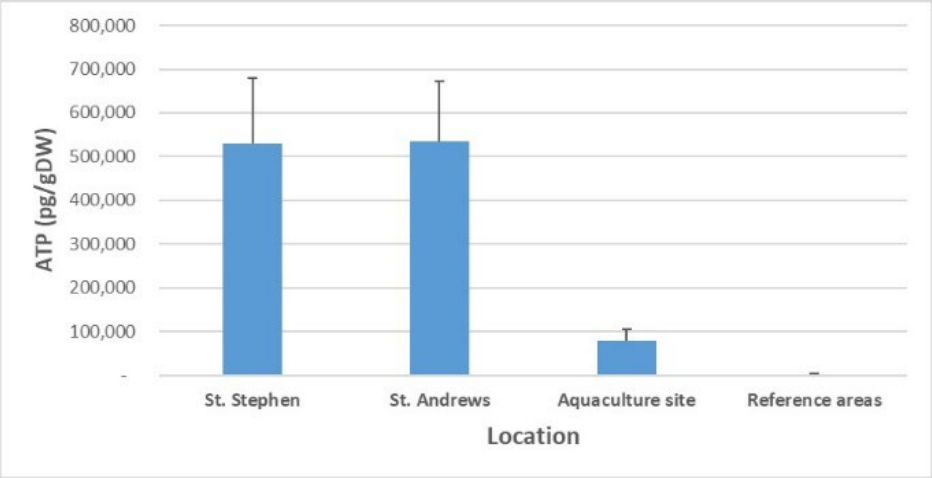

Figure 13. Mean ATP concentrations at reference, farm and sewage treatment plant locations. St. Stephen and St. Andrews represent the sewage treatment plants. All error bars are 1 standard error.

There were substantially more reference stations taken in the November time period in order to provide more context for the ATP concentrations in relation to the farm. No comparable samples were taken in the summer. The ATP microbial patterns show distinct groupings with regard to bacterial densities. Sewage treatment plants had an order of magnitude more ATP (approximately 500,000 pg ATP/g DW) than the benthic samples from the aquaculture farms (approximately 79,000 pg ATP/g DW) (Fig. 13). The salmon farms had an order of magnitude more ATP than the general reference sites outside of 200 m from the salmon farm (2,200 pg ATP/g DW).

eDNA results

Overall distributions

The meta-genomic analyses of the benthic sediment samples showed 70 different classes of bacteria present, although the bulk of the bacteria (based on number of reads) were represented by approximately 10 different classes. There were distinct patterns of bacterial classes at the farms and the reference sites (Fig. 14). Farms were often dominated by Deltaproteobacteria, Epsilonproteobacteria, and Flavo bacteriia classes where they could represent up to 60% while at the reference stations, Gammaproteobacteria were usually dominant along with Deltaproteobacteria, alpha Proteobacteria and the cyanobacteria Oscillatoriophycideae. The unclassified number of classes identified in the benthic samples ranged from 6 to 12%.

The Davidson's head site was the only farm that was sampled in both summer and winter. In the summer, Deltaproteobacteria dominated at the farm over Gammaproteobacteria by a factor of two, while in the winter Gammaproteobacteria was more dominant by approximately 50%. Epsilonproteobacteria remained high at both seasons and represented approximately 10 to 12% of the bacteria present.

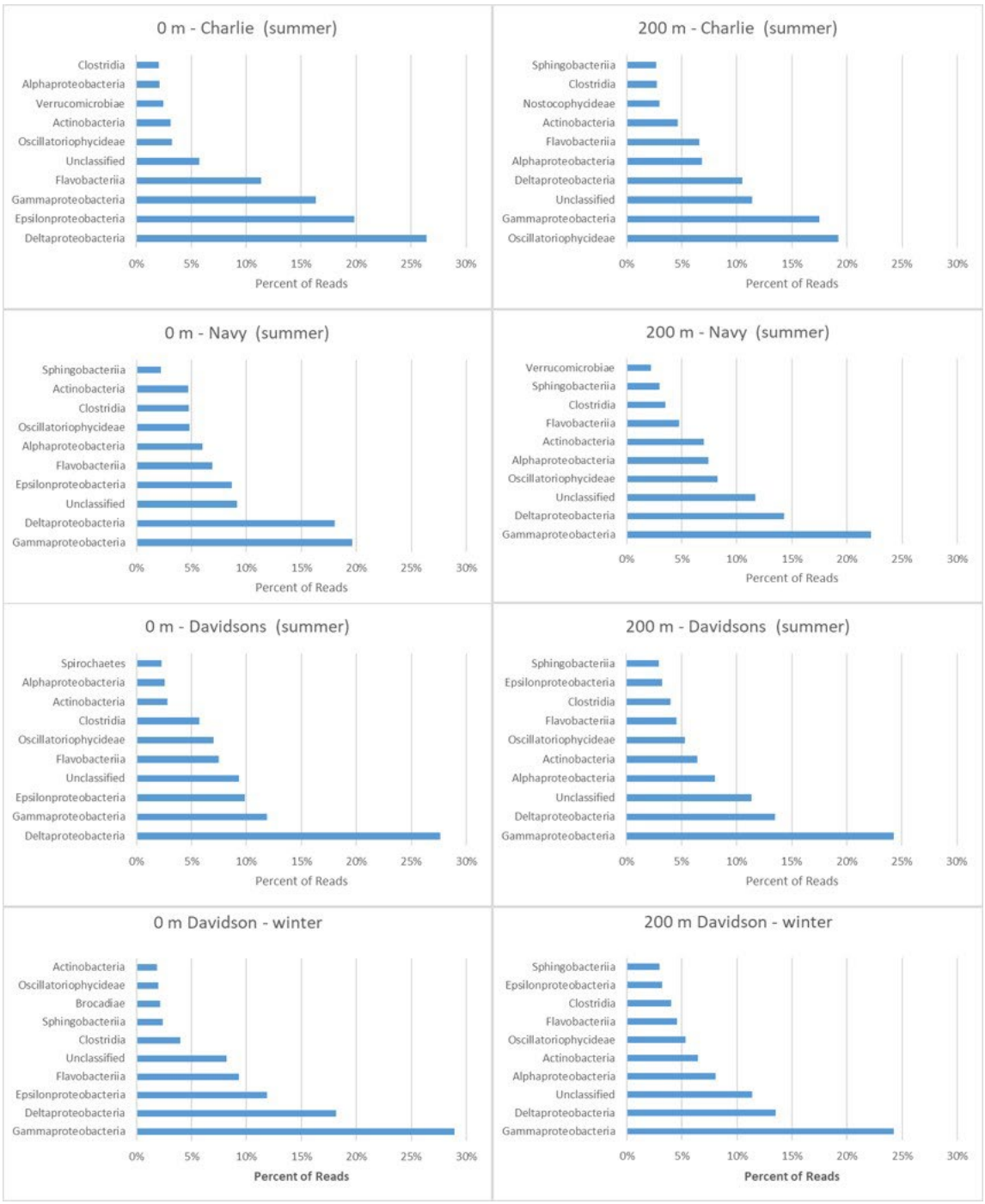

Figure 14. Proportion of the top 10 bacterial classes (including unclassified) found in the benthic sediment samples at the 0 m cage edge and the 200 m reference station for the three farms studied in this project. Summer samples were taken in August 2018 and the winter samples were taken in late November.

Spatial trends

The transect data from the three farms in the summer months showed that the dominant classes (Deltaproteobacteria, Epsilonproteobacteria and Flavobacteriia all were more abundant at the farm and dropped off quickly in the samples farther away from the fish cages (Fig. 15). A lesser abundant class, the Spirochaetes also suggested a decrease in abundance away from

farms, although the Charlie Cove farm was a bit more unclear in this trend. Conversely, the Oscillatoriophycideae and Nitrospira had the opposite trend with increasing abundance away from the farms.

The eDNA survey showed 15 pathogenic bacterial genera for fish present in the benthic samples around the salmon farms (Table 9). Abundances of the genera often differed by two orders of magnitude and some of the genera were quite rare (e.g., *Piscirickettsia, Renibacterium* and *Yersinia*).

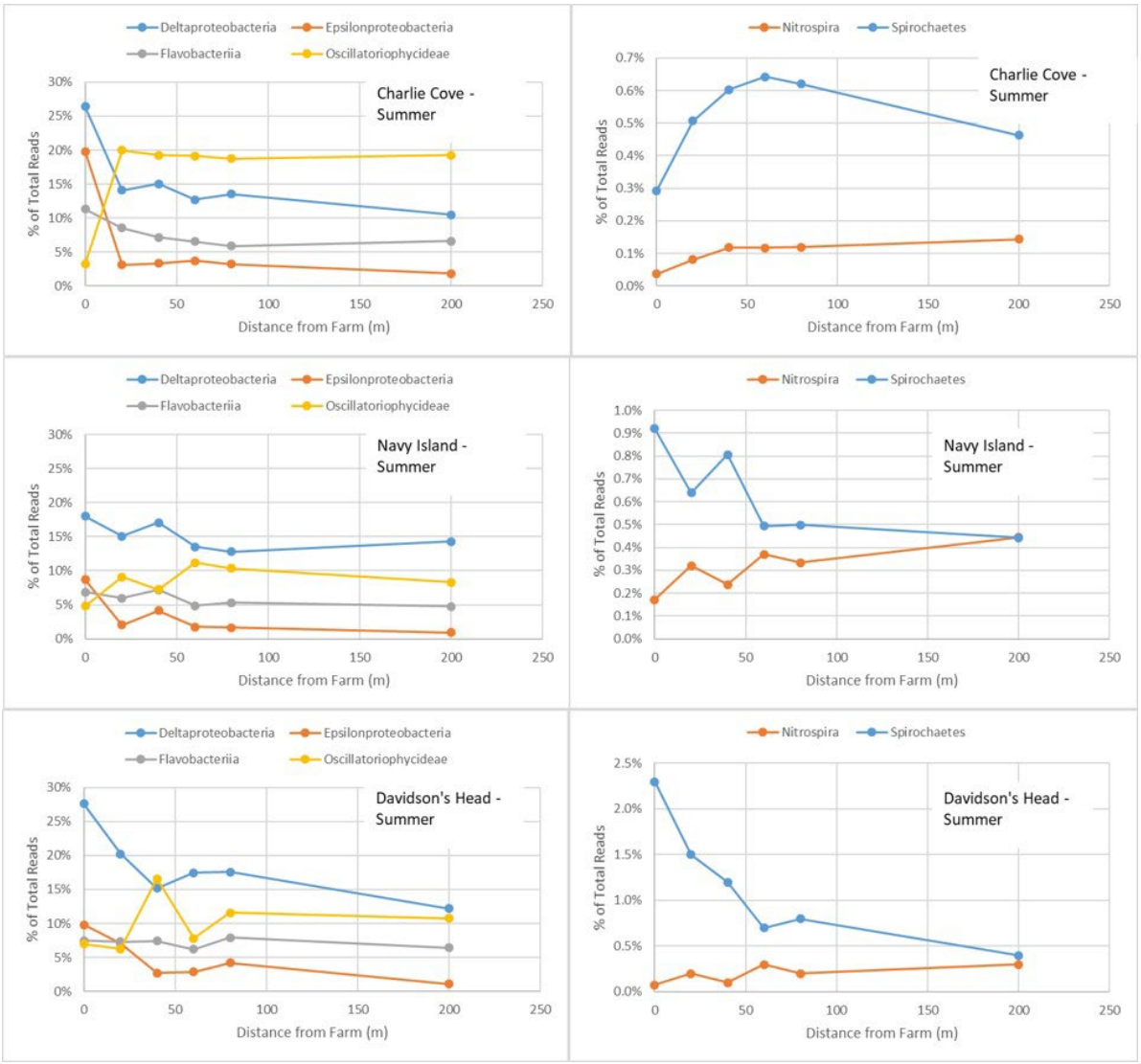

Figure 15. Distribution of the dominant bacterial classes along a 200 meter transect in August 2018 from three salmon farms in the Bay of Fundy. Percentage numbers are the percentage of the mean reads from five replicates in the sample for that distance (e.g., the average of five replicates taken at 20 m and then calculated as a percentage for all the classes found at that distance).

Table 9. Presence/absence list of bacterial genera of interest for fish health found in benthic sediment samples taken from transects away from three salmon farms and the polled reference areas in the Bay of Fundy in August 2018 and in Nov 2018. Note that most of the species within these genera are not pathogenic, but their presence suggests that environmental conditions are suitable for these types of bacteria to exist.

	Farm			
Genus	**Navy**	**Davidsons**	**Charlie**	**Reference Area**
Acinetobacter	P	P	P	P
Aliivibrio	P	P	P	P
Arthrobacter	P	P	P	P
Flavobacterium	P	P	P	P
Francisella	P	P	P	P
Moritella	P	P	P	P
Mycobacterium	P	P	P	P
Photobacterium	P	P	P	P
Piscirickettsia	P*	P*	P*	P*
Pseudomonas	P	P	P	P
Renibacterium	A	A	P*	A
Shewanella	P	P	P	P
Tenacibaculum	P	P	P	P
Vibrio	P	P	P	P
Yersinia	P	P**	P	P*

* Low # reads
** not present in winter samples

Antibiotic Resistant Genes (ARGs)

The survey results for ARGs show that they were all present in different amounts in virtually all the samples that were taken common ranging from 16% to 100% depending on the antibiotic. The relative concentrations of ARGs varied several orders of magnitude between samples depending on their location.

Florfenicol

The florfenicol ARG was found in 79% of all of the samples that we analysed and concentrations ranged approximately 3 orders of magnitude. It was found in high concentrations

in the St. Stephen sewage treatment plant and to a lesser extent in the St. Andrews sewage treatment plant (Fig. 16). There seemed to be a trend towards an increase in the abundance of florfenicol genes away from the farms, although at Davidson's Head, there was a reasonably strong signal at 0 m and there was no strong trend at Navy island. There was a drop in the florfenicol abundance from the summer to the winter samples at Davidson's Head. At the 0 meter mark in August, the ARGs dropped 6.3×10^{-6} to 1.3×10^{-6} in November. At the 200 meter reference mark, the florfenicol ARGs dropped from 1.7×10^{-6} to zero in November. On the grid, the ARGs dropped from 4.8×10^{-7} in August to zero in November. The midwater samples at the reference station showed no signs of florfenicol ARGs in either the summer or winter.

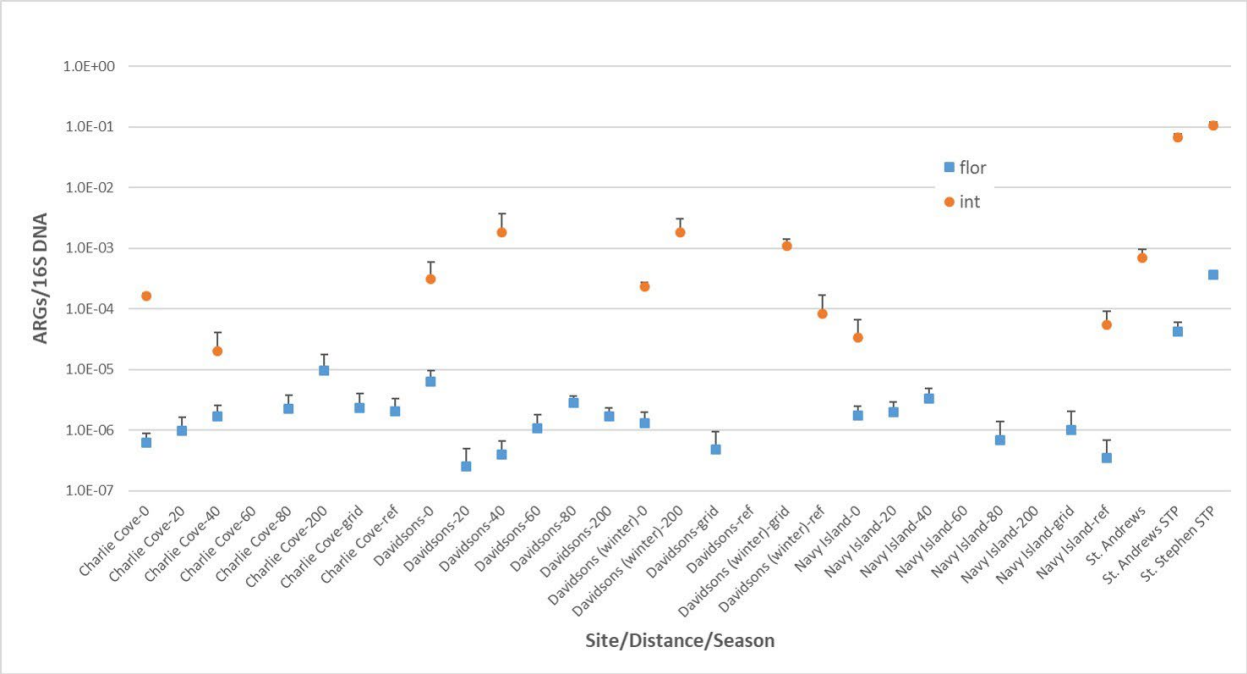

Figure 16. Relative abundance of florfenicol and integrase ARGs from the sampling stations at three salmon farms in the Bay of Fundy and at two periods of the year, August and end of November (denoted by "winter") 2018. Error bars represent one standard error.

Integrase

The integrase gene was found in 45% of all samples tested and ranged almost 4 orders of magnitude. The sewage treatment plants at St. Stephen (1.1×10^{-1}) and St. Andrews (6.7×10^{-2}) had the highest levels of the gene (Fig. 16). The ARG was present at the 0 m station at each of the 3 farms, but there was no pattern of decreasing abundance with distance as some of the more distant stations from the farm had higher levels of ARGs. Davidson's Head had levels ranging from zero to 1.8×10^{-3} while Charlie Cove ranged from zero to 1.6×10^{-4} and Navy Island ranged from zero to 5.5×10^{-5}.

Sulfonamides

The sulfonamide sul1 ARG was found in 77% of all of the samples that were analysed and the sul2 ARG was found in 97%. The concentration of these ARGs ranged 6 orders of magnitude among all the stations. For sul1, there was a positive relationship between concentration and distance from the farm at Charlie Cove, but this relationship was not as strong at the other two sites, although a trend was there. The sul2 ARG showed no real trend with distance at the Charlie Cove site, but there was a negative relationship between concentration and distance at

the other two sites (Fig. 17). At the Davidson's Head site, the winter sample concentrations tended to be lower than the summer concentrations in the su2 ARG.

The other high signal was from the St. Stephen sewage treatment plant with the sul2 ARG at 3.2×10^{-3}. Midwater samples from the grid and reference stations were all low in comparison to the benthic samples.

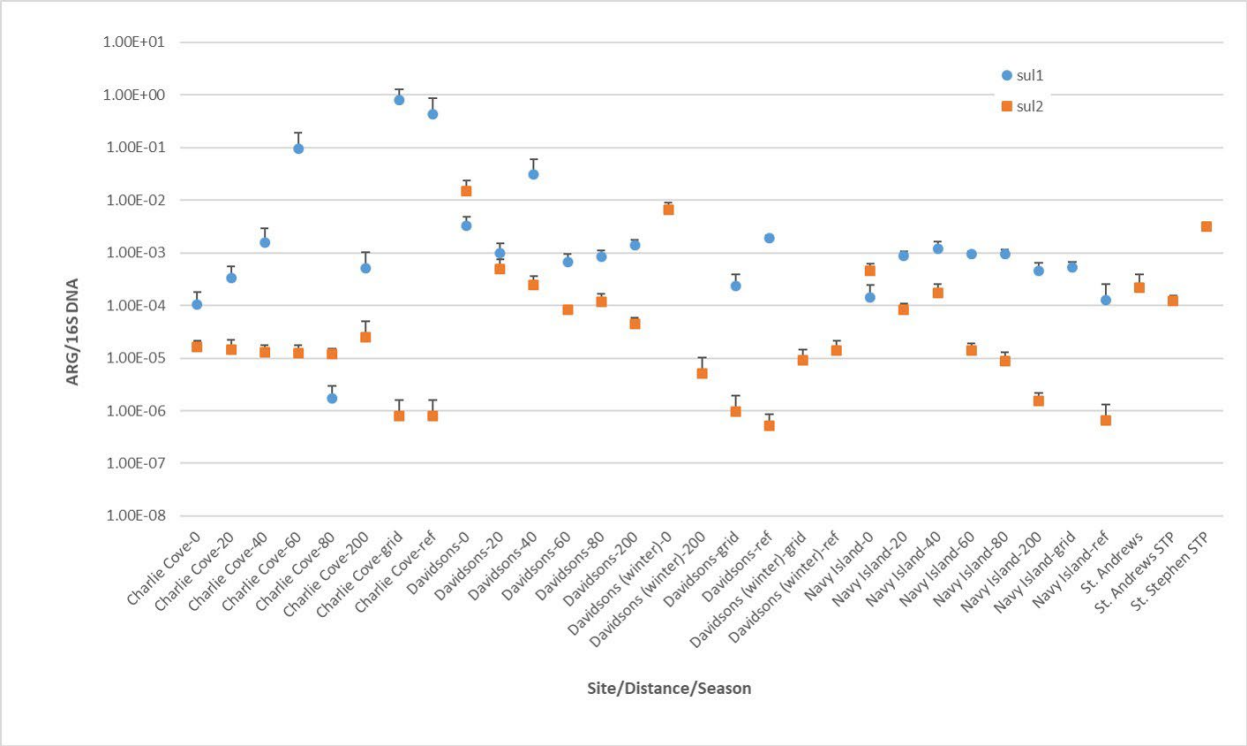

Figure 17. Relative abundance of sulfonamide sul1 and sul2 ARGs from the sampling stations at 3 salmon farms in the Bay of Fundy and at two periods of the year, August and end of November (denoted by "winter") 2018. Error bars represent one standard error.

Tetracycline

The percentage of samples that tested positive for tetracycline ARGs varied depending on the gene; tetA (84%), tetB (16%), tetK (68%) and tetM (68%) in the range for all the ARGs was approximately 5 orders of magnitude. The highest abundance of tetracycline genes were found for tetA (3.3×10^{-4} ARG/16S DNA) and tetM (2.1×10^{-3}) at the 0 m station at the Davidson's Head farm in both the summer and winter sample (8.2×10^{-4}) as well as tetA at the St. Stephen sewage treatment plant (1.4×10^{-3}) (Fig. 18). There was a decreasing trend of tetracycline ARGs away from the farm dropping quite quickly from 0 to 20 m and beyond. This decreasing trend was found in tetA, tetK and tetM. Mean values at the 200 meter station were quite low at all of the farms Charlie Cove (3.4×10^{-7}), Davidson's Head (4.6×10^{-6}) and Navy Island (2.4×10^{-7}). The midwater samples at either the grid or the reference station had very low levels of tetracycline ARGs.

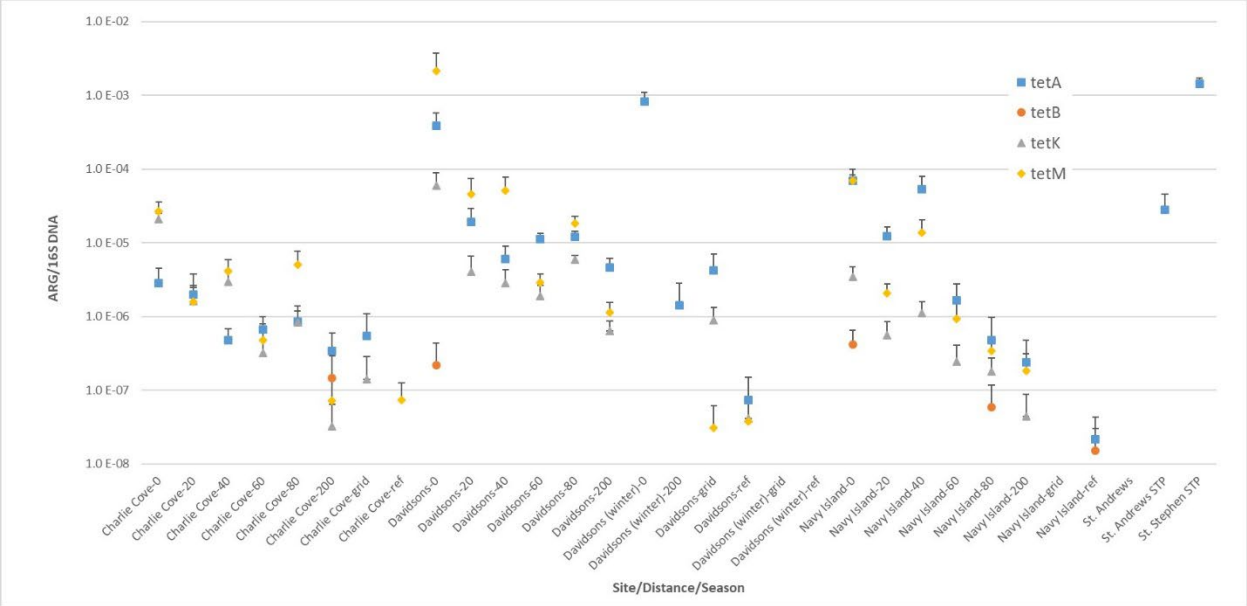

Figure 18. Relative abundance of for tetracycline ARGs (tetA, tetB, tetK, tetM) from the sampling stations at 3 salmon farms in the Bay of Fundy and at two periods of the year, August and end of November (denoted by "winter") 2018. Error bars represent one standard error.

Analysis of the relationship between the different ARGs showed some genes were well correlated (Table 10). The class 1 integrase gene was correlated with floR and tetA. The sul2 gene was correlated with tetA, tetK and tetM, but not as well with tetB. tetK and tetM were correlated.

Table 10. Correlation matrix of the eight ARGs investigated in this study.

	sul1	sul2	floR	tetA	tetB	tetK	tetM	int1
sul1	1							
sul2	-0.08	1						
floR	-0.05	0.16	1					
tetA	-0.09	0.52	0.83	1				
tetB	-0.09	0.36	-0.05	0.05	1			
tetK	-0.08	0.83	-0.05	0.14	0.40	1		
tetM	-0.05	0.90	-0.03	0.18	0.44	0.94	1	
int1	-0.07	0.10	0.90	0.70	-0.09	-0.08	-0.05	1

DISCUSSION

ATP trends

The ATP data clearly show that the microbial populations are substantially enhanced around aquaculture farms in comparison to the reference areas which are more representative of the natural ecosystem. This amplification of bacterial populations, as inferred by the ATP data, is approximately an order of magnitude higher than reference areas as the bacteria are

opportunistically feeding on the organic carbon that is being deposited by the farm. The concentrations of ATP are only elevated near the cages as the samples from 20 m outwards to 200 m all show a dramatic drop in the ATP concentrations. This observation was consistent in both summer and winter samples, although the latter comparison was only done between 0 and 200 meter stations. This observation of increased microbial activity near farms, based on ATP, is consistent with observations from the Mediterranean Sea at sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), farms (Karakassis et al. 2000). Their data showed enhancement of benthic activity up to 25 m away from the farm, after which it decreased rapidly to background levels. Similar results on increased bacterial activity in close proximity to salmon farms were also observed in Australia (Bissett et al. 2006, Bissett et al. 2007, Bissett et al. 2008).

The midwater samples at the grid and the reference stations showed different concentrations of ATP per gram of sediment in the summer and about the same in the winter. However, the total amount of sediment needs to be factored in in order to calculate total biomass of the microbes. The ropes on the reference stations were quite clean with very little sediment, while the ropes on the grid were loaded with sediment and fouling organisms (S. Robinson, personal observation). We can infer some information from the relative average difference in the sediment weights in the syringe samples between the farm and the reference sites (4.6:1 (farm:reference). This suggests there were almost five times the total amount of ATP (e.g., bacteria) on the farm grids compared to the reference station mooring lines.

The lower midwater concentrations of ATP on the Charlie Cove site is consistent with the fallowing status of that farm in comparison to the other two active ones. The average ATP concentrations were only 50% of the Davidson's Head or Navy Island site.

The concentrations of ATP from other areas in the region give an insight into the potential contribution of other anthropogenic activities. The reference areas are very similar in ATP concentrations over broad spatial scales, but were only 10% of the levels found on farms or 1% of those found in sewage treatment plants. The ATP levels from sewage treatment plants in both St. Andrews and St. Stephen were similar in concentration and were an order of magnitude higher than the salmon farms. These observations would suggest that either point source (farms or treatment plants) are capable of producing significant quantities of bacteria for interactions with the natural environment. These trends have been shown for terrestrial waste treatment plants in the past (Thiyagarajan et al. 2010, Liu et al. 2016).

eDNA trends

There were very clear trends in the population diversity of bacteria in proximity to salmon aquaculture farms at the taxonomic class level. The best classes for discriminating proximity to the salmon farms were the classes: Deltaproteobacteria, Epsilonproteobacteria, Flavobacteriia, Spirochaetes and Nitrospira. The first three classes would often represent almost 50% of all of the identified OTLs in the samples. These observations showing a clear pattern of specific classes of bacteria existing in close proximity to the nutrient rich conditions of a salmon farm have been replicated in other areas. For example, the class Flavobacteria been found to be quite important in the initial biopolymer degradation of sedimentary organic matter as one of the initial colonizers (Bissett et al. 2008). The overall distribution of bacterial classes in relation to fish farming in different geographical areas is also reasonably consistent (Keeley et al. 2018, Stoeck et al. 2018, Keeley et al. 2020).

One of the reasons it might be important to understand what taxonomic groups are being grown in close proximity to salmon aquaculture farms is that it may be easier to exchange genes between more taxonomically similar species (Kidwell 2001). If a certain suite of species is being

selected for in the vicinity of an aquaculture farm, and those species represent a higher percentage in the microbiome of the cultured organisms (fish, shellfish) which will be sold into the human food market (Wang et al. 2017a), then perhaps there is a higher probability of ARGs being swapped with human pathogens that are in similar taxonomic groups. Some of the human pathogens that belong to the dominant classes around farms that were found in this study can be seen in Table 11.

Table 11. List of human pathogens by taxonomic class and their effects. Note that most of the species within these genera are not pathogenic, but their presence suggests that environmental conditions are suitable for these types of bacteria to exist.

Class	Human Pathogenic Genera	Disease
Gammaproteobacteria	*Enterobacter*	urinary and respiratory tract infections in hospitalized patients
	Klebsiella	may cause pneumonia
	Shigella	Shiga toxin, which can destroy cells of the gastrointestinal tract
	Vibrio	may cause serious wound infections
	Yersinia	human pathogens; *Y. pestis* causes bubonic plague and pneumonic plague; *Y. enterocolitica* can be a pathogen causing diarrhea in humans
Deltaproteobacteria	*Campylobacter*	may cause severe enteritis through eating undercooked meat
	Helicobacter	may cause chronic gastritis, peptic ulcers, and stomach cancer
Alphaproteobacteria	*Brucella*	causes brucellosis in cattle and humans
	Chlamydia	can cause chlamydia, trachoma, and pneumonia
	Rickettsia	may cause Rocky Mountain spotted fever and typhus

Reference

ARG trends

This research demonstrated that ARGs are common in the marine benthic environment although the concentrations in a bacterial population can vary widely, over six orders of magnitude.

In the tetracycline suite of ARGs, tetA was the most abundant followed by tetM. Both of these ARGs decreased away from the farms suggesting that there was a selective pressure maintaining this gene in closer proximity to the farm. This pattern was consistent between farms, although the Charlie Cove farm had lower values (approximately one order of magnitude) than the other two which is consistent with its status as being fallowed at the time. It is also interesting to note that Charlie Cove was also the site with the highest use of antibiotics, based on the historical records from the industry and the province of New Brunswick. Tetracycline ARGs have been found to increase in microcosm studies where tetracycline was added in combination with fish meal (Han et al. 2018). Over a course of 14 days, the ARGs for tetracycline increased in the treatments with fish meal. Tetracycline ARGs are thought to be associated with the effux pump system (tetA) and soluble ribosomal protection proteins (tetM) that results in antibiotic resistance in the marine bacterium *Edwardsiella tarda*, that creates problems in eel culture (Lo et al. 2014). The levels of tetA found in this study were similar to two rainbow trout farms in the Baltic Sea (approximately 10^{-5}), but the tetM concentrations were two orders of magnitude lower than what they found (Tamminen et al. 2011a). Other studies have also found tetracycline ARGs in the sediment in association with fish farming (Miranda and Zemelman 2001, 2002, Miranda et al. 2003, Buschmann et al. 2012b, Tomova et al. 2015, Miranda et al. 2018).

The sulfonamide group of ARGs showed that these genes were fairly ubiquitous since they were found in 77% and 97% of the samples for sul1 and sul2, respectively. The sul1 gene was in higher abundance than the sul2 gene and it seemed to show different dynamics with regard to distance from the fish farm and also whether the site was active or fallowing. Interestingly, the patterns that were seen in the Bay of Fundy fish farms were somewhat comparable to those found in three fish farms studied near Singapore. Both studies found the sul1 gene was somewhat higher away from the farm while the sul2 gene seem to be enhanced closer to the farms in the Bay of Fundy while there was no difference between the farm and reference sites in Singapore (Ng et al. 2018). Both of the genes have been found in several studies associated with the impacts of fish farming (Muziasari et al. 2014, Capkin et al. 2017, Su et al. 2017, Jang et al. 2018, Ng et al. 2018).

There were a number of interesting correlations found in this study. The class 1 integrase gene (int1) was found to be strongly correlated with ARGs for florfenicol (floR) and tetracycline (tetA). Interestingly, it was not well correlated with any of the other tetracycline ARGs. The int1 gene is commonly linked to genes conferring resistance to antibiotics, disinfectants and heavy metals and has also been suggested as a good marker for anthropogenic impacts (Gillings et al. 2015). The results from this study indicate that the highest int1 concentrations were found generally closest to the farms and also at the two sewage treatment plants which were almost two orders of magnitude higher than the other sampling stations. It is generally acknowledged that the int1gene is strongly associated with the development of antibiotic resistance problems in cultured animals, both terrestrial and aquatic (Čížek et al. 2010, Ndi and Barton 2011, Jechalke et al. 2013, Gillings et al. 2015, Jang et al. 2018, Sáenz et al. 2019).

CONCLUSIONS

The overall conclusions from this early empirical study are that salmon farms and other points of large organic accumulation (such as sewage treatment plants) are “hotspots” for creating large

numbers of bacteria. These bacteria grow and evolve quickly and create spatial patterns in both abundance (due to the abundance of organic carbon available from the waste streams) and in taxonomic diversity (due to the environmental conditions created at the site). Antibiotic resistant genes (ARGs) are a natural phenomenon in marine bacteria and they are present in almost all bacterial populations we sampled, although we cannot trace them back to a specific taxonomic group at this point. While these ARGs are natural, they are enhanced at farm sites and sewage treatment plants likely vertically through natural selection and also horizontally through the exchange of plasmids based on the presence of the class I integrase gene (int1). The risk the ARGs present to either the fish health management on the farm or the human food supply coming from the farmed species (or wild species in the vicinity that are affected) is dependent on the probability of transfer of the ARGs to pathogens of either the fish or humans. We have no regionally relevant data available on this, but other international studies have suggested that it is possible that farms can enhance ARGs over background environmental levels that have human health implications. Further research into this topic is warranted so that a proper risk assessment can be done on the probability of the introduction of ARGs into human or fish pathogen resistome.

OVERALL CONCLUSIONS

The spread of antibiotic resistance genes associated with the use of antibiotics in aquaculture poses a threat not only to the aquaculture industry, but also potentially to human health, as it has been demonstrated by reviews of various studies that antibiotic resistance genes can be passed to human pathogens via this vector. Many of the antibiotics used in aquaculture are also used in human medicine, which enhances the danger when genes conferring resistance to these drugs enter the microbial resistome. This study showed empirically that the process of ARG enhancement in bacterial populations associated with salmon farming is present in the Bay of Fundy, although the scale at which this is happening on a spatial and temporal basis and the implications to the food supply coming from the industry is still unknown. For the overall aquaculture industry, efforts should be made to reduce the usage of antibiotics wherever possible through a series of alternate strategies. This is already happening at the industry and government management levels in Canada and internationally. As described above, this has been achieved in certain areas through legislation against non-therapeutic antibiotic usage and through vaccination efforts (Norway is a good example of this). There have also been successful attempts at research scales to reduce antibacterial usage by implementing phage therapy, quorum sensing inhibitors, probiotics, immunostimulants and herbal medications. In the future, more research should be done assessing the temporal and spatial scales of antibiotic microbial resistance at the aquaculture farms, the surrounding environment, the food supply coming from the industry and from wild commercial species that are associated with point sources of antibiotic input into the environment through anthropogenic activities. This objective will fit into the One Health initiative that is implementing non-antibiotic practices/treatments to control bacterial diseases in order to slow the spread of antibiotic resistance genes to human pathogens through the treatment of disease in cultured organisms. Once a better understanding is gained on these aspects, a proper risk assessment can be done on aquaculture activities to answer questions such as: appropriate treatment regimes, probable impacts on the environment, implications of site selection and overall risk to human health.

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