ASSESSMENT OF ANTIMICROBIAL RESISTANCE IN MARINE ENVIRONMENTS OF PRINCE EDWARD ISLAND USING A SELECTED BACTERIAL GROUP

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ABSTRACT

Antimicrobial resistance (AMR) in the aquatic environment is a common concern associated with urban, industrial and farm effluents. Naturally resistant bacteria have also been reported from environments without anthropogenic influence. Different approaches have been done to evaluate AMR in marine environments mostly assessing the frequency of resistance (FR) of a wide variety of heterotrophic bacteria. The study of a particular group of bacteria and the understanding of the different factors that influence AMR in marine environments would contribute in developing a more accurate method of assessment. The main objective of this thesis was to select a bacterium or a specific group of bacteria able to be used for assessing the level of AMR in different marine environments. Bacterial flora from 18 duplicated samples of sediments and pooled mussels from 3 different marine sites of Prince Edward Island, obtained during 2 seasons, were prescreened in the selective media Thiosulphate Citrate Bile Sucrose (TCBS) containing 50 µg/mL oxytetracycline (OTC) and FRs were obtained. Screened bacteria were identified by means of 3 different methods: conventional, automated (Biolog®) and analysis of 16S rDNA sequence similarity. Finally, the susceptibility of selected bacteria to 19 antibiotics was assessed using disk diffusion tests and interpreted according to season and the bacterial genus. The FR values significantly varied with the site ($P < 0.001$), type of sample ($P < 0.001$) and season ($P = 0.01$). During the warm season, FR values were highest in sediments of the urban-influenced site (78.9%) and in mussels of the mussel-abundant site (68.1%). Anthropogenic pollution, in the former site, and mussel bacterial selection, in the latter site, are believed to influence the FR values. However, in general, cold temperatures appeared to select for OTC-resistant bacteria. From these results, the bacterial flora from sediments appears to be a more accurate indicator of the FR to OTC in marine environments. Complementary information gained from different methods allowed the identification of the bacterial isolates to level of genus in most cases. The genera *Vibrio* and *Shewanella* constituted the majority of the bacterial flora from mussels and sediments, respectively, in which *V. splendidus* appeared to be common at all sites. More than 90% of all isolates showed non susceptibility to streptomycin. Almost 45% of the isolates had intermediate susceptibility to erythromycin, whereas up to 25% of the isolates showed non susceptibility to ampicillin, amoxicillin/clavulanic acid, carbenicillin, cephalothin, oxolinic acid, gentamicin and amikacin. All isolates were susceptible to cefotaxime, some fluoroquinolones, tetracyclines, potentiated sulfonamides, and phenicols. The percentage of non susceptible isolates to streptomycin was high and constant regardless of the season and bacterial genus in contrast to other antibiotics. Among *Shewanella* isolates, higher percentages of non susceptibility were mostly observed in those recovered during the warm season whereas among *Vibrio* isolates the percentages of non susceptible isolates were similar in both seasons and generally higher than for *Shewanella* isolates. *Vibrio* isolates were more frequently non susceptible to multiple antibiotics than *Shewanella* isolates. The susceptibility of all the isolates to the tetracyclines suggests that although 50 µg/mL OTC exerted some degree of selectivity in TCBS the selected flora has only a low level resistance. The E test showed that the activity of OTC is significantly decreased in TCBS in comparison to Mueller-Hinton agar 1.5% NaCl and that *Shewanella* tolerates higher concentrations of OTC than *Vibrio* isolates in both media but more efficiently in TCBS.

DEDICATION

To my wife, Rina, and to my parents, Enrique and Nelly, for their encouragement, support and love.

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LIST OF ABBREVIATIONS

ANOVA	-	Analysis of variance
ATCC	-	American Type Culture Collection
BUG	-	Biolog Universal Growth media
CFU	-	Colony forming unit
CDC	-	Center for Disease Control
DNA	-	Deoxyribonucleic acid
FR	-	Frequency of resistance
GN	-	Gram-negative
kbp	-	Kilobase pairs
LPS	-	Lipopolysaccharides
MHA	-	Mueller-Hinton agar
MIC	-	Minimum inhibitory concentration
NCCLS	-	National Committee for Clinical Laboratory Standards
OTC	-	Oxytetracycline
PCR	-	Polymerase chain reaction
PEI	-	Prince Edward Island
ppt	-	Parts per thousand
RNA	-	Ribonucleic acid
rDNA	-	Ribosomal DNA
rRNA	-	Ribosomal RNA
SIM	-	Degree of similarity
TCBS	-	Thiosulphate citrate bile sucrose agar
TSA	-	Trypticase or Tryptone soy agar
TSCA	-	Tryptone soy citrate agar
TSI	-	Triple sugar iron agar

CHAPTER 1: GENERAL INTRODUCTION

1.1 ANTIBIOTICS

Antibiotics are low-molecular-weight compounds that prevent bacterial growth. Their effect can be bacteriostatic, by stopping the bacterial cells from dividing, or bactericidal, by killing them. Many antibiotics are produced by bacteria or fungi. Some of them have been chemically modified, to make them more active or capable of overriding bacterial resistance mechanisms, to become classified as semisynthetic or completely synthetic antibiotics. Some antibiotics are of narrow spectrum, effective against only a limited range of infectious agents; others are of broad spectrum, effective against a wider range covering both gram-positive and gram-negative pathogens (JETACAR, 1999; Saylers, 2002).

The early pioneers of microbiology, Pasteur, Koch and Ehrlich, made references to antibiosis but it was during the late 1920's that Fleming reported the discovery of what eventually became known as penicillin, produced by the fungus *Penicillium* (Wheat, 2001). Many years later, in 1940, Florey and Chain reported the first successful use of this antibiotic in treating infected animals after purifying it (Prescott et al., 1990). The discovery and development of new agents continued in the successive years especially from the 1960s to the 1990s after the introduction of chloramphenicol, erythromycin, and tetracycline. During this period a number of antibiotics, such as vancomycin, nalidixic acid, trimethoprim/sulfamethoxazole, fluoroquinolones, four generations of cephalosporins as well as extended spectrum macrolides, tetracyclines and fluoroquinolones, among others, entered into the pharmaceutical market (Blondeau and Vaughan, 2000).

1.1.1 Antibiotic groups

As reviewed by Blair (1999) and Saylers (2002), the main groups of antibiotics include the β-lactams, aminoglycosides, macrolides, tetracyclines, phenicols, quinolones, and sulfonamides. Beta-lactam antibiotics inhibit the last step in peptidoglycan synthesis, and also bind to and inhibit the action of other inner membrane proteins that may have a role in peptidoglycan synthesis. These antibiotics are normally bactericidal and more effective against gram-positive bacteria. Beta-lactams are most effective when bacteria are actively growing or multiplying as cell walls are being formed. Aminoglycosides, tetracyclines, phenicols and macrolides are protein synthesis inhibitors. They target the bacterial ribosome and, in general, are more effective against rapidly multiplying organisms. These antibiotics bind to specific ribosomal subunits, 30S or 50S after using a membrane transport to reach them. Aminoglycosides are bactericidal, and especially effective against gram-negative bacteria. Tetracyclines target the 30S subunit, binding it transiently, causing it to be bacteriostatic but effective against both gram-positive and gram-negative organisms, rickettsiae and chlamydiae. Phenicols act by binding to a peptidytransferase enzyme located on the 50S subunit. These antibiotics are also bacteriostatic, and also act against gram-positive and gram-negative bacteria, rickettsiae and chlamydiae. Macrolides bind the 50S subunit. They are bacteriostatic for most bacteria but are bactericidal for some gram-positive bacteria, mycoplasmas, and some rickettsiae. Quinolones inhibit bacterial DNA replication. Fluoroquinolones were produced after adding fluorine to the original quinolone molecule. Quinolones and the fluoroquinolones inhibit the activity of two topoisomerase enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV, respectively, which results in suppression of

DNA replication. The quinolones are bactericidal and are more effective against gram-negative bacteria and some gram-positives. Sulfas competitively inhibit the production of folic acid which is needed by bacteria for the synthesis of purines required for the construction of RNA and DNA. Sulfa drugs are usually bacteriostatic but can be bactericidal at high concentrations. They are effective against both gram-positive and gram-negative bacteria. Potentiated sulfonamides are sulfa drugs with the addition of agents within the diaminopyrimidine group (ormetoprim, trimethoprim), which in combination block enzyme systems of two sequential steps of folic acid metabolism causing bacterial death. Potentiated sulfonamides are effective against both gram-positive and negative organisms.

1.2 RESISTANCE

Antibiotic resistance occurs when a bacterium that is normally susceptible to an antibiotic grows in the presence of antibiotic levels that would normally suppress growth or kill susceptible organisms (JETACAR, 1999). The increasing prevalence of antibiotic resistant bacteria is a public health issue of major concern since antibiotics are becoming less effective and there are fewer alternatives available for treatment (JETACAR, 1999). Resistant bacteria appeared very soon after the introduction of the first antimicrobial agents (Houvinen, 1999). This underlines the extraordinary capacity of bacteria to quickly and efficiently respond to the selective pressure imposed by antibiotic use (Schwarz and Chaslus-Dancla, 2001). However, the emergence of antimicrobial resistance globally has grown at disproportionate rates between countries, within

countries, regions and cities in direct relation to the antibiotic use (Blondeau and Vaughan, 2000; Catry et al., 2003).

1.2.1 Mechanisms

The mechanisms of antibiotic resistance can be grouped into three major types based upon their biochemical function as described elsewhere (Schwarz and Chaslus-Dancla, 2001; Saylers, 2002; Rice et al., 2003). Briefly, one mechanism is restricting the access of the antibiotic to its target. This is achieved by the restriction of the diffusion of antibiotics across the outer membrane due to alteration of porins or a switch in the charge of cell wall lipopolysaccharides (LPS) in gram-negative bacteria; through the presence of transporter proteins in the cytoplasmic membrane (efflux pumps) that expel antibiotic from the bacterial cytoplasm; and by the inactivation of transporter proteins across the cytoplasm. A second type of resistance mechanism is the presence of enzymes that inactivate the antibiotic, either by hydrolyzing it or by adding chemical groups to an important part of the antibiotic. A third type is through the modification of the antibiotic target in a way that the antibiotic no longer binds it but their function is not altered.

Resistance to β-lactams is mainly due to enzymatic degradation by β-lactamases in both gram-positive and gram-negative bacteria. In gram-negatives restriction and alterations of porins, or export by multidrug transporters reduces β-lactam uptake; and in gram-positives the alteration of penicillin binding proteins protects them. For aminoglycosides, enzymatic inactivation by aminoglycosides modifying enzymes confers resistance mainly to Enterobacteria. Also, changes in the outer membrane permeability and LPS charge as well as anaerobic environments decrease their uptake in

some gram-negatives. Efflux systems confer protection to some gram-positives. Resistance against tetracyclines is mainly by the presence of efflux pumps and also by the production of proteins that bind the ribosome altering the conformation of the active site. These mechanisms are distributed among gram-positive and gram-negative bacteria. Changes in the outer membrane permeability and alteration or overproduction of the antibiotic target enzymes due to mutations are responsible for resistance to potentiated sulfonamides. Resistance against quinolones is due to mutational alteration of the topoisomerase enzymes in enteric gram-negative and efflux systems in gram-positives and negatives. Efflux systems and decreased uptake due to porin mutations are also believed to confer resistance to quinolones. Resistance to phenicols is mainly due to enzymatic degradation. Permeability barriers and multidrug efflux systems also play a role in gram-negative resistance. Resistance against macrolides is intrinsic in several gram-negative bacteria due to the reduced permeability in their outer membrane. In gram -positives methylation of the ribosome and efflux of the antibiotic by membrane pumps confer resistance (Schwarz and Chaslus-Dancla, 2001; Saylers, 2002; Rice et al., 2003).

1.2.2 Ecological approach

The occurrence of resistant bacteria is possible through selection by the presence of antibiotics in a particular environment. In the presence of antibiotics, susceptible bacteria are unable to multiply in contrast to resistant ones which increase in number (amplification) and also in proportion of the total population (enrichment), leading to an imbalance in prior relationships between susceptible and resistant bacteria (Levy, 1997; JETACAR, 1999). Moreover, under the exposure to antibiotics resistant

genes are commonly induced and transferred not only to daughter organism but also to susceptible bacteria of the microbial community (Khachatourians, 1998; Rooklidge, 2004). The presence of antibiotics in low concentrations for a prolonged period of time is probably the most common pattern that promotes development of resistant bacteria. This allows some bacteria to survive through resistance mechanisms but also permit them to compensate for the metabolic expense associated with the maintenance of resistance mechanisms (Salyers, 1999). The acquisition of resistance by most bacterial pathogens usually carries a cost on fitness and virulence however compesatory mutations acquired by natural selection may restore them without loss of resistance (Stratton, 2002). This suggests that once resistance has developed, even in the long term, is difficult to eradicate it (Franklin, 1999). Resistance mechanisms have been developed along with evolution as a consequence of the contact of susceptible bacteria and natural antimicrobial producers, however the intensity and frequency of antibiotic use by humans have accelerated this process (Schwarz and Chaslus-Dancla, 2001; Besser at el., 2003).

1.3 ANTIBIOTICS USE AND RESISTANCE PROBLEM

Antibiotics were initially developed for the treatment of human infections but their successful application led to their use for the treatment of animals and eventually plants (Levy, 1997). However, due to the widespread use in human medicine, agriculture and veterinary medicine, antibiotic resistant genes have been spread among bacteria giving rise to resistance related problems in these fields (Khachatourians, 1998; Davidson, 1999).

1.3.1 Human medicine

The use of antibiotics has drastically reduced the cases of morbidity and mortality in humans (Blondeau and Vaughan, 2000). The proportion of hospitalized patients due to infections decreased from 70-80%, in the 1920's, to now only about 10-15% (Huovinen, 1999). However, the use and overuse of antibiotics in human medicine is recognized as the major factor contributing to the development of antibiotic resistance (JETACAR, 1999). In industrialized regions such Europe and North America, in which a prescription is required, physicians tend to prescribe antibiotics without further analysis of their need. In other countries antibiotics are available to the general public in pharmacies and their misuse is also feasible (Levy, 2002). The economic cost associated with treating clinical patients having antimicrobial resistant bacterial infections is greater than US $4 - 5 million (McGowan, 2001).

The most prominent medical examples of resistance in human pathogens are vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and multi-resistant pneumococci, pseudomonads and Enterobacteriaceae (Swartz, 1994; Kümmerer, 2004). These opportunistic pathogens, which mainly affect immunocompromised patients, were not considered important pathogens 20-30 years ago, but now cause serious infections in hospitals worldwide (Levy, 2002). In addition to resistant bacteria present in hospitals, strains of multidrug resistant bacteria, such as *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *Escherichia coli*, also cause serious community acquired infections (Levy, 1998).

1.3.2 Veterinary medicine and animal husbandry

The use of antibiotics in animals is not only limited to therapy but also for prophylaxis, metaphylaxis and growth promotion. Treatment is carried out on the basis of animal health and welfare, and to prevent economic losses associated with death or decreased productivity as a result of illness. Prophylactic treatment is used to prevent anticipated disease whereas metaphylaxis is used to treat initial disease in individuals and to prevent further outbreaks in the group. Antimicrobials can also be used in low concentrations to increase the rate of growth and to optimize feed conversion rate for the rearing of food-producing animals (Snary et al., 2004). For economic reasons, old but still efficient molecules are extensively used such as penicillins and tetracyclines whereas new antibiotics are mainly used in human medicine (Schwarz and Chaslus-Dancla, 2001). However, their significant structural similarities lead to the potential of resistance occurrence in human treatment (Khachatourians, 1998).

Heavy antimicrobial drug selective pressure in overcrowded populations of production animals creates favorable environments both for the emergence and the spread of antibiotic resistant genes among animal and zoonotic microorganisms (Franklin, 1999). Zoonotic bacteria such as salmonellae and campylobacters and commensals such as *Escherichia coli* and enterococci spread easily between animals and humans. In Europe and US resistant strains of these bacteria have been associated with the use of antibiotics in animals (JETACAR, 1999). As a consequence, the use of antibiotics in animals, particularly as growth promotants, has recently received increasing attention as a contributing factor in the international emergence of antibiotic resistant bacteria in

humans (JETACAR, 1999). A complete ban of their use is expected in European countries by 2005 (Kümmerer, 2004).

1.3.3 Aquaculture

With the intensification of aquaculture aquatic organisms are kept in dense populations which increase susceptibility to infectious diseases. Control of bacterial infections with antibiotics has been common on fish and shrimp farms (Sørum, 1999; Tendencia and de la Pena, 2001), and mollusk hatcheries (Alderman, 1992). They are used therapeutically, when an outbreak of disease occurs in the system and sometimes prophylactically, at times of disease risk (Inglis, 2000). However, in several countries, the range of drugs legal for use in aquaculture is very limited due to regulatory and market problems (Alderman and Michel, 1992). The first antibiotics used were sulphonamides and tetracyclines, and later, along with the industry growth, antibacterial chemotherapy became generalized with the use of potentiated sulphonamides (Inglis, 2000). The use of antibiotics continued to increase until the introduction of effective vaccines in salmonid farming reduced drastically antibiotic usage (Grave et al., 1999; Inglis, 2000). Alternative methods to fight against diseases in aquaculture such as the use of immunostimulants and probiotics will also probably contribute to reductions in antibiotic usage (Verschuere et al., 2000; Brincknell and Dalmo, 2005).

Among the antibiotics in use, oxytetracycline has become the most popular drug therapy in aquaculture due to its demonstrated efficacy, low cost and low toxicity (Alderman and Michel, 1992; Shao, 2001). However, due to binding with seawater borne divalent cations (Mg^{2+} and Ca^{2+}), the bioavailability of tetracyclines in salmonids is low

in the marine environment (Burka et al., 1997). Besides tetracyclines and the potentiated sulphonamides, other antibiotics such as the quinolones oxolinic acid and flumequine, and florfenicol are used for all types of bacterial infections including classical vibriosis, cold water vibriosis, furunculosis, and yersiniosis (Burka et al., 1997; Shao, 2001). Macrolides have limited use since the majority of fish bacterial pathogens are gram-negative, however erythromycin is of some use against bacterial kidney disease (Treves-Brown, 2000). The nitrofurans and chloramphenicol were used in the past but due to mutagenic properties and cases of aplastic anemia, respectively, their use is now discouraged (Burka et al., 1997).

The use of antibiotics in aquaculture has been associated with the development of resistant pathogens (Alderman and Michel, 1992; Sørum, 1999; Aoki, 2000; Inglis, 2000; Tendencia and de la Peña, 2001, 2002). In Japan, Europe and US fish pathogens such as *Vibrio anguillarum, V. salmonicida, Aeromonas salmonicida, A. hydrophila, Edwardsiella tarda* have been reported to be resistant to different antibiotics and their resistance has been associated with the presence of resistance genetic elements (plasmids) coding for resistance (Sørum, 1999). Multiple resistant *V. harveyi* has also been associated with the use of antibiotics in Asian shrimp farms (Tendencia and de la Peña, 2002). In addition, since the most common use is via medicated feed, during therapy a great proportion of antibiotics enter into the environment as a result of leaching from feces and uneaten medicated feed. This results in high local concentrations of antibiotics in the water compartment and adjoining sediments of fish and shrimp farms exposing the bacterial flora from sediments and the wild fauna to selective pressure for resistance (Samuelsen et al., 1992b; Ervik et al., 1994; Treves and Brown, 2000; Tendencia and de

la Peña, 2002; Kümmerer, 2004; Lalumera et al., 2004). Some antibiotics, such as tetracyclines, can accumulate and persist for long periods of time in marine sediments (Samuelsen et al., 1992a; Lalumera et al., 2004; Kümmerer, 2004). This situation likely triggers the selection and dissemination of resistance in the autochthonous bacterial flora. In addition, there is a risk of transmission of resistant genes from the autochthonous flora to human pathogens (Toranzo et al., 1983; Furushita et al., 2003). However, the risk of transfer of such resistance to general human consumers by any of the possible routes appears to be low and would be rather focalized in raw fish consumers from places with tropical climates or in people with close contact with fish (Smith et al., 1994; Alderman and Hastings, 1998).

1.3.4 Horticulture

In horticulture, streptomycin and oxytetracycline are the most used antibiotics (McManus and Stockwell, 2001). They are mainly used on apple, pear, and related ornamental trees. Also, gentamycin, in Latin America, and oxolinic acid, in Israel, are used to control different bacterial diseases of vegetable and fruit crops (McManus et al., 2002). The efficacy of these antibiotics has been diminished in some regions due to the emergence of antibiotic-resistant strains of pathogens. The greatest concern is that spraying antibiotics in the open environment and over large areas of land might increase the frequency of resistance genes, with the risk of these genes finding their way into human pathogens (McManus and Stockwell, 2001; McManus et al., 2002). In addition, fruits and some vegetables are often consumed uncooked thus resistant bacteria are not killed (Salyers, 1999). Nevertheless, antibiotics are still the most effective and economic

tools for the management of some of most devastating plant diseases (McManus and Stockwell, 2001).

1.4 GENETIC BASIS FOR ANTIBIOTIC RESISTANCE TRANSFER

Resistance can be either inherent or acquired. Inherent resistance is a result of the normal genetic, structural, or physiological state of a microorganism. Acquired resistance occurs when the organism has been able to either develop resistance by spontaneous mutation or has acquired a resistance mechanism from an external source (Low, 1999). The resistance mechanisms can arise in bacteria by one of two means: chromosomal mutation (*de novo* resistance) or by any of the mechanisms of horizontal gene transfer. The transferable resistance genes, or determinants, originate from mutated bacterial housekeeping genes and from natural resistant genes originating from antibiotic-producing microorganisms (Catry et al., 2003).

1.4.1 Chromosomal or *De Novo* resistance

De novo resistance is often the result of a single or multiple genetic mutations. Such mutations are called spontaneous or growth dependent (Low, 1999) and can occur at the rate of one per million to one per billion (JETACAR, 1999). Mutations can cause chromosomal genes that usually code for antibiotic sensitivity to start coding for resistance, like the alteration of the antibiotic target such that the antibiotic is no longer inhibitory, or the alteration of the permeability of the cell, reducing intracellular concentration of the antibiotic below that needed for inhibition of growth or killing (Khachatourians, 1998; JETACAR, 1999).

1.4.2 Horizontally acquired resistance

The mechanisms of horizontal transfer include: transformation, a process whereby a naked piece of DNA is taken up by a competent bacterial cell; transduction, whereby the donor DNA is packed into the capsid of a bacteriophage and injected into the recipient cell upon subsequent infection; and conjugation, in which plasmids, and transposons, can mediate their own transfer from donor to recipient cells by a process which requires cell-to-cell contact through a sex pilus (Trevors et al., 1986). The latter mechanism is considered to be the main process by which antibiotic-resistance genes disseminate among bacterial populations (Rowe-Magnus and Mazel, 1999).

1.4.2.1 Elements of horizontal transmission

Plasmids (2 kbp - > 100 kbp) are capable of autonomous replication and can be spread to member of the same species and other species, as well as vertically, to daughter cells (Lewin, 1992; Schwarz and Chaslus-Dancla, 2001). In addition, conjugative plasmids may also mediate the transfer of non conjugative plasmids, which are usually smaller and exist in the host in a higher copy number than conjugative plasmids, when they both reside in the same donor cells (Trevors et al., 1987). Some bacteria are considered "promiscuous", because once they have acquired antibiotic-resistant plasmids, intra- and inter-species transfer of resistance occurs irrespective of the environment, whether or not antibiotics are present (Khachatourians, 1998). Transposons and integrons also play an important role in the horizontal transmission of antibiotic resistant genes. Transposons (< 1 kpb – 60 kbp) act as mobile elements that "jump" from one DNA molecule to another (Olsen, 1999). They do not posses replication systems and therefore

must integrate for their stable maintenance into replication-proficient vector molecules such as chromosomal DNA or plasmids in the cell (Schwarz and Chaslus-Dancla, 2001). Integrons act as insertion sites for resistant genes that form mobile cassettes which encode resistance mechanisms to several drugs, and even heavy metals, giving rise to multiple resistant strains (Olsen, 1999). These gene cassettes represent small mobile elements of less than 2 kbp. The transference of conjugative mobile elements is triggered by SOS responses to DNA damage in the presence of antibiotics (Beaber et al., 2004).

1.5 HYPOTHESIS AND OBJECTIVES

The main hypothesis of the thesis is that antimicrobial resistance in marine environments can be assessed and interpreted more accurately using a specific group of bacteria. Secondary hypotheses are that frequencies of resistance (FR) are influenced by the sampling site, sample type and season, and that the susceptibility of marine bacteria to several antibiotics is influenced by the season and bacterial genus.

To test these hypotheses 3 main objectives had to be fulfilled:

1. To prescreen a bacterial group using a selective culture media containing antibiotic, and to determine the FR of the marine bacterial flora of 2 types of samples obtained from 3 different environments during 2 seasons.

2. To identify the bacterial flora selected previously by means of the complementary information obtained from phenotypic and genotypic methods.

3. To assess the susceptibility of the selected bacterial flora to different antibiotics and to interpret the results according to the season and bacterial genera.

CHAPTER 2: PRESCREENING OF MARINE BACTERIA USING TCBS MEDIUM WITH OTC AND ASSESSMENT OF THE FREQUENCY OF RESISTANCE OF BACTERIAL FLORA OF SEDIMENTS AND MUSSELS FROM 3 MARINE ENVIRONMENTS IN 2 SEASONS

2.1 INTRODUCTION

The frequency of resistance (FR) is a value that shows the level of resistance of the bacterial population to a particular antibiotic in an environment. This value is obtained by culturing bacteria in media with and without the addition of an antibiotic and calculating the percentage of bacteria able to grow in the presence of such antibiotic. This method has two main difficulties: culture methods have the limitations that less than 1% of the total viable microflora is actually cultured and secondly, any culture media allow the growth of quantitatively and qualitatively different subpopulations. Thus, the choice of media influences the FR detected (Smith et al., 1994). However, it is suggested that even with the shortcomings, this method remains the best way available for understanding the impact of antibacterials on the composition of marine microbial communities (Herwig and Gray, 1997) and may be more sensitive than chemical analysis for antibacterial residues in the environment (Herwig et al., 1997).

This technique requires that a selective breakpoint concentration of the agent be empirically established. Resistance is then defined as the ability to form colonies on media containing the breakpoint concentration of antibiotic (Pursell et al., 1996). In theory, three approaches could be used to set a suitable breakpoint concentration for studies in aquatic environments: 1) by reference to the distribution of levels of resistance present in undisturbed microflora of the environment being examined; 2) by reference to a breakpoint concentration previously established for the agent in fish; and 3) by setting

the concentration at such a level that would select all cells containing resistance plasmids (Smith et al., 1994). In studies of human pathogens these breakpoint concentrations have been based on the distribution of minimum inhibitory concentrations (MIC) and the levels of resistance conferred on them by genes encoding specific resistance (Pursell et al., 1996). There is less data available in this respect to determine FR in natural aquatic populations. In practice, most studies of the frequency of resistance (FR) to oxytetracycline (OTC) in these environments have employed a nominal concentration of 25 μg/mL, which is the concentration used in studies of the ecology of resistance to tetracyclines in human pathogens (Smith et al., 1994; Pursell et al. 1996).

The degree of selection exerted by a nominal concentration of an antimicrobial agent will also be influenced by the composition of the medium and the temperature and the time of its incubation (Pursell et al., 1996). In studies of marine microflora, the inclusion of divalent cations such as Mg^{2+} and Ca^{2+} are considered essential to maximize the number of colony forming units. However, these ions are known to inhibit the biological activity of OTC (Lunestad and Goksøyr, 1990). Thus media suitable for the enumeration of marine sediment microflora almost inevitably contains components that reduce the activity of many antimicrobial agents (Pursell et al., 1996). Norwegian studies of the frequency of resistance to OTC of bacterial flora from sediments associated with fish farms have used Tryptone Soya Agar (TSA) and Tryptone Soya Citrate Agar (TSCA) with the inclusion of 70% sea water (Husevåg et al., 1991; Samuelsen et al., 1992; Sandaa et al., 1992; Hansen et al., 1993). Irish studies have used a medium designated 2216V which contains 6% sea water (Kerry et al., 1994, 1995, 1996). The most frequently used breakpoint concentration in these studies has been 25 μg/mL.

However it has been suggested that in TSCA this concentration may only exert a selection pressure equivalent to 5 μg/mL (Sandaa et al., 1992). The use of a breakpoint concentration of 25 μg/mL in Mueller-Hinton Agar (MHA) for the selection of human pathogens assumes that all bacteria containing genes encoding specific resistance mechanisms will be capable of colony formation under this degree of selection. A reduction in the breakpoint concentration below 25 μg/mL would result in an increase in the number of resistant bacteria whose resistance is mediated via mechanisms other than the possession of a specific resistance gene (Pursell et al., 1996). Thus, the selection pressure exerted by 25 μg/mL OTC in culture media with marine salts is probably significantly lower than that required to select for strains possessing specific resistance genes. In order to compensate for the inhibitory effects of the divalent cations in the sea water, Pursell et al. (1996) suggested that a suitable breakpoint for OTC for both TSCA and 2216V media would be 100-200 μg/mL. Herwig et al. (1997) used 150 μg/mL OTC in MHA with the addition of 70% seawater but found that this concentration missed low level resistant bacteria.

This study determines whether MHA prepared with 70% seawater (24 ppt) or Thiosulphate Citrate Bile Sucrose agar (TCBS) are suitable media for obtaining representative bacteria which could be used as indicators of the level of antimicrobial resistance in marine environments. Additionally, a suitable breakpoint of OTC to be used in these media for the determination of frequencies of resistance (FR) is obtained. Finally, the examination of the FR of 3 representative marine sites is assessed and the suitability of using the bacterial flora from sediment and mussels as indicators of antimicrobial FR of those environments during different seasons is investigated.

2.2 MATERIAL AND METHODS

2.2.1 Sampling

2.2.1.1 Selection of the OTC breakpoint concentration and culture media for FR assessment

Five samples of subtidal sediments were obtained, by means of a Birge-Eckman type grab sampler, from the bottom of Hillsborough River near a municipal outlet pipe that discharges treated sewage from the city of Charlottetown (population of 35000). Only the upper layer (up to 2 cm in depth) of sediment was sampled. All samples were placed in sterile bags and transported in an insulated container with ice packs to the laboratory. The collection was done on May 21st of 2004 when the water temperature was 9.6°C and the salinity was 26 ppt.

2.2.1.2 Assessment of the frequency of resistance of representative sites

Three sites representative of different environments in Prince Edward Island (PEI) were chosen for this study including an urban-influenced site, an isolated site and a mussel-abundant site. Sampling was done during two seasons, a warm season during the months of June to September, and a cold season, during the months of November and December of 2004. In each occasion, 6 samples of sediments and 6 samples of blue mussels (*Mytilus edulis*) closely associated with sediments were obtained. Each sample of mussel was represented by a pool of 5 individuals with an average length of 4 to 6 cm. Sediments samples were taken from the upper layer (up to 2 cm in depth).

The location in Hillsborough Rv. described above was chosen as an urban-influenced site. On June 17th (water temperature: 13.7°C; salinity: 27.3 ppt), and

December 15th (water temperature: -1.2°C; salinity: 24.9 ppt), samples were obtained from this location. During the warm season sampling, a Birge-Eckman type grab sampler was used for obtaining the sediments, and oyster tongs were used to obtain the mussels from the bed of the river. During the cold season sediments were obtained using a long metallic spoon, and mussels were collected manually from the inter-tidal bed, during low tide. The shores of Cap Egmont, at the east side of Egmont Bay, were chosen as an isolated site due to its scarce population and the relatively long distance to any industrial center or farms. On August 12th (water temperature: 22.2°C; salinity: 27.9 ppt), and December 3rd (water temperature: 0.2°C; salinity: 26.8 ppt), samples were obtained from that location. Both types of samples, sediments and mussels, were taken from the inter-tidal bed of a rocky crag. Finally, due to its greatest activity of mussel farming in PEI, Murray River was chosen as a mussel-abundant site. On September 10th (water temperature: 20.6°C; salinity: 25.9 ppt), and November 22nd (water temperature: 4.3°C; salinity: 24.2 ppt), samples were obtained from the inter-tidal bed of the river in Kings Castle Provincial Park. Sediment samples from the isolated and mussel-abundant sites (during both seasons) were obtained by means of a long metallic spoon, digging the surface of the bed of the shore, and the mussels were detached from the algae or stones settled on the bed in 6 random points. All samples were placed in sterile bags and transported in an insulated container with ice packs until arrival to the laboratory.

2.2.2 Preparation of the antibiotic stock solution and culture media

The antibiotic tested was oxytetracycline hydrochloride (Sigma). A stock solution containing 1000 μg/mL of active drug was prepared. The antibiotic powder was

dissolved in distilled water and filter sterilized (0.22 μm) (Steritop®), and used within 1 hour of preparation.

For the selection of the OTC breakpoint, Mueller-Hinton Agar (MHA) (pH 7.4, Oxoid Ltd.) prepared with 70% of Instant Ocean® synthetic seawater (24 ppt final salinity), as recommended by Lunestad and Goksøyr (1990), and Thiosulphate Citrate Bile Sucrose (TCBS) (pH 8,6, Oxoid Ltd.) media were used. Both media, MHA prepared with 70% synthetic seawater and TCBS media were prepared in three 1 L glass bottles made up to 432, 456 and 468 mL. Molten media were cooled to 50°C and then amounts of 48, 24 and 12 mL of OTC stock solution were added respectively, in order to bring the total volume to 480 mL. This quantity is required to prepare 24 plates containing 20 mL of media of each concentration (100, 50 and 25 μg/mL, respectively).

For the assessment of FR of representative sites, only TCBS medium containing a concentration of 100 μg/mL OTC was used. The preparation followed was the same as described above.

2.2.3 Preparation of the samples

Mussels were rinsed with sterile synthetic seawater and their digestive glands were removed aseptically. Sediments and mussels (digestive glands) were homogenized, during 3-4 minutes using a Stomacher® blender, and serially diluted in autoclaved Instant Ocean® synthetic seawater. Aliquots of 50 μL of the dilutions were plated, in duplicate, on the culture media with and without OTC, and spread with a glass spatula. The incubation period for the plates from the sediment samples used for selecting an OTC breakpoint was 7 days at 15°C. The incubation period for those plates from sediment and

mussel samples used for assessing FR was 5 days at the same temperature (15°C) since it was observed that after 7 days some bacterial colonies were found not to be viable for subsequent experiments. After the incubation, the mean values of two replicates were determined and expressed as CFU/g taking into account the dilution factor. The FR value was calculated by dividing the number of colonies in the plates containing OTC by the number of colonies in the plates without OTC and multiplying this factor by 100.

2.2.4 Statistical analysis

Differences among the FR values obtained from the different OTC concentrations used for the selection of the OTC breakpoint were determined by a one-way analysis of variance (ANOVA). Differences among factors involved on the FR from the representative sites: type of site, type of sample, and season were determined by a three-way ANOVA. In both cases a statistical significance level of 0.05 was used. Also, in both cases, the data was square root transformed prior to analysis to meet the model assumptions of normal distribution.

2.3 RESULTS

2.3.1 Selection of OTC breakpoint concentration and culture media for FR assessment

The number of colony forming units (CFU) obtained from the sediment samples corresponding to each OTC concentration and their respective frequency of resistance (FR) are indicated in Appendix A.1 and Tables 2.1 and 2.2. The results show an expected decrease of the FR as the concentration of OTC in both media increase. On TCBS medium, even at the concentration of 100 μg/mL of OTC, bacterial colonies could

grow but their variety was limited to one or two distinct types of colonies. These colonies were 3 mm diameter on average and characterized to be dark green with a dark or black spot in the center. Plates at concentrations of 25 and 50 μg/mL of OTC showed higher numbers and quite similar varieties of colonies. Those colonies seemed to belong to different bacterial species, some were green, green with black centered spot, yellow or cream color colonies. The FR value obtained from the plates at concentration of 25 μg/mL of OTC (60.0%) was significantly higher ($P = 0.02$) than that obtained from the plates at 100 μg/mL of OTC (33.7%), and the FR value obtained from plates at the concentration of 50 μg/mL of OTC (42.9%) was higher but not significantly different from that one obtained at 100 μg/mL of OTC. On the MHA plates there was scarce colony growth at all concentrations of OTC, and in many plates there was no growth at all. Therefore the FR was very low even at the lowest OTC concentration.

A great difference was noticed in the effect of the OTC on the colony growth in these two media. Colony growth was higher in MHA plates that did not contain OTC in comparison with TCBS plates, but the opposite situation occurred with the plates containing OTC. The colony counts in TCBS plates containing OTC were much higher than the ones in MHA plates, thus, the differences in FR on both media are large (Tables 2.1 and 2.2).

It was observed that after an incubation period of 7d at 15°C colony growth was excessive since some colonies were overlapping and some of them appeared not to be viable making further analysis difficult.

2.3.2 Frequency of resistance of representative sites

The ANOVA showed a statistically significant three-way interaction between site, type of sample and season ($P = 0.03$). Overall tests including both main effects and interactions were strongly significant for the factors site and type of sample ($P < 0.001$) and also significant for season ($P = 0.01$). Pairs of means were compared by t-tests with a Bonferroni adjustment for a total of 24 comparisons.

In Hillsborough Rv., the FR found in sediments (78.9%) was significantly higher ($P = 0.04$) than that obtained from mussels (9.7%), during the warm season (Tables 2.3, 2.4 and Appendices A2, A3). During the cold season, again, sediments showed higher but not significantly different ($P > 0.36$) levels of resistance than mussels (FR=41.2% and 18.3%, respectively) (Tables 2.5, 2.6 and Appendices A4, A5) (Figure 2.1).

In Cap Egmont, the FR found in sediments (8.6 %) was lower, but not significantly different ($P > 0.36$), than the obtained from mussels (17.3 %), during the warm season (Tables 2.7, 2.8 and Appendices A6, A7). During the cold season, sediments and mussels showed similar levels of resistance (FR = 36.9 % and 37.1 %, respectively) (Tables 2.9, 2.10 and Appendices A8, A9) (Figure 2.1).

In Murray Rv., the FR found in sediments (8.0 %) was significantly lower ($P = 0.02$) than the obtained from mussels (68.1 %), during the warm season (Tables 2.11, 2.12, Appendices A10, A11). During the cold season, sediments and mussels showed similar levels of resistance (FR = 56.2 % and 54.2 %, respectively) (Tables 2.13, 2.14 and Appendices A12, A13) (Figure 2.1).

Table 2.1. Colony counts on average and resistance frequency of sediment samples from Hillsborough Rv. cultured in TCBS with and without different concentrations of OTC

Sample number	0 µg/mL OTC	25 µg/mL OTC		50 µg/mL OTC		100 µg/mL of OTC	
	CFU/g mean	CFU/g mean	FR (%)	CFU/g mean	FR (%)	CFU/g mean	FR (%)
1	$1.23x10^4$	$9.80 x10^3$	79.84	$4.48 x10^3$	36.45	$5.35 x10^3$	43.58
2	$5.40x10^3$	$2.32 x10^3$	43.06	$2.55 x10^3$	47.22	$1.62 x10^3$	30.09
3	$1.60x10^4$	$8.25 x10^3$	51.56	$6.08 x10^3$	37.97	$5.28 x10^3$	32.97
4	$1.03x10^4$	$4.02 x10^3$	39.08	$5.12 x10^3$	49.76	$2.48 x10^3$	24.03
5	$1.45x10^4$	$1.25 x10^3$	86.21	$6.25 x10^3$	43.10	$5.50 x10^3$	37.93
Mean			**59.95**		**42.90**		**33.72**
Sd			21.66		5.74		7.46

Sd: standard deviation

Table 2.2. Colony counts on average and resistance frequency of sediment samples from Hillsborough Rv. cultured in MHA with and without different concentrations of OTC

Sample number	0 µg/mL OTC	25 µg/mL OTC		50 µg/mL OTC		100 µg/mL of OTC	
	CFU/g mean	CFU/g mean	FR (%)	CFU/g mean	FR (%)	CFU/g mean	FR (%)
1	$1.38x10^5$	$5.0 x10^2$	0.36	$2.5 x10^2$	0.18	0	0
2	$9.45x10^4$	$2.5 x10^2$	0.26	0	0	0	0
3	$7.68x10^4$	$7.5 x10^2$	0.98	0	0	0	0
4	$4.78x10^4$	$2.5 x10^2$	0.52	0	0	0	0
5	$2.93x10^5$	$5.0 x10^3$	1.71	$7.5 x10^2$	0.25	$2.5 x10^2$	0.08
Mean			**0.77**		**0.09**		**0.02**
Sd			0.60		0.12		0.04

Sd: standard deviation

Table 2.3. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Hillsborough Rv. during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	2.78×10^4	2.62×10^4	94.59
2	5.72×10^4	4.75×10^4	82.97
3	1.38×10^5	2.25×10^5	163.04
4	5.68×10^4	3.58×10^4	63.00
5	2.15×10^4	1.50×10^4	69.77
6	8.75×10^3	0.00	0.00
Mean			**78.90**
Sd			52.73

Sd: standard deviation

Table 2.4. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Hillsborough Rv. during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	2.49×10^5	2.85×10^4	11.44
2	2.66×10^5	2.45×10^4	9.19
3	2.09×10^5	2.15×10^4	10.29
4	2.37×10^5	2.15×10^4	9.07
5	2.91×10^5	1.56×10^4	4.98
6	9.70×10^5	5.81×10^4	12.99
Mean			**9.66**
Sd			2.72

Sd: standard deviation

Table 2.5. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Hillsborough Rv. during cold season

Sample number	0 µg/mL OTC Mean CFU/g	50 µg/mL OTC Mean CFU/g	FR (%)
1	1.87×10^3	1.31×10^3	69.74
2	3.32×10^2	9.25×10	27.82
3	2.30×10^3	7.98×10^2	34.67
4	1.61×10^3	8.78×10^2	54.50
5	2.82×10^3	7.60×10^2	26.90
6	2.51×10^3	8.50×10^2	33.89
Mean			**41.25**
Sd			17.15

Sd: standard deviation

Table 2.6. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Hillsborough Rv. during cold season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	4.5 x10	5.00	11.11
2	0.00	0.00	0.00
3	9.98×10^2	5.8×10^2	58.65
4	6.25 x10	2.5 x10	40.00
5	2.75 x10	0.00	0.00
6	6.25 x10	0.00	0.00
Mean			**18.29**
Sd			25.12

Sd: standard deviation

Table 2.7. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Cap Egmont during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	1.80×10^4	5.00×10^2	2.78
2	1.38×10^4	2.50×10^2	1.82
3	2.50×10^3	6.70×10^2	26.68
4	1.75×10^4	2.50×10^2	1.43
5	1.62×10^4	1.50×10^3	9.23
6	1.02×10^4	1.00×10^3	9.75
Mean			**8.62**
Sd			9.59

Sd: standard deviation

Table 2.8. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Cap Egmont during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	7.52×10^5	5.25×10^5	69.77
2	1.85×10^5	2.75×10^4	14.86
3	8.68×10^5	5.00×10^4	5.76
4	5.30×10^6	5.00×10^5	9.42
5	6.32×10^5	2.67×10^4	4.22
6	1.88×10^5	2.33×10^5	0.00
X			**17.34**
Sd			26.17

Sd: standard deviation

Table 2.9. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Cap Egmont during cold season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	1.20×10^2	3.50 x10	29.17
2	5.00 x10	7.50	15.00
3	9.50 x10	7.67 x10	80.74
4	1.68×10^2	0.00	0.00
5	1.60×10^2	7.50 x10	46.88
6	4.20×10^2	2.08×10^2	49.40
Mean			**36.86**
Sd			28.56

Sd: standard deviation

Table 2.10. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Cap Egmont during cold season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	0.00	3.00 x10	-
2	1.05 x10^2	6.00 x10	57.14
3	9.75 x10	2.50 x10	25.64
4	4.75 x10	1.00 x10	21.05
5	5.00 x10	3.50 x10	70.00
6	6.28 x10^2	7.33 x10	11.68
Mean			**37.10**
Sd			25.09

Sd: standard deviation

Table 2.11. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Murray Rv. during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	8.48×10^4	1.68×10^4	19.76
2	6.88×10^4	4.33×10^3	6.30
3	5.26×10^5	5.10×10^4	9.70
4	5.80×10^4	1.25×10^3	2.16
5	6.88×10^4	3.00×10^3	4.36
6	23.68×10^5	5.05×10^3	6.02
Mean			**8.05**
Sd			6.25

Sd: standard deviation

Table 2.12. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Murray Rv. during warm season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	2.88×10^5	9.25×10^4	32.17
2	2.62×10^5	1.40×10^5	53.33
3	1.02×10^5	5.00×10^4	48.78
4	8.00×10^4	5.75×10^4	71.88
5	2.12×10^5	8.67×10^4	40.78
6	9.75×10^4	1.58×10^5	161.53
Mean			**68.08**
Sd			47.69

Sd: standard deviation

Table 2.13. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each sediment sample, cultured in TCBS with and without OTC, obtained from Murray Rv. during cold season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	6.65×10^2	3.50×10^2	52.63
2	3.10×10^3	2.52×10^3	81.30
3	6.55×10^2	4.30×10^2	65.65
4	1.15×10^3	4.68×10^2	40.65
5	2.82×10^3	1.14×10^3	40.37
6	2.54×10^3	1.44×10^3	56.83
Mean			**56.24**
Sd			15.65

Sd: standard deviation

Table 2.14. Mean and standard deviation of the colony counts and frequency of resistance (FR) values of each mussel sample, cultured in TCBS with and without OTC, obtained from Murray Rv. during cold season

Sample number	0 µg/mL OTC mean CFU/g	50 µg/mL OTC mean CFU/g	FR (%)
1	4.75×10^2	1.75×10^2	36.84
2	1.50×10^2	0.00	0.00
3	3.50×10^2	2.00×10^2	57.14
4	4.75×10^2	5.50×10^2	115.79
5	1.38×10^3	3.75×10^2	27.27
6	6.5×10^2	5.75×10^2	88.46
Mean			**54.25**
Sd			42.28

Sd: standard deviation

Table 2.15. Summary table of the frequency of resistance (FR) values found in sediments and mussels from 3 different environments of PEI during 2 seasons using TCBS with and without 50 μg/mL OTC

Type of sample	Hillsborough Rv.		Cap Egmont		Murray Rv.	
	warm	cold	warm	cold	warm	cold
Sediment	78.9 ± 52.7	41.2 ± 17.2	8.6 ± 9.6	36.9 ± 28.5	8.0 ± 6.2	56.2 ± 15.6
Mussel	9.7 ± 2.7	18.3 ± 25.1	17.3 ± 26.2	37.1 ± 25.1	68.1 ± 47.7	54.2 ± 42.3

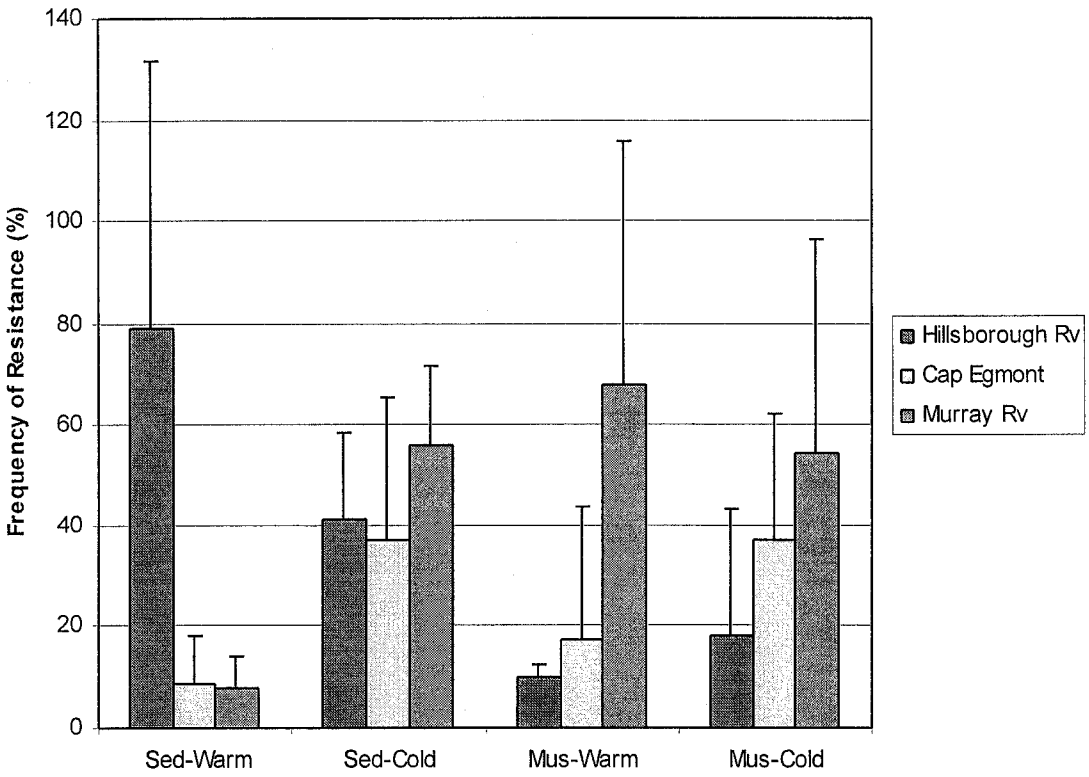

Figure 2.1 Levels of FR to 50 µg/mL OTC in TCBS of the bacterial flora of sediments (n = 18) and (pooled) mussels (n = 18) from 3 different marine environments of PEI obtained during 2 seasons (warm and cold) (Sed: sediments; Mus: mussels)

2.4 DISCUSSION

2.4.1 Selection of OTC breakpoint concentration and culture media for FR assessing

It is known that marine salts containing Ca^{2+} and Mg^{2+} reduce the antibacterial effect of OTC by chelation (Lunestad and Gøksøyr, 1990). Despite the fact that MHA was supplemented with synthetic sea water, which contains Ca^{2+} and Mg^{2+} ions, there was limited bacterial growth in the MHA plates containing OTC. Although a percentage of the OTC might have been neutralized by those cations, the remaining active component of OTC was able to inhibit the growth of most of the sediment bacteria. There are very few published reports on frequencies of resistance in natural environments and specifically in sewage outlet areas. Most research on frequencies of resistance in marine environments has been carried out in areas influenced by aquaculture activities and using different culture media with different OTC concentrations. Herwig et al. (1997) used modified MHA media, prepared with 70% seawater salt solution, containing 150 µg/mL of OTC to assess the frequency of resistance of the bacterial flora from sediments of fish farm sites and other sites distant from fish farm sites but with a probable influence from wastewater effluent leakage. In sites distant to fish farms they found frequencies of resistance ranging from 0.2 to 1.6%. The OTC concentration of 100 µg/mL used in this study resulted in a FR of 0.2% for sediments from a site influenced by a sewage outlet.

In this study, higher values of FR were obtained for OTC using TCBS than MHA media. MHA is a general medium that allows the growth of a wide spectrum of aerobic heterotrophic bacteria whereas TCBS is a selective medium. The high concentrations of bile salts, thiosulfate and citrate and the strong alkalinity in TCBS largely inhibit the growth of Enterobacteriaceae and other bacterial groups while

promoting the growth mainly of vibrios, and other genera to a lesser degree such as *Aeromonas, Photobacterium, Shewanella, Pseudoalteromonas, Pseudomonas* (Bolinches et al., 1988; Thompson et al., 2004). It is possible that bacteria with the ability to grow in the presence of such inhibitors in TCBS have developed resistance mechanisms that also confer protection from OTC. One of the resistance mechanisms found to be activated in *E. coli* in the presence of bile salts are efflux pumps required to tolerate the enteric environment (Thanassi et al., 1997). Some efflux pumps are known to have wide substrate activation, including antibiotics, biocides, detergents, and other antibacterials (Nikaido, 1998; Van Bambeke et al., 2003; Kumar and Schweizer, 2005). Genes encoding OTC resistance by means of efflux mechanisms have been found in several bacterial genera including *Vibrio, Aeromonas*, and *Pseudomonas* (Chopra and Roberts, 2001). Resistance to bile is also associated to changes in the outer membrane proteins in vibrios (Wang et al., 2003) which may confer low level resistance to OTC. However, the higher counts of CFU/g obtained from the TCBS media in comparison with those from MHA plates containing OTC also suggests that the antimicrobial activity of OTC might have been inhibited by the components of the TCBS media. This was also suggested by Tendencia and de la Peña (2002) who compared bacterial counts obtained from 2216E Zobell's medium, TCBS and *Pseudomonas-Aeromonas* selective agar containing 25 μg/mL of OTC.

The main objectives of this study were to determine the media best suited to finding representative marine environmental bacteria that could be used as indicators of resistance and to obtain a suitable OTC concentration to be used as a breakpoint. The diversity of bacterial colony types on TCBS medium was similar when using 50 or 25

μg/mL of OTC. Since the FR values were not significantly different between plates at 50 and 100 μg/mL OTC, 50 μg/mL was selected as the most suitable breakpoint concentration for subsequent samplings. Mueller-Hinton agar prepared with 70% seawater (24ppt) was not selected due to the scarce colony growth.

Mueller-Hinton agar is recommended for sensitive tests due to the lack of interference to antibiotics (Barry 1976; Herwig, 1997; NCCLS, 2003). This medium has also been used in other studies assessing frequencies of resistance (Herwig et al., 1997). Mueller-Hinton agar is a medium used to recover a wide range of heterotrophic bacteria whereas TCBS is used for selecting presumptive vibrios. Pre-selecting a specific group of bacteria to assess the level of resistance in the environment minimize changes in FR due to variation in bacterial populations, therefore the interpretation of differences in FR, from different sites or from the same site during different seasons, using selective culture media might be more the consequence of acquisition of mechanisms of resistance of a particular population.

2.4.2 Frequency of resistance of representative sites

In general, the values of CFU/g (from plates without OTC) in mussels were higher than those in sediments during the warm season, with the exception of Murray Rv where the values in mussels and sediments were similar. A similar situation occurred for the OTC-resistant bacteria, with the exception of Hillsborough Rv where the values in mussels and sediments were also similar. This general trend may be due to the fact that mussels are sessile filter-feeders (or suspension-feeders) that concentrate particulate matter including bacterial cells (Kueh and Chan, 1985; Stabili et al., 2005). It is probable

that the abundance of suspended organic matter in farm sites, such as Murray Rv, saturates the potential filter capacity of mussels which may explain the similar bacterial densities found in mussels and sediments. The filtration activity of the mussel *M. edulis* decreases as the concentration of suspended food increases (Schulte, 1975). The lack of difference in the CFU/g of resistant bacteria from sediments and mussel samples from Hillsborough Rv might be explained considering the likely great proportion of resistant bacteria filtered by mussels from that environment that may also saturate their filter capacity.

The values of CFU/g in the samples decreased during the cold season, as expected. A decrease in bacterial numbers has been reported in sediments, seawater and bivalves during fall and winter (Cavallo et al., 1999; La Rosa et al., 2001; Croci et al., 2001; Stabili et al., 2005). In mussels the decrease was much more marked than in sediments, either in the plates that did not contain OTC (representing the whole population of bacteria able to grow in TCBS) as in the ones that did contain OTC (representing the subpopulation of OTC-resistant bacteria). The physiological activity and hence the filtration rates of bivalves are closely related to temperature. It has been noticed that at low temperatures the filtration rate of *M. edulis* drops markedly (Schulte, 1975).

The FR values obtained from the bacterial flora of sediments and mussels, sampled at each of the 3 representative sites during two seasons, showed marked differences during the warm season especially within Hillsborough Rv. and Murray Rv. The FR values among the 3 sites also varied markedly during the warm season. During the cold season the differences were smaller. The FR differences of the bacterial flora

from sediments and mussels seem to be due to different reasons that depend mainly on the site, and secondarily to the season. It means that all the three factors analyzed in this study: sample type, site and season, have a significant effect individually and in combination on the values of FR obtained.

Hillsborough Rv. is an urban-influenced site that receives the treated discharges of Charlottetown city's sewage. Municipal waste water usually receives chemical compounds used in medicine that are only partially metabolized by patients which, together with multiple resistant bacteria, are not fully eliminated during the sewage purification process and, thus, released in the aquatic environment (Bell et al., 1983; Hirsch et al., 1999; Kümmerer, 2004). Bacterial flora of sediments from sewage contains a complex composition of resistant genes (Andersen and Sandaa, 1994). Industrial wastes, containing heavy metals, also favor multiple resistant bacteria (Baya et al., 1986). Hence, it was expected that the highest levels of FR were going to be in this site and, therefore, the FR values in sediments found during the warm season (78.9%) and cold season (41.2%) were not surprising since they reflect the selective pressure exerted by antibiotics and other chemical compounds most probably present in this kind of environment. In this site, markedly lower levels of FR were found in mussels than in sediments in both sampling seasons, warm and cold. Thus, the FR values obtained from mussels apparently do not reflect the selective pressure from urban pollution compared to sediments. Mussels filter planktonic bacteria from the surrounding water column (Stabili et al., 2005). However, ingested bacteria might be in a microenvironment distinct from the outside sedimental environment. In fact, mussels have low bio-concentration potential for most antibiotics, including OTC (Le Bris and Pouliquen, 2004). Coyne et al. (1997)

found a shorter half-life for persistence of OTC in mussels than in sediments, from an environment associated with finfish aquaculture and considered this drug has extremely transient retention inside these invertebrates. In contrast, OTC in marine sediments is very stable and can persist months or even years (Samuelsen et al., 1992; Weston, 1999, Lalumera et al., 2004). Therefore, the pressure exerted by those compounds might be much lower inside mussels than outside them in any environment that contains antimicrobials.

In Cap Egmont and Murray Rv. similar trends in OTC resistance patterns were in contrast to the trend in Hillsborough Rv. During the warm season, the FR found in sediments was 8.6% and 8.0% in Cap Egmont and Murray Rv., respectively. These values were significantly lower than that one from Hillsborough Rv. (78.9%). Neither of these environments with low FR is exposed to chemical pollutants or domestic wastewaters to a considerable degree. In the mussel-abundant site (Murray Rv.), a significant difference in the FR values between the bacterial flora from sediments (8.0%) and mussels (68.1%) was found during the warm season. This difference of FR is probably the reflection of the difference in the composition of the bacterial flora, able to grow in TCBS, between sediments and mussels. Some degree of selectivity in the stomach bacterial flora filtered by bivalves, including mussels has been reported (Kueh and Chan, 1985). This selection in mussels is partly a consequence of the presence of nutritional bacteriolytic lysozymes (Birkbeck and McHenery, 1982) and also probably due to the presence of natural inhibitors involved in host defense. Mussels produce antimicrobial peptides as part of its innate host defense mechanism for the destruction of bacteria in phagocytes and hemolymph (Mitta et al., 2000b). These antimicrobial peptides

are commonly found in tissues such as gut and respiratory tissues where exposure to pathogenic microorganisms is possible (Tincu and Taylor, 2004). It is probable that a considerable proportion of the bacterial flora that survives in the digestive gland of mussels possesses resistance mechanisms that confer protection against a wide variety of inhibitors including lysozymes and antimicrobial peptides. These resistance mechanisms might also be effective against some antibiotics, such as OTC. Wide spectrum defense conferred by the synergy between reduced permeability of the outer membrane and broadly specific multi-drug efflux systems are found in gram-negative bacteria (Nikaido, 1998; Butaye et al., 2003; Van Bambeke, 2003; Kumar and Schweizer, 2005). Vibrios, which are commonly found in mussels (Hariharan et al., 1995; Croci et al., 2001), and are mainly selected by TCBS, also possess genes encoding for efflux system mechanisms that confer resistance against OTC (Chopra and Roberts, 2001; Teo et al., 2002). Some of the efflux systems found in bacteria are capable of pumping out a wide range of substrates (Nikaido, 1998; Van Bambeke et al., 2003), including antimicrobial peptides (Bell and Gouyon, 2003).

The significant higher value of FR found in mussels from Murray Rv. (68.1%) than that of mussels from Hillsborough Rv. (9.7%) and also higher than those of mussels from Cap Egmont (17.3%) might be explained by the characteristics of mussel-abundant sites during the warm season. Sediments and water from mussel-abundant sites are rich in organic matter due to the large biomass of invertebrates producing faeces and pseudo-faeces that enhance bacterial growth and multiplication (Mirto et al., 2000). The innate immune system of mussels living in such environments may be highly challenged by the abundant bacterial flora. Antimicrobial peptides in the hemolymph would be greater in

higher organic loads than in those living in oligotrophic sites like Cap Egmont. Therefore, the selection of bacterial flora of mussels living in mussel abundant sites would favor more resistant strains than those from other environments.

The values of FR of sediments in Cap Egmont and Murray Rv. increased from 8.6% and 8.0% during the warm season to 36.9% and 56.2% during the cold season, respectively. This suggests a seasonal change in the environmental bacterial flora that favors resistant bacteria. La Rosa et al. (2001) observed a significant correlation between temperature and the occurrence of vibrios in sediments, and Thompson et al. (2004) also noticed seasonal changes in coastal water bodies that lead to distinct vibrio communities. In this study, it was noticed that the variety of colony growth recovered by TCBS media was reduced to one or two types from the samples obtained during the cold season. In contrast to what was observed during the warm season, FRs from sediments and mussels from the mussel-abundant site and the isolated site were very close in the cold season (56.2% and 54.2% in Murray Rv; 36.9% and 37.1% in Cap Egmont, respectively). This suggests that the level of OTC resistance of the bacterial flora from the mussels does not suffer distortion from what occurs in the outer environment during the cold season. It is possible that this happens as a consequence of a decreased activity of antimicrobial peptides in mussel hemolymph during cold season. Hernroth (2003) reported that antibacterial response of mussel antimicrobial peptides decreases at low temperatures. The bacterial flora from Hillsborough Rv., however, appears not to follow the same seasonal pattern as in the other 2 sites. This, most probably happens due to the nature of this site which is exposed to anthropogenic pollution by the sewage discharges. The composition of these discharges might constantly experience changes independent of

seasonal variations which would govern the proportion of resistant bacteria through time in that site.

The FR values found in Cap Egmont and Murray Rv., especially during the cold season, are surprisingly high taking into account the nature of such sites. These levels of OTC resistance are not likely associated with anthropogenic pollution due to the characteristics of the sites. However, several reports have showed elevated levels of antibiotic resistance in natural and confined aquatic environments (Jones, 1986; Magee and Quinn, 1991; Boon and Catanach, 1999; Ash et al., 2002). Bacteria resistant to antibiotics appear to be widespread in the aquatic environment (Ash et al., 2002), with the possibility that resistance genes are exchanged between bacteria in these environments (Davidson, 1999; Kümmerer, 2004). Jones (1986) considered that among the factors that contribute to the elevated occurrence of antibiotic resistance in natural aquatic environments is the species composition of the bacterial communities and the resistance associated with growth in low nutrient environments. The cell membrane protein components of such bacteria likely change to enhance resistance to antibacterial substances (Jones, 1986). Regarding the particular case of OTC, Kapetanaki et al. (1995), working with OTC-free marine mesocosms, demonstrated that the presence of this antibiotic is not a necessary causal condition for the emergence of high frequency of resistance. In a similar experiment, Kerry et al. (1995) found that organic load from non medicated fish pellets promoted OTC-resistant bacteria.

In conclusion, TCBS medium containing 50 μg/mL OTC was suitable for screening bacterial isolates and obtaining FR from 3 different marine environments of PEI. It was demonstrated that the FR values significantly varied according to the type of

sample used, the environment where the samples came from and the season in which they were obtained. During the warm season, FR values were highest in sediments of the urban influenced site (78.9%) and in mussels of the mussel farm site (68.1%). Anthropogenic pollution, in the former site, and mussel bacterial selection, in the latter site, are believed to influence in these FR values. In general, cold temperatures appeared to select for OTC resistant bacteria. From these results, the bacterial flora from mussels appeared to suffer some selection in mussels favoring OTC resistant bacteria. In contrast, the bacterial flora from sediments appears to be a more accurate indicator of the FR to OTC in marine environments.

CHAPTER 3: IDENTIFICATION OF PRESELECTED BACTERIA BY CONVENTIONAL METHOD, BIOLOG AUTOMATED SYSTEM AND ANALYSIS OF THE 16S rDNA SEQUENCE

3.1 INTRODUCTION

Conventional procedures for identification of bacterial isolates are based on classical phenotypic characteristics such as morphological, physiological and biochemical features. In a system-dependent methodology, the utilization of a series of substrates creates a metabolic profile of the isolate tested that is compared with known profiles. Most systems are based on the detection of color changes as a consequence of pH changes resulting from utilization of substrate, enzymatic reactions, and metabolic activity in the presence of carbon sources. Other common systems are based on the detection on chromatography of volatile or nonvolatile acids, or recognition of visible growth (Mohr O'Hara et al., 2003). Due to the limitations that are often encountered in phenotypic characterization and identification genotypic information is being used frequently in bacterial taxonomy and identification. Genotypic information is derived from the nucleic acid sequences (DNA and RNA) characteristic to that species. The ribosomal RNA (DNA) is frequently used in bacterial taxonomy and information about the sequence of the 16S rDNA is gaining more acceptance in bacterial identification (Rodicio and Mendoza, 2004). However, the use of genotypic information is not totally conclusive. Therefore, the polyphasic approach involving genotypic and phenotypic characterization, is recommended for taxonomy and identification purposes (Vandamme 1996, 2003; Venkateswaran et al., 1999; Stackebrandt, 2002).

3.1.1 Phenotypic methods of identification

3.1.1.1 Conventional Methods

These methods include physiological and biochemical reactions in tube media and observation of physical characteristics, such as morphology and odor, and tests for cell wall coloration, agglutination and antimicrobial susceptibility profiles (Mohr O'hara, 2003). The morphological description of a bacterium comprises cellular characteristics, such as shape, presence of endospore, flagella, and inclusion bodies, and gram-staining patterns, and colony characteristics, such as color, dimensions, and form. The physiological and biochemical features comprise data on growth at different temperatures, production of various enzymes, utilization of carbohydrates, amino acids, and other substrates (Vandamme, 2003). All these characteristics together constitute a distinctive feature that allows identifying and classifying bacterial isolates in a taxonomic group.

3.1.1.2 Automated identification system: Biolog®

Biolog Inc. (Hayward, California) has developed a technology that characterizes and identifies microbial isolates by examining their carbon source utilization profiles based upon redox chemistry that produces a change in substrate color. The redox dye tetrazolium violet is used to colorimetrically detect the increased metabolic rate of a cell when a carbon source is oxidized. The metabolism of bacteria increases in the presence of a carbon source they can oxidize and the colorless dye is irreversibly reduced to a purple formazan. Virtually any chemical substrate oxidized by a cell will result in the formation of NADH which donates electrons to the electron

transport chain. Redox dyes, such as tetrazoliums, divert electrons from this flow converting the tetrazolium to a highly colored formazan. Biolog uses microplates with an 8 x 12 matrix of different carbon sources that gives a pattern of purple and colorless wells which constitutes the metabolic "fingerprint" of the test organism which is then compared to a database (Bochner, 1989).

3.1.2 Genotypic methods: analysis of sequence similarity of 16S rDNA

Nucleotide sequence analysis of the bacterial DNA of the 16S rRNA gene is the new standard for bacterial identification as this is a stable genotypic signature. The molecule is approximately fifteen hundred nucleotides in length and contains highly conserved regions that are common to all organisms but also contain variations in specific zones such as the first 500 bases (Rodicio and Mendoza, 2004). The DNA sequencing approach to microbial identification involves extraction of the nucleic acids, amplification of the 16S rDNA by PCR, sequence determination and a computer software-aided search of an appropriate sequence database (Nolte and Caliendo, 2003). The technique provides complementary information to DNA-DNA hybridization. It has been observed that organisms with genomic similarities above 70%, which are considered as the same species, usually share more than 97% 16S rRNA sequence similarity. A 95% 16S rRNA sequence similarity includes bacteria in the same genus (Roselló-Mora and Amann, 2001).

This study determines the identification of the bacteria, recovered in TCBS medium containing 50 μg/mL from PEI marine environments, by complementary use of phenotypic and genotypic methods. Conventional basic characterization, Biolog

automated system and the analysis of the sequence similarity of the 16S rDNA was performed to obtain the bacterial identification. This identification was carried out in order to find bacterial representatives of marine environments for antimicrobial resistance studies.

3.2 MATERIALS AND METHODS

Bacterial flora of sediments and mussels from 3 representative marine sites of PEI, Hillsborough Rv., Cap Egmont and Murray Rv., were studied. Samples were processed as indicated in the previous chapter. Representative colonies of each morphological type cultured on TCBS, with and without the addition of 50 μg/mL OTC, were chosen for identification. Three methods for identification were performed: 1) conventional presumptive identification through staining, morphology and basic biochemical characterization, 2) automated identification using the Biolog system which is also based on phenotypic characterization of the bacterial metabolic pattern, and 3) molecular identification based on the degree of similarity of the bacterial 16S rDNA sequence.

3.2.1 Conventional identification

Representative colonies from TCBS plates with or without 50 μg/mL OTC were streaked onto Trypticase Soy agar (TSA) (pH 7.3, Oxoid Ltd.) supplemented with 2% NaCl and incubated for 24h at 22°C to obtain pure cultures. Isolates were tested by gram-staining and confirmed with KOH test. The production of catalase was detected by flooding and mixing bacterial mass with 3% H_2O_2 on a glass slide. The fermentation of glucose and lactose or sucrose and H_2S production were tested in triple sugar iron agar (TSI) (pH 7.4, Difco™) supplemented with 2% NaCl. The production of oxidase was detected by contact between oxidase reagent (tetramethyl-p-phenylenediamine) and bacterial mass on filter paper. Sensitivity to the vibriostatic O129 (2,4-diamino-6, 7-diisopropylpteridine phosphate) was detected with 150 μg discs (Oxoid Ltd.) on TSA

plates supplemented with 2% NaCl. The hemolytic character of the isolates was observed in Biolog Universal Growth media (BUG) (ph 7.3, Biolog Inc.) supplemented with 5% sheep blood. Isolates were stored in 20% glycerol at -80°C for further tests.

3.2.2 Biolog automated identification

3.2.2.1 Selection of suitable culture media and incubation conditions

Eighteen randomly chosen marine isolates recovered from sediments of Hillsborough Rv. were prepared following the Biolog GN technique (Biolog, 2001). These isolates were recovered in MHA prepared with 70% seawater (24 ppt) plates and TCBS, with or without different concentrations of OTC, transferred to Biolog Universal Growth media (BUG) (pH 7.3, Biolog Inc.) plates supplemented with 5% sheep blood, with and without the addition of 1.5% of NaCl, and incubated for 24 h at 22° and 30°C to determine optimal conditions for bacterial growth. Inocula were prepared, from the plates that showed better growth, in Inoculating Fluid (Biolog Inc., Hayward, CA., USA), and the cell density standardized at a transmittance level of 52 ± 2 % using a Biolog turbidimeter. Each well of the Biolog GN microplates (Biolog Inc., Hayward, CA., USA) was inoculated with 150 µL of cell suspension, and the microplates were incubated at 30°C for 24-48 h. Changes in color were measured using the Biolog MicroStation™ and the identification was achieved with assistance of the Biolog MicroLog™ release 4.2. Good identifications were considered for those whose similarity (SIM) values are 1 or close. SIM values below 0.5 were not considered.

To determine if the salinity and the incubation temperature have an influence in the growth in GN microplates, isolates 3 and 14 (whose colonies were the most

representative on the MHA and TCBS plates respectively). Each of these isolates was tested using Inocultaing Fluid with and without the addition of 1.5% NaCl and incubated at both 22° and 30°C degrees for 24-48 h.

To verify the accuracy of the Biolog identification system, some known species of vibrios from the collection of the Fish Health Research Laboratory of the Atlantic Veterinary College were tested. These strains were cultured in BUG media supplemented with 5% sheep blood and inoculated into the microplates using regular Inoculating Fluid (without the addition of NaCl) and incubated at 22° or 30°C. The strains used were *V. alginolyticus* incubated at 22° and 30°C, *V. parahaemolyticus*, and *V. ordalii*, incubated at 30°C, and *V. anguillarum* incubated at 22°C.

3.2.2.2 Identification of isolates from different marine environments

The identification of the marine isolates obtained from 3 different representative sites was performed using a modified form of the Biolog GN technique (Biolog, 2001), similar to methods described by Austin et al. (1995). Isolates were grown for 24-48h in Biolog Universal Growth media (BUG) supplemented with 5% sheep blood and with the addition of 1.5% NaCl. Inocula were prepared in Inoculating Fluid (Biolog Inc., Hayward, CA., USA) with the addition of 1.5% NaCl, and the cell density standardized at a transmittance level of 52 ± 2 % using a Biolog turbidimeter. Each well of the Biolog GN microplates (Biolog Inc., Hayward, CA., USA) was inoculated with 150 µL of cell suspension, and the microplates were incubated at 22°C for 24 h. Changes in color were measured using the Biolog MicroStation™ and the identification was achieved with assistance of the Biolog MicroLog™ release 4.2 software. Due to the slow

growth of some isolates some microplates were incubated for 48 hours, or in exceptional cases for 72 hours in order to obtain the highest similarity (SIM) value possible.

3.2.3 Molecular identification through 16S rDNA sequence similarity

3.2.3.1 DNA extraction

The molecular identification of the marine isolates obtained from 3 different representative sites was performed on those isolates recovered from the TCBS plates containing OTC. The method used was based on the Qiagen (2003) protocol. The DNA was extracted by adding 20 µL of proteinase K and 180 µL of ATL buffer to each microtube containing a single isolate colony in 100 µL of sterile water. The microtubes were mixed and put in water bath at 55°C for 1 h to assure cellular lysis. To each sample 200 µL of AL reagent was added, mixed and placed in a water bath at 70°C for 10 min. Subsequently, 210 µL of ethanol (96-100%) was added and mixed vigorously. This was transferred into a Qiagen® column and centrifuged at 10,000 x g for 1 min. Five hundred µL of washing buffer AW1 was added to the column, after discarding the collection tube with the eluant, and centrifuged for 1 min at 10,000 x g. The same procedure was repeated with the washing buffer AW2 with a subsequent 3 minutes centrifugation. The column was then placed in a new microtube and 200 µL of buffer AE was added and centrifuged for 1 min at 10,000 x g. The eluant was retained for the PCR reaction.

3.2.3.2 Polymerase chain reaction (PCR) reaction and detection of amplified products

The master mix consisted of 5 µL of extracted DNA, 5 µL of dNTPs, 4 µL of $MgCl_2$, 5 µL of buffer 10X, 2,5 µL (10 pmol) of primer R and F each, and 0.5 µL of Taq

polymerase adjusted to a volume of 50 µL with sterile water. The forward primer used was 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and the reverse primer was 519R 5'-GWA TTA CCG CGG CKG CTG-3'. The PCR reaction consisted of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 48°C for primer annealing, and 30 seconds at 72°C for elongation, and a final step of 7 min at 72°C. The product was stored at 4°C until needed for electrophoresis. Detection of the amplified products was made using an agarose gel at 1% in TAE 1X on a electric field of 90 volts for 60 minutes and subsequently read under UV light.

3.2.3.3 Sequencing and identification

The PCR product for sequencing was approximately 500 base pairs in length. The isolated products were submitted for sequencing to the Laboratoire de Diagnostic Moléculaire in the Faculty of Veterinary Medicine, University of Montreal. Then sequences obtained were subsequently analyzed using the BLAST program for sequence similarity searching in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) and significant alignments were obtained allowing identification of the bacteria.

3.3 RESULTS

3.3.1 Conventional identification

With this method, 4 genera were presumptively identified from the representative sites. Gram-negative bacteria that produced oxidase and catalase, fermented sugars present in TSI, but did not produce H_2S, and were sensitive to the vibriostatic agent O129 were considered presumptive *Vibrio* spp. Bacteria that had the

same pattern but did not ferment sugars in TSI nor produced H_2S were considered presumptive *Pseudomonas* spp. Those bacteria that produced H_2S, regardless of the sugars fermentation, were considered presumptive *Shewanella* spp. Bacteria that fermented sugars in TSI but did not produce H_2S and were resistant to the vibriostatic agent O129 were considered presumptive *Aeromonas* spp. The results of the biochemical identification are shown in detail in Appendices B.1-B.6 and summarized in Table 3.1.

The genera *Vibrio* and *Shewanella* were most frequently identified followed by the genus *Pseudomonas* and *Aeromonas*. All of these genera were recovered on TCBS plates with or without 50 μg/mL OTC from the 3 representative sites during both seasons with the exception of presumptive *Aeromonas* which was not recovered from Hillsborough Rv. Isolates identified as presumptive vibrios were recovered from sediments and mussels from the 3 representative sites. Presumptive *Shewanella* was recovered from Hillsborough Rv sediments and sediments and mussels from Cap Egmont and Murray Rv. Presumptive *Pseudomonas* were recovered from sediments and mussels from the 3 representative sites as well as presumptive *Aeromonas*, with the exception of Hillsborough Rv. where no presumptive *Aeromonas* were recovered from mussels and the only one from sediments was recovered from plates without OTC.

Some vibrios were found to be hemolytic and the isolates varied in the ability to ferment glucose, lactose and sucrose. The colonies in TCBS were mainly yellow, indicating the utilization of sucrose, but green and cream color colonies were also observed. Microscopically, these bacteria were mostly short thick rods. Most presumptive *Shewanella* isolates were non-fermenters, others were only glucose fermentative and others glucose, lactose or sucrose fermentative. The common feature of the colonies was

that they were dark green with a dark or black spot in the center or were completely black with transparent edges. None of them were hemolytic. Microscopically, these bacteria were medium to long rods. Presumptive *Pseudomonas* isolates were characterized by their non-fermenting property which differentiated them from vibrios. They were differentiated from *Shewanella* by the lack of H_2S production, however there are some few exceptions. Characteristics of the colonies and the bacterial microscopic morphology of the presumptive *Pseudomonas* were very similar to those for *Shewanella*. The presumptive *Aeromonas* were primarily differentiated on the basis of its resistance to the vibriostatic agent O129. Their colonies were mainly yellow, green yellowish or light green.

3.3.2 Biolog automated identification

3.3.2.1 Selection of suitable culture media and incubation conditions

Growth of a higher number of isolates was achieved in the BUG media with the addition of 1.5% NaCl and incubated at 22°C. Sixteen of the isolates (all except isolates 15 and 17) could grow under those conditions while only 10 isolates with BUG media without NaCl incubated at 22°C, 5 isolates with BUG media with NaCl incubated at 30°C, and 3 isolates with BUG media without NaCl incubated at 30°C could achieve growth. Isolates 15 and 17 did not grow on any culture media or incubation conditions. Despite the growth of most of the isolates on BUG plates under the conditions mentioned 16 isolates did not show growth in the GN microplates and only two isolates could be identified. Isolate 2 was identified as *Psychrobacter immobilis* (SIM = 0.55) and isolate 18 was identified as *Burkholderia glumae* (SIM = 0.61).

From the test performed to determine the best temperature and salinity conditions for growth, only isolate 14 tested in Inoculating Fluid with 1.5% NaCl and incubated at 22°C was identified by the Biolog system. This isolate was identified as *Burkholderia glumae* (SIM = 0.74).

Among the known samples of vibrios, *V. alginolyticus* was well identified (SIM = 0.71) when incubating the microplates at 30°C but it was identified as *V. proteolyticus* at 22°C. *V. parahaemolyticus* and *V. ordalli* were only identified as *Vibrio* sp when incubated at 30°C., and *V. anguillarum* had no identification at 22°C.

3.3.2.2 Identification of isolates from different marine environments

A considerable number of isolates could not be identified by the Biolog system because the similarity value (SIM) was insufficient to be considered adequate or due to the lack of growth in the microplates. The results of the Biolog identification are shown in detail in Appendices B.7-B.12 and summarized in Table 3.1. Among those isolates that could be identified by the Biolog system, 24 hours of incubation was required in most cases to obtain a similarity distance greater than 0.5. Sometimes, 48 hours and occasionally 72 hours was needed to grow for some isolates. Exceptionally 72 hours was required for some few isolates. There were 2 cases in which the Biolog system gave 2 different identifications after different incubation times for the same isolate. The isolate 3MMRW was identified as *Vibrio aestuarianus* after 24 h and as *V. splendidus* after 48 h, and the isolate 3MCEC was identified as *V. splendidus* after 24 h and as *V. vulnificus* after 72 h. Eight different species of vibrios were identified: *V. splendidus, V. tubiashi, V. aestuarianus, V. carcariae, V. harveyi, V. alginolyticus, V. vulnificus,* and *V.*

proteolyticus. Among them, *V. splendidus* and *V. tubiashi* were most frequently isolated in the 3 representative sites. However, most isolates identified as *V. tubiashi* were recovered in TCBS plates without OTC. The isolates obtained from sediments recovered on TCBS plates containing 50 μg/mL OTC were identified as *V. splendidus, V. tubiashi, V. alginolyticus*, or *V. vulnificus*. The isolates obtained from mussels that were recovered from TCBS plates containing 50 μg/mL OTC were identified as *V. splendidus, V. tubiashi, V. alginolyticus, V. vulnificus, V. proteolyticus*, or *V. harveyi*. *Burkholderia glumae* was also frequently identified in samples from Hillsborough Rv. and Murray Rv., but not in Cap Egmont. Isolates identified as *B. glumae* were obtained only from sediments on TCBS plates with and without 50 μg/mL OTC. Other isolates were identified as *Shewanella putrefaciens* A, recovered from sediments, *Aminobacter aminovorans* recovered from mussels, both from TCBS plates without OTC. Also, CDC group II-E subgroup A, obtained from sediments, *Aeromonas veronii* DNA group 10 obtained from mussels, both from TCBS plates with 50 μg/mL OTC.

3.3.3 Molecular identification through 16S rDNA sequence similarity

The 16S rDNA sequences of the bacterial isolates obtained for BLAST analysis are shown in Appendix B.13. With this method three genera were identified among the representative isolates recovered in TCBS plates with 50 μg/mL: *Vibrio, Shewanella* and *Pseudoalteromonas* (see details in Appendices B.14-B.19 and summarized in Table 3.1). Among the genus *Vibrio,* different species were identified, especially *V. splendidus*, which was isolated from sediment and mussels. Other species identified were *V. halioticoli, V. tasmaniensis, V. gallicus, V. pomeroyi, V. alginolyticus,*

V. supersteus, V. harveyi, from mussels; and *V. campbelli* from sediments. In general, most *Vibrio* isolates were recovered from mussels. There was no single species that could be obtained from the 3 representative sites, probably due to the low number of isolates, but *V. splendidus* appeared to be the most common species. It could be isolated from the samplings in the warm and cold season. Within the *Shewanella* genus, there was no single common species since most sequences that produced significant alignments corresponded to undetermined species. The species of *Shewanella* identified were *S. colwelliana, S. baltica, S. gaetbuli, S. woodyi, S. affinis* and *S. frigidimarina* isolated from sediments; and S. *waksmanii* isolated from mussels. In general, most *Shewanella* isolates were obtained from sediments. As in the case of vibrios, there was not a single species that could be isolated from the 3 representative sites. Isolates identified as *Pseudoalteromonas* sp. were recovered from sediments and mussels. In some instances, more than one species had significant alignments with the isolates sequenced. Therefore, this method was not considered a definitive identification tool to the species level.

Table 3.1. Identification, by different methods, of bacterial isolates from sediments and mussels obtained from 3 representative marine sites from PEI during 2 seasons

Isolate		Identification methods		
Code	Origin[1]	Conventional	Biolog	Molecular
01SHRW	Sediment - OTC 0	*Vibrio* sp.	No growth	
02SHRW	Sediment - OTC 0	*Vibrio* sp.	No ID	
03SHRW	Sediment - OTC 0	*Vibrio* sp.	*V. tubiashi*	
04SHRW	Sediment - OTC 0	*Shewanella* sp.[+]	*B. glumae*	
05SHRW	Sediment - OTC 0	*Shewanella* sp.	*S. putrefaciens* A	
06SHRW	Sediment - OTC 0	*Vibrio* sp.	No growth	
07SHRW	Sediment - OTC 50	*Vibrio* sp.	*V. splendidus*	*V. splendidus*
08SHRW	Sediment - OTC 50	*Vibrio* sp.	No growth	*V. splendidus*
09SHRW	Sediment - OTC 50	*Shewanella* sp.	*B. glumae*	*Shewanella* sp.
10SHRW	Sediment - OTC 50	*Shewanella* sp.[+]	*B. glumae*	*Shewanella* sp.
11SHRW	Sediment - OTC 50	*Vibrio* sp.	No growth	
01MHRW	Mussel - OTC 0	*Vibrio* sp.	No growth	
02MHRW	Mussel - OTC 0	*Vibrio* sp.	*V. splendidus*	
03MHRW	Mussel - OTC 0	*Vibrio* sp.	*V. splendidus*	
04MHRW	Mussel - OTC 0	*Vibrio* sp.	No ID	
05MHRW	Mussel - OTC 0	*Vibrio* sp.	*A. aminovorans*	
06MHRW	Mussel - OTC 50	*Vibrio* sp.	*V. splendidus*	*V. pomeroyi/ V. splendidus*
07MHRW	Mussel - OTC 50	*Pseudomonas* sp.	No ID	*Shewanella sp.*
01SCEW	Sediment - OTC 0	*Vibrio* sp.	No growth	
02SCEW	Sediment - OTC 0	*Pseudomonas* sp.	No ID	
03SCEW	Sediment - OTC 0	*Vibrio* sp.	*V. aestuarianus*	
04SCEW	Sediment - OTC 0	*Vibrio* sp.	No ID	
05SCEW	Sediment - OTC 0	*Vibrio* sp.	*V. carcariae*	
06SCEW	Sediment - OTC 0	*Vibrio* sp.	*V. carcariae*	
07SCEW	Sediment - OTC 50	*Pseudomonas* sp.	No growth	

01MCEW	Mussel - OTC 0	*Vibrio* sp.	No growth	
02MCEW	Mussel - OTC 0	*Vibrio* sp.	No growth	
03MCEW	Mussel - OTC 0	*Vibrio* sp.	No growth	
04MCEW	Mussel - OTC 0	*Vibrio* sp.	*Vibrio*	
05MCEW	Mussel - OTC 0	*Vibrio* sp.	No growth	
06MCEW	Mussel - OTC 0	*Aeromonas* sp.	No growth	
07MCEW	Mussel - OTC 50	*Vibrio* sp.	No growth	*V. tasmaniensis/ V. supersteus*
08MCEW	Mussel - OTC 50	*Aeromonas* sp.	No growth	*V. haliticoli*
09MCEW	Mussel - OTC 50	*Shewanella* sp.	No growth	*S. waksmanii*
10MCEW	Mussel - OTC 50	*Vibrio* sp.	No growth	
11MCEW	Mussel - OTC 50	*Vibrio* sp.	No growth	*Artic sea bacterium/ marine sediment bacterium*
12MCEW	Mussel - OTC 50	*Aeromonas* sp.	No growth	
1SMRW	Sediment - OTC 0	*Vibrio* sp.	*V. tubiashi*	
2SMRW	Sediment - OTC 0	*Vibrio* sp.	No growth	
3SMRW	Sediment - OTC 0	*Shewanella* sp.[+]	*V. tubiashi*[§]	
4SMRW	Sediment - OTC 0	*Vibrio* sp.	No ID	
5SMRW	Sediment - OTC 0	Lost	Lost	
6SMRW	Sediment - OTC 0	*Shewanella* sp.	*V. tubiashi*	
7SMRW	Sediment - OTC 0	*Shewanella* sp.[+]	No growth	
8SMRW	Sediment - OTC 50	*Vibrio* sp.	*B. glumae*	*S. gaetbuli*
9SMRW	Sediment - OTC 50	*Shewanella* sp.[+]	CDC group II-E subgroup A	*S. affinis/ S. colwelliana*
1MMRW	Mussel - OTC 0	*Vibrio* sp.	No growth	
2MMRW	Mussel - OTC 0	*Vibrio* sp.	*V. tubiashi*	
3MMRW	Mussel - OTC 0	*Vibrio* sp.	*V. aestuarianus V. splendidus*	
4MMRW	Mussel - OTC 0	*Vibrio* sp.	No growth	
5MMRW	Mussel - OTC 0	*Vibrio* sp.	No growth	

6MMRW	Mussel - OTC 0	*Vibrio* sp.	No growth	
7MMRW	Mussel - OTC 50	*Vibrio* sp.	No growth	*V. halioticoli*
8MMRW	Mussel - OTC 50	*Vibrio* sp. or *Aeromonas* sp.	No ID	*V. harveyi/ V. alginolyticus*
9MMRW	Mussel - OTC 50	*Vibrio*	No growth	
10MMRW	Mussel - OTC 50	*Pseudomonas* sp.	*V. splendidus*	
11MMRW	Mussel - OTC 50	*Vibrio* sp.	No growth	
12MMRW	Mussel - OTC 0	*Vibrio* sp.	No growth	
13aMMRW	Mussel - OTC 0	*Vibrio* sp.	*Vibrio splendidus*	
13bMRW	Mussel - OTC 0	*Vibrio* sp.	No ID	
14MMRW	Mussel - OTC 50	*Vibrio* sp.	*A. veronii* DNA group 10	*Vibrio sp.*
15aMMRW	Mussel - OTC 0	*Vibrio* sp.	*V. tubiashi*	
15bMMRW	Mussel - OTC 0	*Pseudomonas* sp.	*V. tubiashi*	
1SHRC	Sediment - OTC 50	*Shewanella* sp.	*B. glumae*[§]	*Shewanella sp.*
2SHRC	Sediment - OTC 50	*Pseudomonas* sp.	No growth	*Shewanella sp.*
3SHRC	Sediment - OTC 50	*Shewanella*	*B. glumae*[§]	*Shewanella sp.*
4SHRC	Sediment - OTC 50	*Vibrio* sp.	No ID	
5SHRC	Sediment - OTC 50	*Pseudomonas* sp.	*B. glumae*[§]	*Shewanella sp.*
6SHRC	Sediment - OTC 50	*Shewanella* sp.	No ID	*S. frigidimarina/ S. baltica*
7SHRC	Sediment - OTC 50	*Shewanella* sp.	*B. glumae*	*Shewanella sp.*
8SHRC	Sediment - OTC 50	*Vibrio* sp.	*V. vulnificus*	*V. splendidus*
9SHRC	Sediment - OTC 0	*Aeromonas* sp.	*V. tubiashi*	
10SHRC	Sediment - TCBS 0	*Vibrio* sp.	*V. tubiashi*	
11SHRC	Sediment - OTC 50	*Pseudomonas* sp.	No ID	*S. woodyi*
1MHRC	Mussel - OTC 50	*Vibrio* sp.	No ID	*V. splendidus*
2MHRC	Mussel - OTC 0	*Pseudomonas* sp.	No ID	
3MHRC	Mussel - OTC 0	*Vibrio* sp.	No ID	
4MHRC	Mussel - OTC 50	*Vibrio* sp.	*V. proteolyticus*	*V. tasmaniensis*
5MHRC	Mussel - OTC 0	*Vibrio* sp.	*V. alginolyticus*	

1SCEC	Sediment - OTC 50	*Shewanella* sp.	*V. tubiashi*[§]	*S. baltica*
2SCEC	Sediment - OTC 50	*Aeromonas* sp.	No ID	*Pseudoalteromonas sp.*
3SCEC	Sediment - OTC 50	*Vibrio* sp.	*V. vulnificus*	*V. splendidus*
1MCEC	Mussel - OTC 50	*Shewanella*	No growth	*Shewanella sp.*
2MCEC	Mussel - OTC 50	*Pseudomonas* sp.	*V. tubiashi*	
3MCEC	Mussel - OTC 50	*Vibrio* sp.	*V. splendidus* *V. vulnificus*	*V. splendidus*
1SMRC	Sediment - OTC 50	*Pseudomonas* sp.	No growth	*Shewanella sp.*
2SMRC	Sediment - OTC 50	*Pseudomonas* sp.	No growth	*Shewanella sp.*
3SMRC	Sediment - OTC 50	*Shewanella* sp.	*B. glumae*[§]	*Shewanella sp.*
4SMRC	Sediment - OTC 50	*Pseudomonas*	*V. tubiashi*	*S. colwelliana*
5SMRC	Sediment - OTC 50	*Aeromonas* sp.	No ID	*Pseudoalteromonas sp.*
6SMRC	Sediment - OTC 50	*Aeromonas* sp.	No ID	*Pseudoalteromonas sp.*
7SMRC	Sediment - OTC 50	*Vibrio* sp.	*V. alginolyticus*	*V. campbelli*
1MMRC	Mussel - OTC 50	*Pseudomonas* sp.	No growth	*Shewanella sp.*
2MMRC	Mussel - OTC 50	*Shewanella* sp.	No ID	*Shewanella sp.*
3MMRC	Mussel - OTC 50	*Vibrio* sp.	*V. harveyi*	
4MMRC	Mussel - OTC 50	*Aeromonas* sp.	No ID	*Pseudoalteromonas sp.*
5MMRC	Mussel - OTC 50	*Vibrio* sp.	No ID	*V. gallicus*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; [+] : *S. putrefasciencis* according to RapID NF Plus System; [§]: Biolog ID with too few positives matches

3.4 DISCUSSION

Each of the methods used for bacterial identification have their limitations but the combination of phenotypic methods and the analysis of genomic information, described as polyphasic approach, is the most recommended for taxonomic classification and identification of a bacterial isolate (Vandamme, 1996, 2003; Stackebrandt, 2002).

The conventional phenotypic characterization gave a taxonomical framework from which presumptive identification to the level of genus could be obtained. One of the disadvantages of analyzing the phenotype is that just part of the genome is expressed since it depends directly on the environmental growing conditions offered in the laboratory (Roselló-Mora and Amann, 2001). However phenotypic characterization delineates the taxonomic position and gives useful information about the physiological and ecological functions of bacteria (Vandamme, 2003). There was no attempt to fully characterize the isolates biochemically since metabolic characterization, using the Biolog system, was also performed. The presumptive genera identified are commonly found in marine environments. Bacteria of the genera *Vibrio, Pseudomonas, Shewanella*, and *Aeromonas* have been isolated from marine water, sediments and mussels (Hariharan et al., 1995; Cavallo et al., 1999; Maugeri et al, 2000; Croci et al., 2001; Hervio-Heath et al., 2002; Ivanova et al., 2003a,b).

The Biolog system has been used for many years in bacterial identification but, its use has been more oriented to bacteria of clinical importance. There are, however, some reports on the use of Biolog identification system in marine environmental bacteria (Ivanova et al., 1998; Makemson et al, 1998), and in vibrios of importance for aquaculture (Austin et al., 1995; Noble and Gow, 1998; Vandenberghe et al., 1999,

2003). Biolog identification of bacteria should be interpreted with caution since results can be affected by the inoculum density, the composition of the agar medium from which the cells are taken (Makemson et al., 1998), and the salt composition of the inoculum fluid (Noble and Gow, 1998).

There were several isolates that could not be identified because of the lack of growth in the GN microplates or because the degree of similarity (SIM) was too low. It is difficult to explain the reason for the lack of growth in the GN microplates of some isolates since reasonable growth occured in the BUG medium at the same incubation temperature. Since most of the isolates required sodium chloride for their growth, the addition of 1.5% NaCl was used in both the BUG medium and inoculating fluid and an incubation temperature of 22°C appeared optimal. This is in accordance to the environmental conditions to which the isolates are naturally exposed. The supplementation of the media and inoculating fluid with marine salts such as calcium salts was not attempted since such modifications are reported to decrease the reliability of the identification (Makemson et al., 1998; Noble and Gow, 1998).

Among the isolates that could be identified there are several species of vibrios which are reported to be commonly found in marine environments, in water, sediments and associated with marine invertebrates (Venkateswaran et al., 1989; Hariharan et al., 1995; Maugeri et al., 2000; Croci et al., 2001; Hervio-Heath, 2002). The most commonly identified species were *V. splendidus* and *V. tubiashi,* mostly recovered from mussels and sediments, respectively. Hariharan et al. (1995) also reported *V. splendidus* to be one of the predominant species isolated from mussels from marine environments of Prince Edward Island (PEI). In fact, *V. splendidus*-like species are distributed widely in coastal

areas, constituting a considerable proportion of culturable vibrios in low temperature environments (Urakawa et al., 1999). *V. tubiashi* was also previously found in PEI mussels but not as part of the predominant flora (Hariharan et al., 1995).

An interesting aspect from the identification of the vibrios was that two isolates recovered from mussels were identified as two different species after different incubation periods. The isolate 3MMRW was identified as *V. aestuarianus* after 24 h of incubation and as *V. splendidus* after 48 h of incubation. Likewise, the isolate 3MCEC was identified as *V. splendidus* after 24 h of incubation and as *V. vulnificus* after 72 h of incubation. Occasional changes of bacterial identification as the incubation progressed were also noted previously (Klinger et al., 1992). This suggests that some species have similar metabolic patterns in the Biolog GN microplates which are difficult to differentiate. Misidentification of some *Vibrio* species by the Biolog system has been reported and it is considered nearly impossible to distinguish species based solely on the phenotypic profile (Thompson et al., 2004). It appears that the single carbon source approach of Biolog does not have sufficient resolution to differentiate between *Vibrio* species (Vandenberghe et al., 2003). A species frequently identified by the Biolog identification system was *Burkholderia glumae*. Bacteria of the genus *Burkholderia* (formerly *Pseudomonas*) are mainly plant pathogens and soil bacteria, but also include some animal pathogens. *B. glumae,* in particular, is a plant pathogen associated with the rot of rice grains and seedlings (Coenye and Vandamme, 2003). Therefore it is most likely that this identification was erroneous. Most of the isolates identified as *B. glumae* by the Biolog system were presumptively identified, according to the conventional phenotypic characterization and to the 16S rDNA sequence similarity, as *Shewanella* sp.,

which are commonly found in marine environments (Ivanova et al., 2003a,b). Some species of *Shewanella* not included in the database of the Biolog system and *B. glumae* likely have very similar metabolic patterns. Other bacterial groups identified were *Shewanella putrefaciens* A, *Aminobacter aminovorans*, *Aeromonas veronii* DNA group 10, and CDC group II-E subgroup A. The isolate 5SHRW identified as *Shewanella putrefaciens* has the basic phenotypic characteristics of the genus *Shewanella*. Although automated identification systems are reportedly unable to distinguish *S. putrefaciens* from *S. algae*, the identification database of gram-negative aerobic bacteria of Biolog (release 4.01) includes both species. This identification was confirmed through its non hemolytic character, which is one of the primary differences between *S. putrefaciens* and *S. algae* (Holt et al., 2005). Species that belong to the genus *Aminobacter* can produce acids from sugar only oxidatively but not fermentatively (Kampfer et al., 2002), whereas the isolate 5MHRW identified as *A. aminovorans* by the Biolog system could ferment sugars, which indicates that the identification was doubtful. Species of *A. veronii* belonging to different biogroups are able to produce H_2S from thiosulfate (Joseph et al., 1991), while the isolate 14MMRW identified as *A. veronii* was H_2S negative in TSI agar, which indicates that the identification was again doubtful.

Molecular identification based upon the degree of similarity of the 16S rDNA sequence is being more frequently used. The degree of similarity of 16S rDNA sequences produces numerical values that can be used as circumscription limits of taxa. Species are considered to include strains with approximately 70% or greater DNA-DNA relatedness (Wayne et al., 1987), which has been found to be equivalent to 97%, or more, similarity of the 16S rDNA sequence (Stackebrandt and Goebel, 1994). However, it is worth

mentioning that these cut-off values are not absolute since there are strains that share a DNA relatedness lower than 50% and hence are classified as different species but have a similarity of 99-100% in their 16S rDNA sequence (Roselló-Mora and Mendoza, 2004). This lack of concordance between similarity degrees in sequences of 16S rDNA and DNA homology has been found in some *Shewanella* species (Toffin et al., 2004; Ivanova et al., 2004a).

By sequencing and comparing the 16S rDNA of the marine isolates three different genera, which included some species, could be identified. The majority of the isolates were identified as *Vibrio* sp., which were mostly recovered from mussels, and *Shewanella* sp., which were mostly recovered from sediments. A few isolates recovered from mussels and sediments were identified as *Pseudoalteromonas* sp.. Among *Vibrio* species identified, *V. splendidus* was the most frequently identified species which is in concordance with the Biolog's most frequently identified species. However not all the isolates identified as *V. splendidus* by the molecular method agreed with the Biolog identification. The isolates 7SHRW, 8SHRW, 6MHRW, 8SHRC, 1MHRC, 3SCEC and, 3MCEC were identified as *V. splendidus*. The Biolog system had also identified the isolates 7SHRW, 6MHRW and 3MCEC as *V. splendidus* whereas the isolates 8SHRC and 3SCEC had been identified as *V. vulnificus* and the isolates 1MHRC and 8SHRW could not be identified. These isolates, with the exception of 8SHRW that did not show growth in the microplates, had a metabolic profile (according to Biolog GN microplates) similar to that reported for both biotypes of *V. splendidus* (Alsina and Blanch, 1994). In contrast to the isolates 7SHRW, 8SHRW, 8SHRC, 3SCEC, and 3MCEC, the isolate 1MHRC could not grow in medium without sodium chloride which is in accordance to

the description of this species (Alsina and Blanch, 1994; Austin and Austin, 1999). The phenotypic profile of *V. splendidus* is heterogenic (Thomson et al., 2005) which makes its identification difficult (Austin and Austin, 1999).

The isolate 6MHRW had high 16S rDNA sequence similarity (98%) with *V. pomeroyi*. Its metabolic pattern (according to Biolog GN microplates) differs with what is reported for *V. pomeroyi* in that it could utilize D-glucoronic acid but not succinic acid (Thompson et al., 2003a). The isolate 8MCEW identified as *V. halioticoli* was resistant to the vibriostatic agent O129 which indicates that it cannot belong to this species (Sawabe et al., 1998). Another isolate identified as *V. halioticoli,* 7MMRW, could utilize sucrose or lactose (fermentatively) but this species is reported not to utilize any of these sugars (Sawabe et al., 1998). The isolates 7MCEW, identified as *V. tasmaniensis* or *V. supersteus,* and 4MHRC, identified as *V. tasmaniensis*, were able to ferment glucose and sucrose. This feature suggests they are not *V. tasmaniensis,* a species that ferments glucose but not sucrose (Thompson et al., 2003b). The isolate 8MMRW identified as *V. harveyi* or *V. alginolyticus* could grow in the absence of sodium chloride in contrast to most vibrios including these two species (Alsina and Blanch, 1994; Austin and Austin, 1999).

There were several isolates identified as *Shewanella* sp. and some were speciated. Among the species identified *S. colwelliana, S. baltica, S. gaetbuli, S. woodyi, S. frigidimarina* and *S. affinis* were obtained from sediments while *S. waksmanni* was obtained from mussels. None of these species constituted a majority. The isolate 4SMRC identified as *S. colwelliana*, did not produce H_2S (in TSI agar), which is in accordance with this exceptional characteristic of this species (Venkateswaran et al., 1999). This

isolate could not utilize oxidatively D-glucose, D-galactose, D-L acid lactic, succinic acid or citric acid (according to Biolog GN microplates) which is all in concordance with *S. colwelliana* (Ivanova 2003a). The isolate 9SMRW identified as *S. affinis* or *S. colwelliana* produced H_2S, which does not correlate with an identification of *S. colwelliana*. It was not hemolytic and therefore not *S. affinis* (Ivanova et al 2004a). The isolate 6SHRC identified as *S. baltica* or *S. frigidimarina* was unable to utilize neither glucose fermentatively (in TSI agar) nor sucrose oxidatively (according to Biolog GN microplates). The isolate 1SCEC identified as *S. baltica* was unable to utilize sucrose oxidatively. *S. frigidimarina* is reported to be one of the species of *Shewanella* that ferments glucose (Ivanova et al., 2003c), while *S. baltica* is able to utilize sucrose oxidatively (Ziemke et al., 1998). This indicates that these isolates do not belong to either of these species. The isolate 11SHRC identified as *S. woodyi* was not able to ferment glucose (in TSI agar), and in general had a metabolic pattern (according to Biolog GN microplates) that matched the description of this species with the exception of the utilization of the substrates D-glucoronate and N-acetylglucosamine (Makemson et al., 1997). The isolate 9MCEW identified as *S. waksmanii* was not hemolytic which disagrees with the character of this species (Ivanova 2003b). The isolate 8SMRW, identified as *S. gaetbuli*, could grow in a medium without sodium chloride which is in contrast to what is reported for this species (Yoon et al., 2004). There were four isolates that were identified as *Pseudoalteromonas* sp., 2SCEC, 5SMRC, 6SMRC and 4MMRC. However, all of them were glucose and sucrose fermentative which is in contrast to one of the main features of this genus that is the absence of fermentative metabolism (Gauthier et al., 1995).

The inaccuracy in the identification to the level of species found in several isolates is not uncommon since the genomic information obtained in the 16S rRNA sequence is considered to lack resolving power at the species level (Roselló-Mora and Amman, 2001). It was found in this study that bacteria that had 97% 16S rDNA sequence similarity with the isolates do not necessarily share the same species identification, as suggested in other reports. Kita-Tsukamoto et al. (1993), working with a broad collection of isolates of the *Vibrionaceae* family, considered that the circumscription for species is at least 99.2% 16S rDNA sequence similarity. An alternative method to the 16S rDNA sequence similarity analysis for taxonomical studies, especially for very closely related bacteria, is the analysis of housekeeping genes such as the DNA gyrase B subunit gene, *gyrB,* which is consistent with DNA-DNA hybridization. This analysis has been used to obtain a clearer taxonomic differentiation among species of the genus *Vibrio* (Le Roux et al., 2004) and *Shewanella* (Venkateswaran et al., 1999), among other genera. Another alternative method considered to be reliable for identification of *Vibrionaceae* is the amplified fingerprint length polymorphism (AFLP) analysis (Thompson et al., 2004).

In conclusion, methods including phenotypic characterization and genomic information were used in this study for the identification of marine bacterial isolates. The conventional method gave a general taxonomic frame for the isolates. The Biolog system's inability to identify several of the isolates was probably because of the limited environmental marine bacteria in the database. However, the metabolic information obtained was helpful for a more complete phenotypic characterization. The analysis of the 16S rDNA sequence similarity gave a partial identification complemented by the phenotypic characterization to obtain a final identification. Among the isolates obtained

from sediments and mussels of three different representative sites of PEI, recovered on TCBS plates containing OTC, two genera were identified most consistedly, *Vibrio* and *Shewanella.* Within each of these genera, one species was identified with certain degree of confidence, *V. splendidus* and *S. colwelliana.* The isolates whose phenotypic characteristics did not result in identification to a species level were grouped as *Vibrio* sp. or *Shewanella* sp according to their high 16S rDNA sequence similarity.

CHAPTER 4: ANTIMICROBIAL SUSCEPTIBILITY OF *VIBRIO* AND *SHEWANELLA* ISOLATES FROM PEI MARINE ENVIRONMENTS

4.1 INTRODUCTION

Susceptibility testing is mainly performed on bacterial pathogens that contribute to an infectious process which require antibiotic intervention (Barry, 1976). Susceptibility tests are also important in studies of the epidemiology of resistance in different types of environments including the aquatic environment and in studies of new antimicrobial agents (NCCLS, 2002, 2003).

Susceptibility testing may be performed reliably by broth or agar dilution, or agar diffusion methods. Dilution methods are quantitative methods done to determine the minimum inhibitory concentration (MIC) of an agent whereas disk diffusion methods are qualitative methods based on the occurrence and size of zones of inhibition. Both methods are reliable since the size of the zone of inhibition of the disk diffusion method and the MIC values are directly related and both methods have similar interlaboratory and intralaboratory reproducibilities for reference strains. Therefore, the choice of methodology may be based on factors such as relative ease of performance, cost, flexibility in selection of drugs for testing, use of automated or semi automated devices to facilitate testing, and the perceived precision of the methodology (Jorgensen and Turnidge, 2003).

Data generated by laboratory susceptibility tests, regardless of method, must be related to probable clinical efficacy of any therapy. Therefore, critical breakpoint values must be established, so that strains tested can be placed in one of the clinically relevant categories. The laboratory test, dilution MIC or disk diffusion is performed on a large

number of strains of a particular species. Provided that these values show bimodal or polymodal distributions, the data can then be used to establish breakpoint values to differentiate between sensitive, resistant and intermediate strains. In some studies of fish pathogens, these critical values have been established by analysis of laboratory data alone (Smith et al., 1994).

4.1.1 Disk diffusion test

Currently, the disk diffusion test most often used is the Kirby-Bauer method, developed in the early 1960s (Barry 1976; Prescott et al., 1990). Disk diffusion assays are frequently the method of choice in diagnostic laboratories where information is required rapidly on the sensitivity of a limited number of isolates to a range of antibiotics (Smith et al., 1994). To perform the test, filter paper disks impregnated with a specific single concentration of an antimicrobial agent are applied to the surface of an agar medium inoculated with the test organism. The drug in the disk diffuses through the agar creating a gradient of drug concentrations in the agar medium surrounding each disk. Concomitant with diffusion of the drug, the inoculated bacteria multiply until a lawn of growth is visible. In areas where the concentration of drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk. The diameter of the zone of inhibition is influenced by the rate of diffusion of the antimicrobial agent through the agar, which may vary among different drugs depending upon the size of the drug molecule and its hydrophilicity (Jorgensen and Turnidge, 2003).

When performing susceptibility tests using a disk diffusion test, it is very important that the composition, pH, humidity and volume of the medium, the incubation

temperature, the method of production of the inoculum, and the size and method of inoculation are standardized (Smith et al, 1994). Standardized techniques have been described on international and national levels (Barry, 1976). In USA, the National Committee for Clinical Laboratory Standards (NCCLS) has standardized the susceptibility methods for clinical bacterial pathogens of human and animal origin, and also fish pathogens. The latter is based on the Workshop on MIC Methodologies in Aquaculture, Weymouth 1998, and the subsequent publication by Alderman and Smith (2001) regarding the draft protocols developed at the workshop. However, no interpretative breakpoints for bacteria of aquatic environments are currently in use and quality control limits for testing halophilic organisms on media supplemented with sodium chloride have not been established (NCCLS, 2003).

4.1.2 Gradient diffusion method: E test

The E test (AB Biodisk, Solna, Sweden) is a quantitative technique for determination of antimicrobial susceptibility of bacteria. The E test is performed with a non-porous plastic strip applied in an agar plate from which an antimicrobial gradient diffuses into the agar medium inoculated with the test organism. After incubation, the MIC is read directly from a scale in the strip where a symmetrical inhibition ellipse, formed by the growth of the organism, intersects it. This method combines the simplicity and flexibility of the disk diffusion test with the ability to determine the MIC of an antibiotic to the test organism. (Jorgensen and Turnidge, 2003).

In this study the susceptibility of the bacteria isolated from PEI marine environments against several antibiotics belonging to different families is assessed using

the disk diffusion susceptibility test. The susceptibilities were analyzed according to the season in which samples were obtained and the bacterial genus. In addition, the assessment of MIC by means of the E test of OTC in TCBS and MHA with the addition of 1.5% NaCl was performed to marine bacterial isolates in order to evaluate the differences.

4.2 MATERIALS AND METHODS

4.2.1 Disk diffusion test

4.2.1.1 Starting culture and inoculum preparation

Bacterial isolates from mussels and sediments from Hillsborough Rv., Cap Egmont and Murray Rv, PEI, recovered on TCBS plates containing 50 μg/mL OTC and stored frozen at -80°C in glycerol 20%, were used for disk diffusion sensitivity tests. Since the NCCLS (2003) have no quality control organisms suggested for testing halophilic isolates on media supplemented with sodium chloride, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 and *Escherichia coli* ATCC 25922 were used as control organisms. The media and procedure used in this test was in accordance with the NCCLS (2003) guidelines. Marine bacterial isolates were cultured on Tryptic Soy Agar TSA (pH 7.3, Oxoid Ltd.) with the addition of 1.5% NaCl, and incubated for 48 h at 22°C. The control organisms were cultured in Trypticase Soya Agar (TSA) and incubated for 24 h at 22°C. A minimum of three colonies were emulsified in sterile 0.85% saline and optical densities were measured on a Ultrospec II spectrophotometer (LKB Biochrom) at 620 nm until reaching a reading in the range of 0.08 to 0.10 which correspond to a concentration of 1 to 2 x 10^8 colony forming units (CFU)/mL, as confirmed by plate counts.

4.2.1.2 Inoculation of test media and application of disks

The culture medium used was Mueller-Hinton agar (MHA, Oxoid Ltd.) without NaCl addition for the control organisms and with the addition of 1.5% NaCl for the marine bacterial isolates and in one occasion for the control organisms, to evaluate differences caused by different media conditions. The surface of MHA plates was

inoculated by streaking with a sterile cotton swab, dipped into the inoculum suspension. The plates were then rotated 60° and the rubbing procedure repeated, followed by a second rotation, the agar was swabbed for third time. After some few minutes to allow the absorption of the inoculum, a maximum of 4 disks of different antibiotics were applied in each 100-mm plate using a commercial dispenser. The antibiotic disks used were amoxicillin/clavulanic acid (30 μg), ampicillin (10 μg), carbenicillin (100 μg), cephotaxime (30 μg), cephalothin (30 μg), enrofloxacin (5 μg), oxolinic acid (2 μg), ciprofloxacin (5μg), flumequine (30 μg), amikacin (30 μg), gentamicin (10 μg), streptomycin (10 μg), oxytetracycline (30 μg), tetracycline (30 μg), trimethoprim-sulphamethoxazole (25 μg), chloramphenicol (30 μg), erythromycin (15 μg), from Oxoid Ltd., and ormetoprim-sulfadimethoxine (25 μg), florfenicol (30 μg), from BBL™. The plates were incubated at 22°C for 24-48 h and the zones of inhibition were read with a transparent ruler to the nearest millimeter. Despite the NCCLS suggestion to read plates at 24 and 48 h, only a measurement at 48 h was considered for interpretation as suggested by Nic Gabhainn et al. (2004).

4.2.1.3 Interpretation of the results

The inhibition zones of the reference organisms were compared with those quality control ranges established by the NCCLS (2003). Since there are no interpretative standard breakpoints for aquatic bacteria the standard breakpoints determined for animals (NCCLS, 2002) were used instead. It is important to mention that these breakpoints have been established for clinical bacteria of terrestrial animals at their optimal temperature which is higher than the optimal for marine environmental bacteria. Cautious

interpretation of the results is required since reference values used for comparison were not defined for this purpose. Since breakpoints have not been established for all 19 antibiotics used in this study, breakpoints were based on antibiotics of the same family (Appendix C.1) (Jorgensen and Turnidge, 2003). Since *Shewanella* and *Vibrio* isolates constitute the vast majority of the flora recovered in TCBS agar, bacterial isolates belonging to other genera were not considered for interpretation and any isolates whose identification were doubtful.

4.2.2 E test

In order to determine the effect of MHA with 1.5% NaCl and TCBS media in the activity of OTC, the susceptibility of 34 isolates using E test strips (AB Biodisk, Solna, Sweden) were tested. Bacterial isolates, including *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 as the control organism, were cultured on Tryptic Soy Agar (TSA) (pH 7.3, Oxoid Ltd.) with the addition of 1.5% NaCl and incubated for 48 h at 22°C. The inoculum suspension was prepared after emulsifying a minimum of three colonies in sterile 0.85% saline and optical densities were measured on a Ultrospec II spectrophotometer (LKB Biochrom) at 620 nm. A reading in the range of 0.08 to 0.10 corresponding to a concentration of 1 to 2 x 10^8 colony forming units (CFU)/mL was used and confirmed by plate counts. The surface of plates containing TCBS (pH 8.6; Oxoid Ltd.) and MHA (pH 7.4; Oxoid Ltd.) with the addition of 1.5% NaCl were inoculated by streaking with a sterile cotton swab that was dipped into the inoculum suspension. The plates were then rotated 60° and the rubbing procedure repeated, followed by a second rotation the agar was swabbed for a third time. After several

minutes to allow the absorption of the inoculum, an E test strip was placed in each plate. The plates were incubated at 15°C for 5 days and the zones of inhibition were read and compared.

In order to determine any significant difference among the MIC values for OTC obtained from the E tests using 2 main bacterial genera (*Shewanella* and *Vibrio*) in 2 different culture media (TCBS and MHA 1.5% NaCl) a 2-way analysis of variance (ANOVA) was performed at a statistical significance level of 0.05. After the data was Box-Cox analyzed for optimal transformation, to meet the model assumptions of ANOVA, it was power transformed with a power of -0.22.

4.3 RESULTS

4.3.1 Disk diffusion test

The inhibition zones formed by the reference strains against 19 antibiotics, for quality control, are shown in Appendix C.2. The sizes of the inhibition zones are within the quality control range for established ranges (9 antibiotics) by the NCCLS, with very few exceptions. Since those quality control ranges are for organisms tested in media without sodium chloride supplementation, they can not be used for the reference strains cultured in MHA with 1.5% NaCl. However it was noticed that even with sodium chloride supplementation the inhibition zones mostly fit inside the quality control ranges for organisms cultured without this supplementation.

The results of the inhibition zones formed for bacterial isolates from marine environments of PEI against 19 antibiotics are shown in Appendixes C3 - C8. The percentages of bacterial isolates non susceptible to each of the 19 antibiotics tested,

according to the criteria mentioned above, are shown in Table 4.1 and Figures 4.1 - 4.5. There was a high percentage of non susceptible (resistant and intermediate) isolates against 2 antibiotics. There was a low percentage of non susceptible isolates against 7 antibiotics. All the isolates tested were susceptible to the remaining 10 antibiotics (Figure 4.1). The highest percentage of non susceptibility was against the aminoglycoside streptomycin (89.5% resistance, 5.3% intermediate resistance), whereas there were low percentages of non susceptibility to the β-lactams ampicillin, amoxicillin/clavulanic acid, carbenicillin, cephalothin, the quinolone oxolinic acid, and the aminoglycosides gentamicin and amikacin. All isolates were susceptible to the β-lactam cefotaxime, the quinolones enrofloxacin, ciprofloxacin and flumequine, both tetracyclines, both potentiated sulfonamides, and both phenicols. Although the percentage of non susceptibility to the macrolide erythromycin is considerable (44.7%), this corresponds entirely to intermediate resistant isolates. After separating the isolates obtained during the warm season from those ones obtained during the cold season, a clear difference can be noticed between seasons in the percentage of non susceptible isolates against most of the antibiotics (Figure 4.2; Table 4.1). With the exception of amoxicillin/clavulanic acid and streptomycin, to which there was a slightly higher percentage of non susceptible isolates in the cold season, the remaining 7 antibiotics showed a markedly higher percentage of resistant isolates when the bacterial isolates were obtained in the warm season. After separating the isolates according to genus, it was noticed that there were higher percentages of non susceptible bacteria among *Vibrio* isolates in comparison with *Shewanella* isolates to most of the antibiotic tested with the exception of cephalothin and streptomycin, to which there was a slightly higher percentage of non susceptible isolates

among *Shewanella* isolates (Figure 4.3, Table 4.1). Considering bacterial isolates of each genus separatedly according to the season in which they were obtained, similar percentages of non susceptible *Vibrio* isolates occurred during both seasons to most of the antibiotics tested (Figure 4.4, Table 4.1). Among *Shewanella* isolates there is a higher percentage of non susceptible isolates during the warm season and markedly lower (or none) during the cold season to most antibiotics tested with the exception of streptomycin and amoxicillin/clavulanic acid. There was a high percentage of streptomycin resistant isolates in both seasons. With regard to amoxicillin/clavulanic acid, all isolates in the warm season were susceptible and a low percentage was non susceptible during the cold season (Figure 4.5, Table 4.1).

There is a considerable proportion (40%) of bacterial isolates non susceptible to a single drug. Sixteen percent of the bacterial isolates were non susceptible to 4 antibiotics and to 5 antibiotcs, 11% of the bacterial isolates were non susceptible to 2 antibiotics and to 3 antibiotics. Only 5% of the bacterial isolates were non susceptible to 6 antibiotics (Figure 4.6, Table 4.2). Isolates obtained during the warm season were more frequently non susceptible to higher number of antibiotics in contrast to those obtained during the cold season which in general were non susceptible to lower number of antibiotics. The modal value for the isolates obtained in the warm season was 4 antibiotics and for those ones obtained in the cold season was a single antibiotic (Figure 4.7, Table 4.2). After separating the isolates according to their genus, it was noticed that in general *Vibrio* isolates were non susceptible to higher number of antibiotics than *Shewanella* isolates. The exceptions were those isolates non susceptible to 6 antibiotics that belong to the genus *Shewanella*. The modal value for *Shewanella* isolates was only a

single antibiotic whereas for *Vibrio* isolates was 4 antibiotics (Figure 4.8, Table 4.2). The pattern of non susceptibility to multiple antibiotics obtained in warm and cold season for *Vibrio* and *Shewanella* isolates is shown in Table 4.3. The antibiotics to which bacterial isolates were most frequently resistant are streptomycin and erythromycin.

4.3.2 E test

The minimum inhibitory concentrations (MIC) of OTC against the bacterial isolates are indicated in Table 4.4. It can be noticed that some isolates had very high MIC (> 256 µg/mL) especially *Shewanella* isolates tested in TCBS. In contrast, other isolates had very low MIC (0.125 - 0.380 µg/mL), especially *Vibrio* isolates tested in MHA 1.5% NaCl. These variations of the MICs, according to the media used and to the genus of the isolate, were strongly significant ($P < 0.0001$). Using Bonferroni-corrected pairwise comparisons, all experimental conditions were shown to be significantly different. The estimated medians of the MICs and the confidence intervals of each experimental condition are shown in Table 4.5. Minimum inhibitory concentrations are higher when TCBS medium was used and this was more markedly in *Shewanella* isolates.

Table 4.1. Percentages of non susceptible* marine bacterial isolates obtained from representative sites of PEI according to their genus and season

Group	Antibiotic	Non susceptibility (%)								
		Vibrio (n=18)			*Shewanella* (n=20)			Total (n=36)		
		Warm	Cold	Total	Warm	Cold	Total	Warm	Cold	Total
Beta-lactams	AMP	30	25	27.8	40	6.7	15	33.4	20	21 (18.4/2.6)
	AMC	0	0	0	0	6.7	5	0	4.3	2.6 (2.6/0)
	CAR	20	25	22.3	20	6.7	10	20	13	15.8 (10.5/5.3)
	CTX	0	0	0	0	0	0	0	0	0
	KF	20	12.5	16.7	80	25	30	40	13	23.7 (18.4/5.3)
Quinolones	ENR	0	0	0	0	0	0	0	0	0
	OA	40	50	44.5	20	0	5	33.4	17.4	23.6 (2.6/21)
	CIP	0	0	0	0	0	0	0	0	0
	UB	0	0	0	0	0	0	0	0	0
Aminoglycosides	CN	30	37.5	33.3	20	0	5	26.7	13	18.4 (7.9/10.5)
	AK	40	37.5	38.9	20	0	5	33.3	100	21.1 (7.9/13.2)
	S	80	100	88.9	100	100	100	86.7	0	94.8 (89.5/5.3)
Tetracyclines	OT	0	0	0	0	0	0	0	0	0
	TE	0	0	0	0	0	0	0	0	0
Potentiated Sulfonamides	SOR	0	0	0	0	0	0	0	0	0
	SXT	0	0	0	0	0	0	0	0	0
Phenicols	FFC	0	0	0	0	0	0	0	0	0
	C	0	0	0	0	0	0	0	0	0
Macrolide	E	90	75	83.3	40	0	10	73.3	26.1	44.7 (0/44.7)

*: Non susceptible = resistant + intermediate; Ant.: antibiotic; *Shew.*: *Shewanella*; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin; Number in parenthesis correspond to the percentages of resistance and intermediate resistance, respectively.

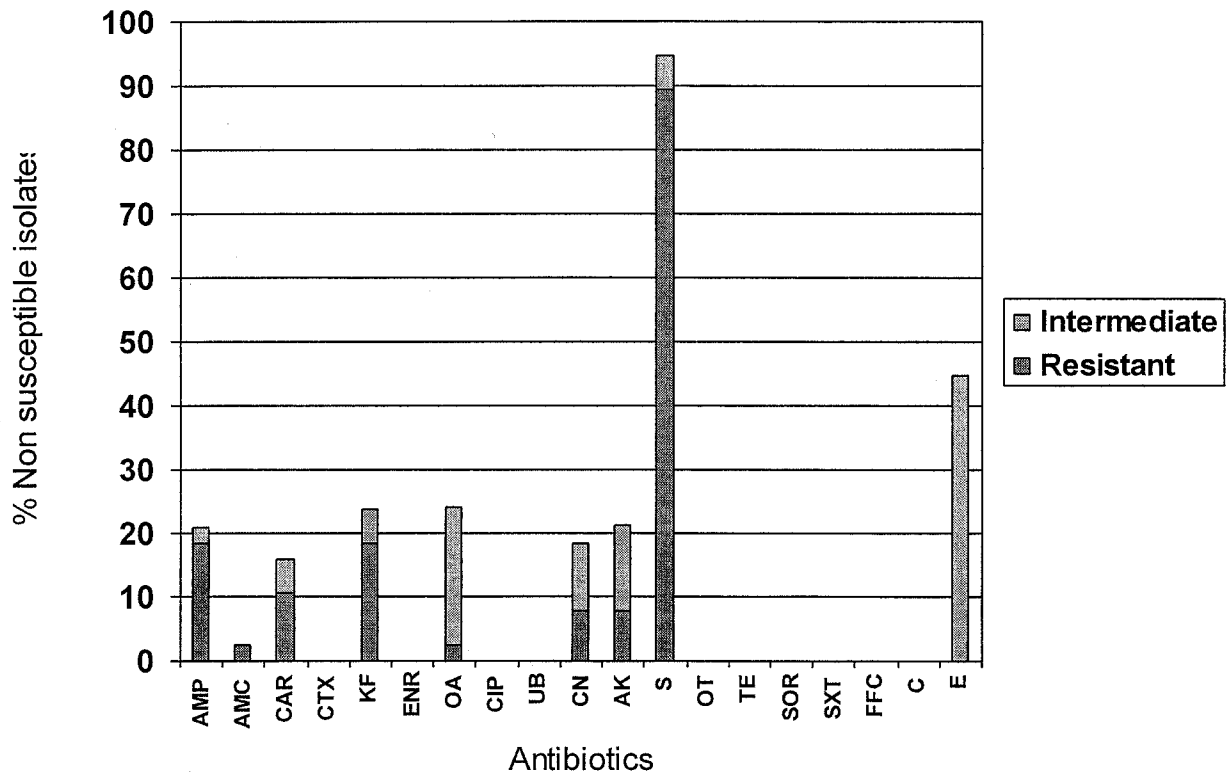

Figure 4.1 Susceptibility to 19 antibiotics of bacterial isolates, obtained from different marine sites of PEI during 2 seasons (n = 38) (AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin)

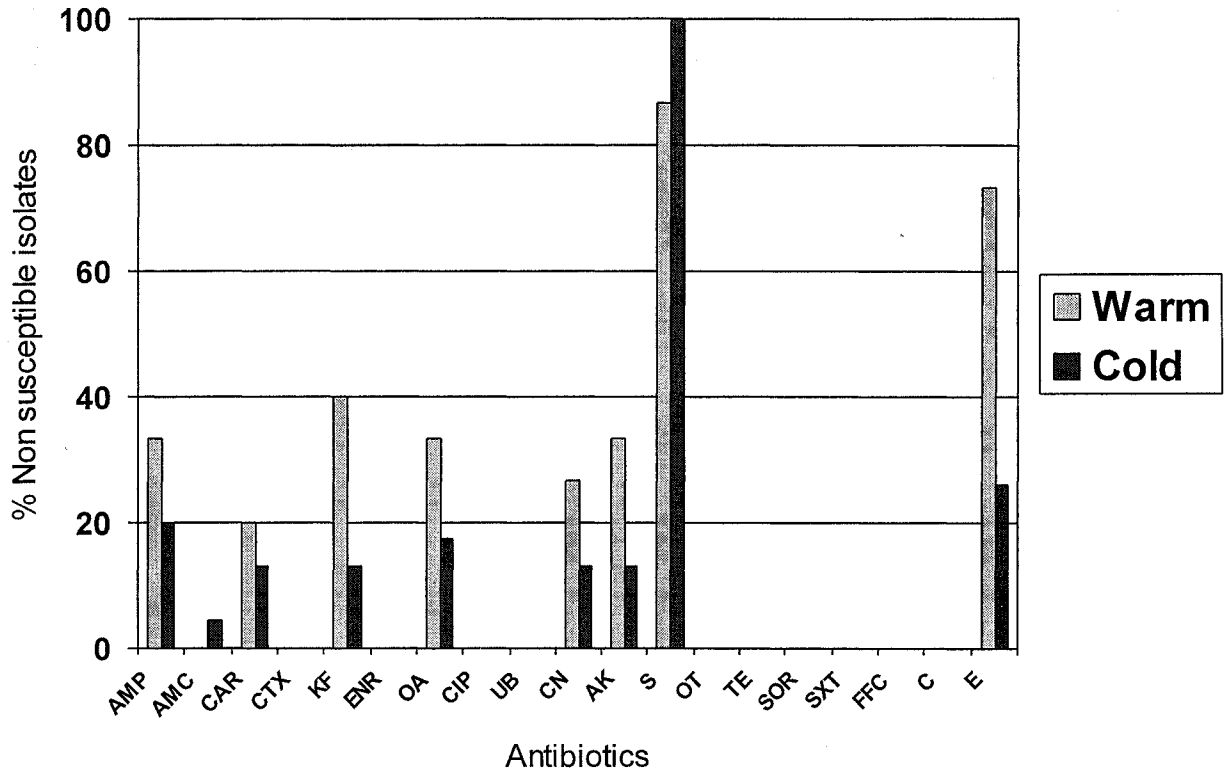

Figure 4.2 Susceptibility to 19 antibiotics by season of bacterial isolates, obtained from different marine sites of PEI in 2 seasons (warm: n = 15; cold: n = 23) (AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin)

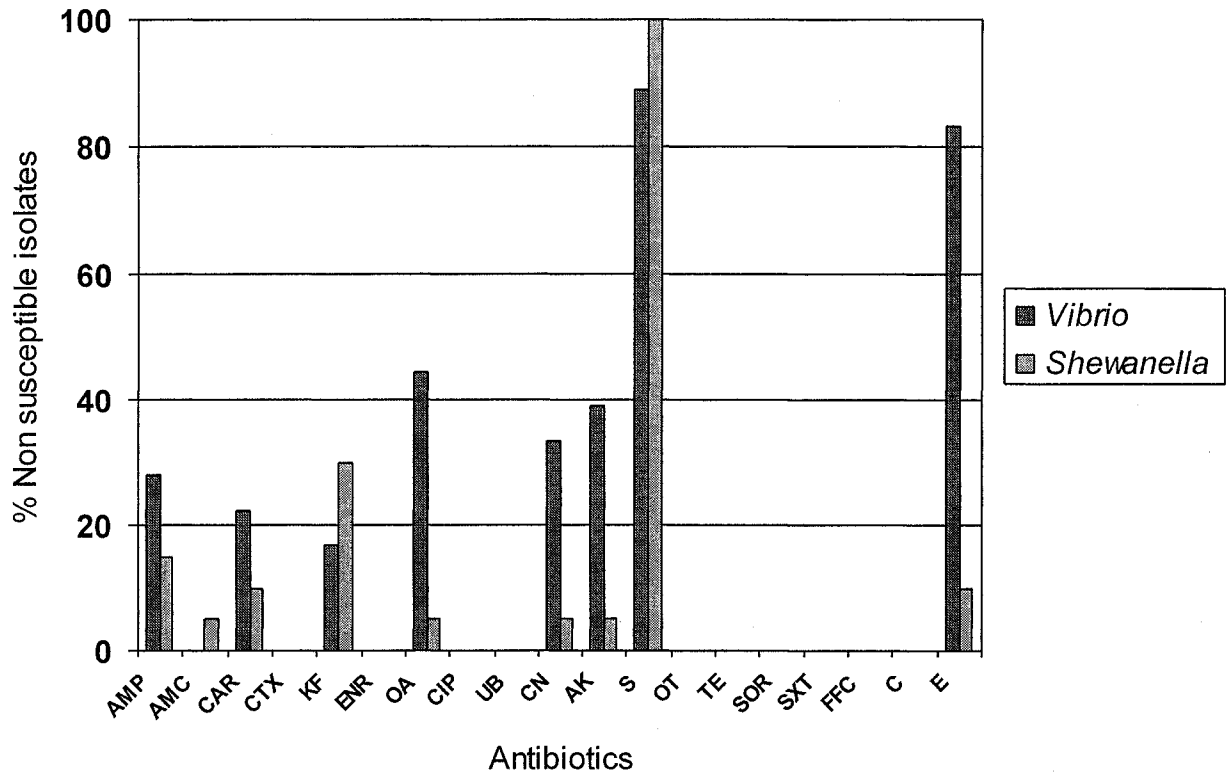

Figure 4.3 Susceptibility to 19 antibiotics by genus of bacterial isolates, obtained from different marine sites of PEI in 2 seasons (*Vibrio*: n = 18; *Shewanella*: n = 20) (AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin)

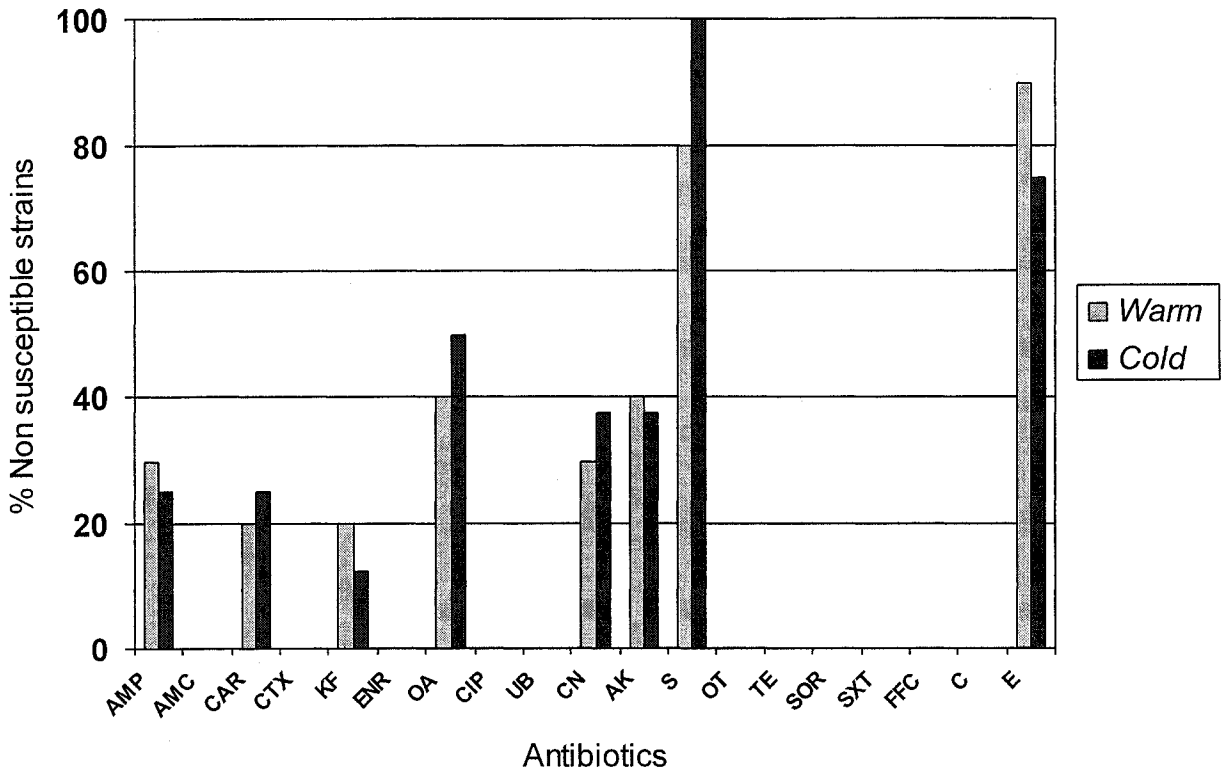

Figure 4.4 Susceptibility to 19 antibiotics by season of *Vibrio* isolates, obtained from different marine sites of PEI during 2 seasons (warm: n = 10; cold: n = 8) (AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin)

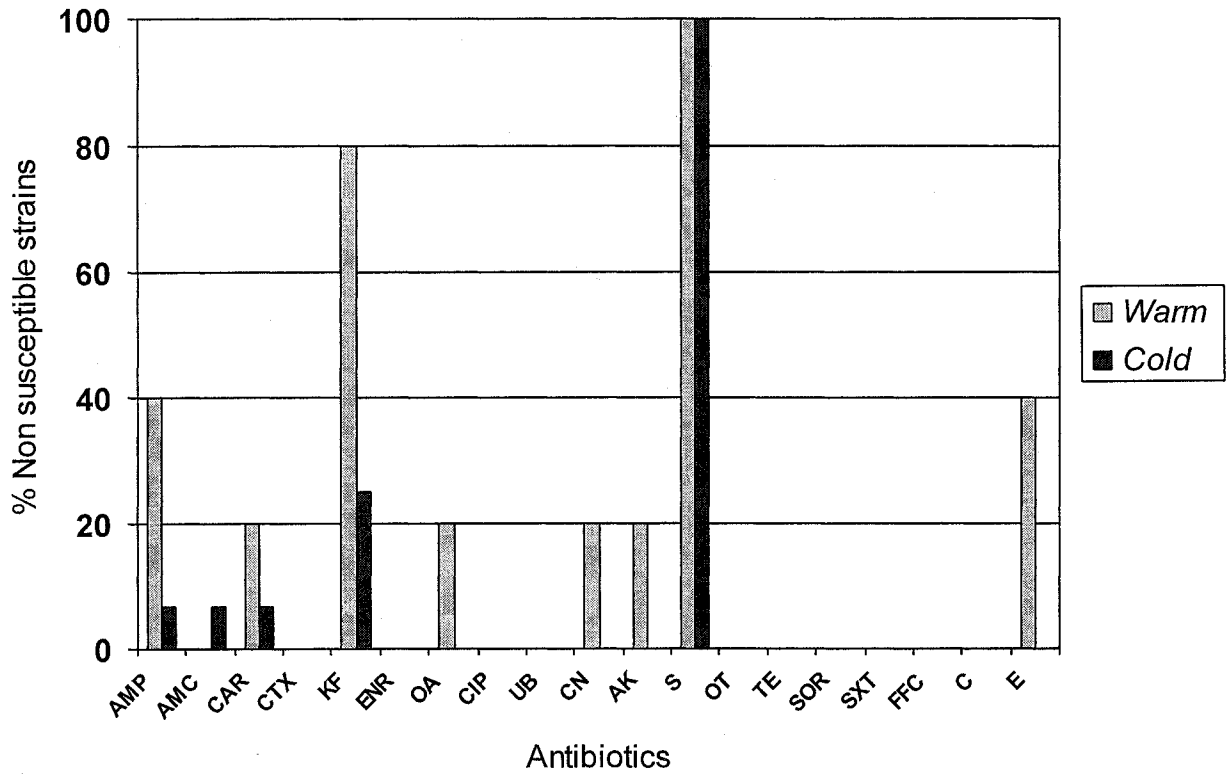

Figure 4.5 Susceptibility to 19 antibiotics by season of *Shewanella* isolates, obtained from different marine sites of PEI during 2 seasons (warm: n = 5; cold: n = 23) (AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin)

Table 4.2. Multiple non susceptibility* of bacterial isolates from PEI marine environments

Number of antibiotics	Total (%) (n=38)	% Non susceptible isolates			
		Total per season		Total per genus	
		Warm (n=15)	Cold (n=23)	*Vibrio* (n=18)	*Shewanella* (n=20)
0	2.6	6.7	0	5.6	0
1	39.5	13.3	56.5	5.6	70.0
2	10.5	13.3	8.7	5.6	15.0
3	10.5	6.7	13.0	22.2	0
4	15.8	26.7	8.7	33.3	0
5	15.8	20	13.0	27.8	5.0
6	5.3	13.3	0	0	10.0

*: Non susceptible = resistant + intermediate

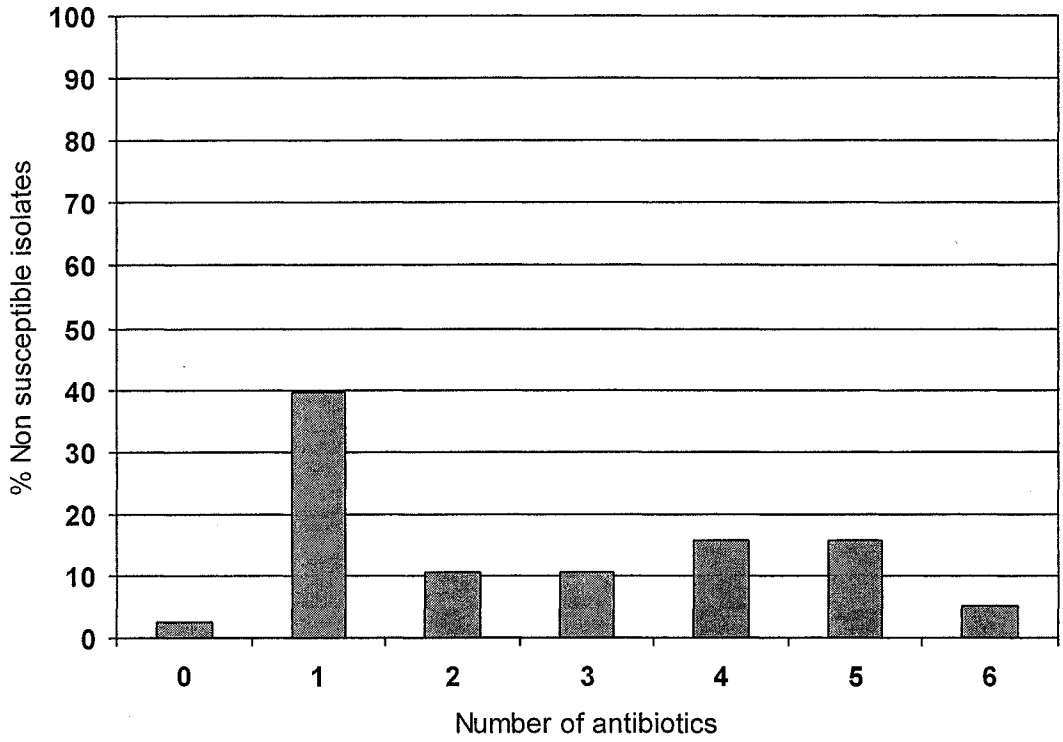

Figure 4.6 Multiple non susceptibility of bacterial isolates, from different marine sites of PEI during 2 seasons, using 19 antibiotics (n = 38)

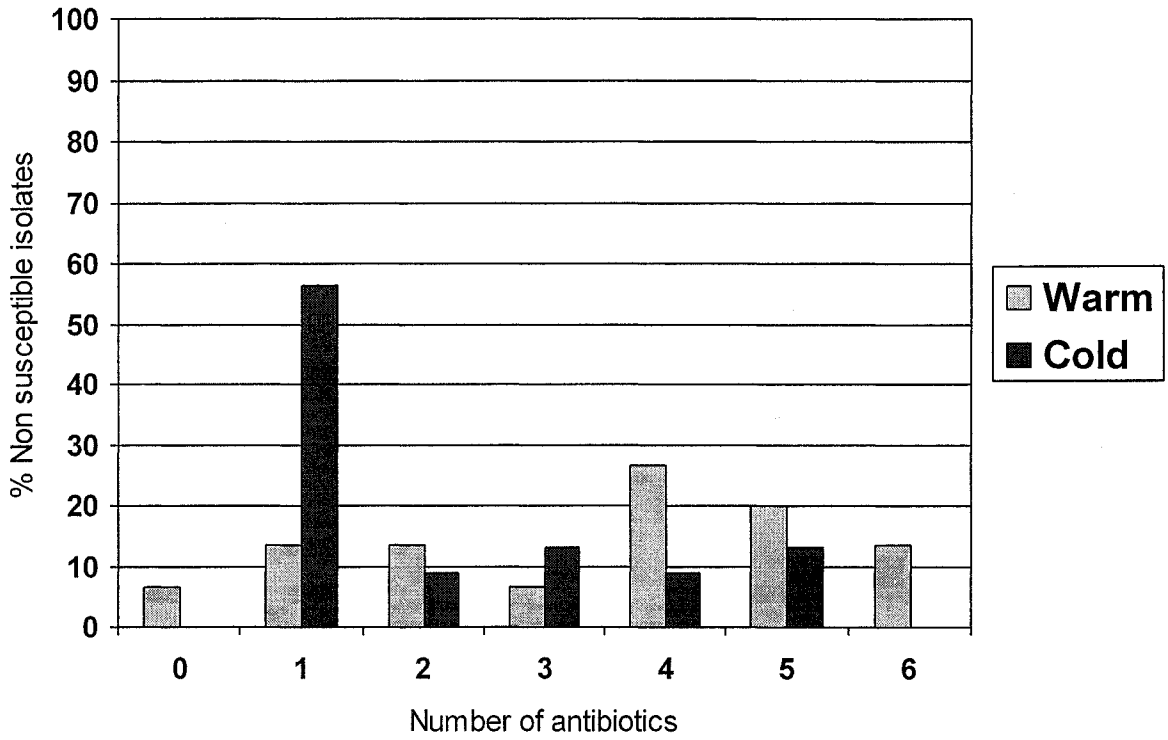

Figure 4.7 Multiple non susceptibility by season, of bacterial isolates from different marine sites of PEI obtained during 2 seasons, using 19 antibiotics (warm: n = 15; cold: n = 23)

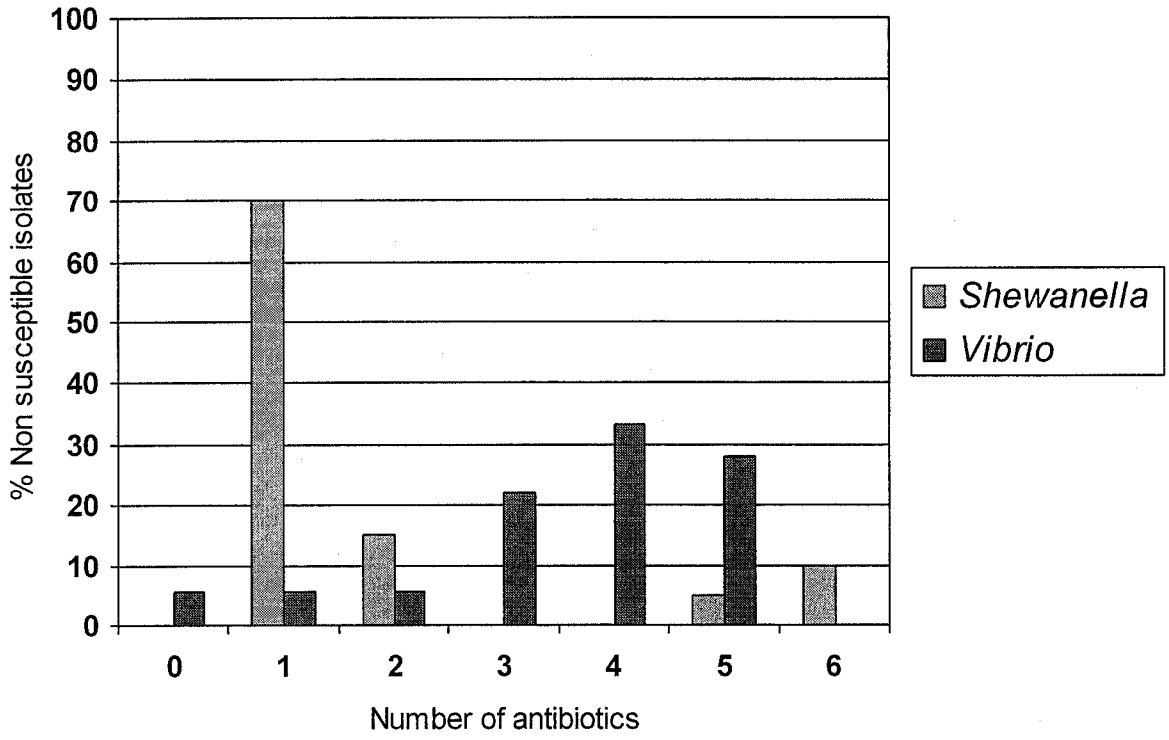

Figure 4.8 Multiple non susceptibility by genus of bacterial isolates from different marine sites of PEI during 2 seasons, using 19 antibiotics (*Vibrio*: n = 18; *Shewanella*: n = 20)

Table 4.3. Multiple non susceptibility* patterns of bacterial isolates from PEI marine environments

Number of antibiotics	Pattern of multiple non susceptibility	*Vibrio* (n=18)		*Shewanella* (n=20)	
		Warm	Cold	Warm	Cold
1	E	1	0	0	0
	S	0	1	1	13
2	S-KF	0	1	2	0
	S-E	0	1	0	0
3	S-E-AK	1	0	0	0
	S-E-OA	0	2	0	0
	S-AK-CN	0	1	0	0
4	S-E-AK-OA	1	0	0	0
	S-E-OA-CN	1	0	0	0
	S-E-KF-AMP	1	0	0	0
	S-E-AK-CN	1	1	0	0
	S-AMP-CAR-KF	0	1	0	0
5	S-E-OA-AMP-CAR	1	1	0	0
	S-E-OA-AK-CN	1	1	0	0
	S-E-AMP-CAR-KF	1	0	0	0
	S-AMP-CAR-KF-AMC	0	1	0	0
6	S-E-OA-AMP-CAR-KF	0	0	1	0
	S-E-AMP-KF-AK-CN	0	0	1	0

*: Non susceptible = resistant + intermediate; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin

Table 4.4. Minimal inhibitory concentrations (MIC) of oxytetracycline to marine isolates from PEI marine environments by E test after 5 days of incubation at 15°C

Bacterial isolate		OTC (μg/mL)	
Genus	Code	MHA (1.5% NaCl)	TCBS
Vibrio	7SHRW	0.500	3.000
Vibrio	8SHRW	0.190	2.000
Shewanella	9SHRW	0.500	No growth
Shewanella	10SHRW	6.000	>256
Vibrio	6MHRW	0.250	2.000
Shewanella	7MHRW	0.500	16.00
Vibrio	7MCEW	0.250	2.000
Undetermined	8MCEW	0.640	1.500
Shewanella	9MCEW	3.000	>256
Undetermined	11MCEW	0.047	0.094
Undetermined	8SMRW	0.500	128
Shewanella	9SMRW	1.500	16.00
Vibrio	7MMRW	0.640	2.000
Vibrio	8MMRW	0.125	2.000
Vibrio	14MMRW	0.190	1.500
Shewanella	1SHRC	0.500	32.00
Shewanella	2SHRC	0.500	48.00
Shewanella	3SHRC	0.125	>256
Shewanella	5SHRC	0.250	3.000
Shewanella	7SHRC	0.470	No growth
Vibrio	8SHRC	0.190	2.000
Shewanella	11SHRC	0.750	3.000
Vibrio	1MHRC	0.380	6.000
Vibrio	4MHRC	0.190	1.500
Shewanella	1SCEC	2.000	>256
Undetermined	2SCEC	0.250	32.00
Vibrio	3SCEC	0.380	6.000
Shewanella	1MCEC	0.500	64.00
Vibrio	3MCEC	0.190	2.000
Shewanella	3SCEC	0.380	>256
Shewanella	1MMRC	0.190	>256
Shewanella	2MMRC	0.380	3.000
Undetermined	4MMRC	0.380	0.750
Vibrio	5MMRC	0.250	6.000
As. salmonicida	Control	0.094	No growth

Table 4.5. Estimated medians and confidence intervals of MICs of *Shewanella* and *Vibrio* isolates tested on MHA with 1.5% NaCl and TCBS

Bacterial genera and Culture media	Estimated Median of MIC (μg/mL)	95% Confidence Interval of MIC (μg/mL)
Shewanella on MHA	0.58	0.40-0.87
Shewanella on TCBS	32.53	13.73-94.36
Vibrio on MHA	0.25	0.18-0.36
Vibrio on TCBS	2.48	1.46-4.50

4.4 DISCUSSION

4.4.1 Disk diffusion test

The occurrence of resistant bacteria in aquatic environments may be the result of different events. It may be acquired as a consequence of the presence of anthropogenic pollution, such as antibiotics freely diluted in water or as active metabolites in faecal material exerting a selective pressure that favors resistant bacteria, or as foreign resistant bacteria transmiting resistance genes to the indigenous flora (Bell et al., 1983; Hirsch et al., 1999). Anthropogenic pollution in aquatic environments may directly originate from urban sewage discharges, aquaculture antibiotic therapies or indirectly through run off from agriculture. Antibiotics or active metabolites in manure from treated animals may filter into ground water that eventually reach rivers and the marine environment (Kümmerer, 2004; Rooklidge, 2004). Natural or intrinsically resistant bacteria may also occur in aquatic environments. This may originate by the natural structure of some bacteria such as lack of permeability in their membranes or due to the production of enzymes that prevent the action of antibiotics (Haydes and Wolf, 1990).

The samples used in this study came from sites that included an urban influenced site, a mussel-abundant site and an isolated site. The first site is more likely affected by anthropogenic pollution whereas the other two sites might be exposed indirectly through agriculture practices. Unfortunately, the low number of bacterial isolates did not allow a comparison of the level of antibiotic resistance among the representative sites but a comparison of certain genera of isolates in different seasons was possible.

Bacterial flora from sediments and mussels, able to grow on TCBS media, obtained from different marine environments in PEI were frequently non susceptible to the aminoglycoside streptomycin (89.5% resistance, 5.3% intermediate). A considerable percentage (44.7%) of bacteria also showed intermediate resistance to the macrolide erythromycin. A low percentage (<25%) of the bacterial flora showed non susceptibility to ampicillin, amoxicillin/clavulanic acid, carbenicillin, cephalothin, oxolinic acid, gentamicin and amikacin and all showed susceptibility to cefotaxime, enrofloxacin, ciprofloxacin, flumequine, both tetracyclines, both potentiated sulfonamides, and both phenicols.

Resistance to aminoglycosides and β-lactams are mostly enzyme-mediated. Aminoglycoside-modifying enzymes and β-lactamases, which represent two of the most widely disseminated antibiotic resistance mechanisms, are responsible for the majority of clinical resistance to their respective antibiotic classes (Bush and Miller, 1998). Other resistance mechanisms includes decreased uptake due to changes in the charge of LPS in some gram-negative bacteria, mutations in ribosomal proteins and efflux systems for aminoglycosides. Reduced uptake and multidrug transporters are also common resistance mechanisms to β-lactams (Schwarz and Chaslus-Dancla, 2001). In aminoglycoside-producing organisms, methylation of 16S rRNA gives high-level resistance to the actions of these antibiotics (Kotra et al., 2000). Resistance to the macrolides among gram-negative bacteria is commonly based on the reduced permeability of the outer membrane, whereas resistance to quinolones depends either on mutations which render the target resistant or on decreased intracellular antibiotic accumulation (Schwarz and Chaslus-Dancla, 2001).

In a variety of aquatic environments, resistance to aminoglycosides has been found among coliforms (Park et al., 2003) and enterococci (Rice et al., 1995). There are some reports on the susceptibility to aminoglycosides of non-cholera vibrios but none of *Shewanella* environmental isolates. Giles et al. (1991) found that *V. ordalii* and *Listonella (Vibrio) anguillarum* isolates from the Canadian Atlantic coast were commonly resistant to streptomycin. In Spain, Balebona et al. (1998) reported high percentages of resistance against streptomycin (98.6%) and erythromycin (83.0%) among *Vibrio* strains isolated from diseased gilt-head sea bream from farms with limited antibiotics use. In Japanese ayu farms, streptomycin-resistant *L. anguillarum* has been reported. However, streptomycin had not been used to treat infections in fish farms in Japan (Sørum, 1999). On the other hand, the occurrence of *V. harveyi* multiple resistant to some antibiotics, including streptomycin, has been associated with the use of antibiotics in shrimp hatcheries of Indonesia (Teo et al., 2002). Ottaviani et al. (2001), studying vibrios isolated from fresh and frozen fish and shellfish, found that more than 90% of the isolates were resistant to streptomycin. The striking high and constant percentage of non susceptible bacteria to streptomycin in *Shewanella* and *Vibrio* isolates suggest that these genera possess an intrinsic mechanism of non susceptibility to this antibiotic. Intrinsic resistance mechanisms are characteristically permanent and with high frequency levels among the cell population (Haydes and Wolf, 1990).

The origin of the resistance to several antibiotics in vibrios appears to be primarily chromosomally mediated. In a study of plasmid profiles and experiments on curing and transferring plasmids, Giles et al. (1995) concluded that resistance to streptomycin was not plasmid mediated in *V. ordalii* and *L. anguillarum*. Li et al. (1999),

studying the susceptibility of vibrios isolated from cultured sea bream, found that resistance to several antibiotics including aminoglycosides, was not always associated with the presence of plasmids, however their presence seemed to increase antibiotic resistance. Ripabelli et al. (2003) found that resistance to ampicillin, carbenicillin and streptomycin among *V. vulnificus* and *V. alginolyticus* isolated from mussels was not associated to the presence of plasmids. The fact that resistance in vibrios is mostly chromosomally mediated might explain the constant percentage of non susceptible isolates to some antibiotics during warm and cold seasons in this study. It is probable that vibrios acquire resistance as a consequence of the insertion of resistance genetic elements in chromosomes that make them more stable. On the other hand, resistance of *Shewanella* isolates might be mostly based on plasmid transfer. It has been reported that, among other aquatic species, *Shewanella algae* represent a potential reservoir of resistance plasmids against quinolones (Poirel et al., 2005). The possibility that resistance in *Shewanella* isolates is mainly plasmid mediated would explain the changes of percentage of resistant isolates during warm and cold seasons. Environmental characteristics during warm seasons might trigger higher rates of exchange of resistance plasmids among marine bacteria. It has been noticed that the frequencies of conjugation are higher in aquatic environments with increased nutrients (Davidson, 1999) and high density bacterial cells (Bale et al., 1988; Sobecky, 2002). Plasmid transformation is also favored in environments with higher organic loads (Paul et al., 1991). These situations occur more frequently during warm months in natural aquatic environments. Moreover, low levels of resistance plasmid conjugation have been noticed at decreased temperatures (Singleton and Anson, 1981).

The occurrence of resistance to a specific antibiotic can be associated with resistance to other antibiotics of the same family or even to unrelated antibiotics. The occurrence of multiple resistance is frequently a consequence of the synergistic effect of reduced uptake due to the outer membrane permeability and active multidrug efflux mediated by pumps. Efflux transporters are widely distributed among bacteria and confer resistance against different types of antibiotics (Van Bambeke et al., 2003). In this study Vibrio isolates were mostly resistant to 4 antibiotics whereas Shewanella isolates were mostly resistant to a single antibiotic. Mostly multidrug efflux transporters are chromosomally encoded whereas specific efflux transporters are associated with mobile genetic elements (Butaye et al., 2003; Kumar and Schweizer, 2005). This would support the hypothesis that resistance in vibrios is more likely encoded in chromosomal genes whereas in *Shewanella* isolates is likely to be encoded in plasmids. Intermediate resistance to erythromycin might be due to naturally low permeability of the cell membranes of *Vibrio* and *Shewanella* isolates to this drug.

Supplementation of culture media with salts is known to have some effect on the antibiotic susceptibility tests of bacteria. Coronado et al. (1995) observed three different patterns of tolerance in moderately halophilic bacteria when salinity varied from 1 to 10% total salts in the testing media. Those patterns involved minimal, remarkable and heterogeneous variation on the susceptibility to different antibiotics depending on the individual strain and antimicrobial tested. Inactivation of the antibiotic, in the case of tetracyclines, or reduced drug uptake, either by alteration of the antibiotic or due to low permeability as a consequence of high osmotic conditions are possible factors involved (Coronado et al., 1995). However, such changes have been noticed with salinities above

4% for vibrios. Salinities in the range 1-4% have not shown differences in sensitivity tests using MHA media (Ottaviani et al., 2001). In this study the salinity used was 1.5% NaCl, therefore minimal influence was suspected on the susceptibility assessments.

4.4.2 E test

All the marine isolates included in this study were previously recovered in TCBS medium containing 50 µg/mL OTC. Since this concentration was observed to have some degree of selectivity (see Chapter 2), it was expected that these bacteria were going to show at least intermediate resistance in the disk susceptibility test. On the contrary all isolates showed to be susceptible to tetracyclines. It appears that although 50 µg/mL OTC in TCBS medium exerts some degree of selectivity among the marine bacterial flora, the selected flora has only low level OTC resistance. An effect of TCBS components in decreasing the activity of OTC has been suggested (Tendencia and de la Peña, 2002). In this study, the level of inhibition of OTC was significantly decreased in TCBS, in relation to MHA with 1.5% NaCl on the E test. This difference was significantly wider in *Shewanella* isolates than vibrios. This is in agreement with the fact of obtaining higher CFU values in the TCBS than MHA (24 ppt seawater) plates containing OTC, and with the observation that only the colonies with characteristics of those from *Shewanella* species could grow in TCBS plates containing 100 µg/mL OTC (see Chapter 2). Therefore, it is difficult to estimate a general equivalence of the effect of 50 µg/mL OTC in TCBS medium for MHA with 1.5% NaCl since the susceptibility to OTC varies according to the genus of the bacteria tested. Rees and Elliot (1998) noticed that bile has influence on the antimicrobial susceptibility in different degrees depending on the

antibiotic and bacteria tested. Activation of efflux systems and increased membrane permeability have been implicated as mechanisms activated by the presence of bile that confers resistance to different antibiotics including OTC (Prouty et al., 2004; Noriega et al., 2005). Probably, similar resistance mechanisms confer a more effective protection to *Shewanella* than vibrios for tolerating the bile salts of the TCBS medium and the presence of OTC.

In conclusion, the genera *Vibrio* and *Shewanella* comprise the majority of the bacterial flora recovered in TCBS from sediments and mussels from PEI. These isolates were frequently non susceptible to streptomycin (> 90%) and intermediately susceptible (44.7%) to erythromycin. This bacterial flora also showed low percentage (< 25%) of non susceptible isolates to ampicillin, amoxicillin/clavulanic acid, carbenicillin, cephalothin, oxolinic acid, gentamicin and amikacin, and full susceptibility to cefotaxime, enrofloxacin, ciprofloxacin, flumequine, tetracycline, oxytetracycline, ormetoprim-sulfadimethoxine, trimethoprim-sulfamethoxazole, florfenicol and chloramphenicol. In the case of streptomycin, non susceptibility is high and constant regardless of the season and genus of bacteria. In case of other antibiotics, the percentages of non susceptibility varied according to the season and the genus of the isolates. Mostly, higher percentages of non susceptibility were observed in those isolates recovered during the warm season among *Shewanella* isolates whereas among *Vibrio* isolates the percentage of non susceptible isolates were similar in both seasons and generally higher than in the case of *Shewanella* isolates. Different genetic sources for resistance are assumed to occur between these genera. In *Shewanella* isolates, plasmid encoded mechanisms might explain the increased non susceptibility in warm season and the specific resistance to a

single antibiotic frequently found. Chromosomically encoded mechanisms might explain the more stable non susceptibility and the non susceptibility to multiple antibiotics observed in *Vibrio* isolates. An intrinsic mechanism of non susceptibility to streptomycin might occur in both genera isolated from PEI marine environments. It was also demonstrated that the activity of OTC is significantly affected by the culture medium used and the bacterial genera tested. The activity of OTC decreased more markedly in TCBS medium and *Shewanella* tolerates better OTC than *Vibrio* isolates, especially when cultured on TCBS medium.

CHAPTER 5: GENERAL DISCUSSION

The main objective of this thesis was to select a bacterial species or a taxonomical group able to be used to assess the level of antimicrobial resistance in the marine environment. For this purpose, bacteria able to grow in a media containing OTC were screened, identified and finally tested for their susceptibility against several antibiotics. In parallel, the assessment and comparison of the level of OTC-resistance, expressed as frequency of resistance (FR), was carried out with the bacterial flora of sediments and mussels obtained from 3 different sites during 2 seasons. This study was done in order to determine if the factors: type of site, type of sample and season of sampling had any effect on the level of OTC-resistance. In other words, a method sensitive enough to show differences among marine sites having different ecological characteristics was sought. As well, suitability was assessed using the bacterial flora from sediment and mussels in different seasons. The bacterial flora selected from PEI marine environments was identified by using 3 different methods, conventional, automated identification system and molecular method. The susceptibility of 50 μg/mL OTC-resistant flora against several antibiotics was assessed using disk diffusion sensitivity tests and interpreted according to season and the genus to which they belong. Finally, the effect of culture media (TCBS and MHA 1.5% NaCl) and bacterial genus (*Shewanella* and *Vibrio*) on the MIC of OTC was analyzed.

The study of the frequency of resistance to a specific antibiotic is considered to be the best approach to evaluate the influence of antibiotics in the bacterial flora of marine environments (Herwig and Gray, 1997). Mostly, these studies have been done to

evaluate the level of OTC resistance in the bacterial flora from sediments associated with fish farms in order to determine the impact of antibiotic therapies on resistant patterns (Husevåg et al., 1991; Samuelsen et al., 1992; Sandaa et al., 1992; Hansen et al., 1993; Kerry et al., 1994, 1995, 1996; Herwig and Gray, 1997; Herwig et al., 1997). Different culture media with different OTC concentrations have been used due to lack of standardized protocols (Smith et al., 1994). Modified general culture media have been used in most studies. However the FR obtained in such studies might not only reflect the selective pressure of antibiotics but could also be the consequence of different proportions of bacterial taxa with inherent antimicrobial susceptibilities in different environments. Therefore, the use of a selective culture medium would decrease the bacterial diversity effect on the FR found in an environment.

In this study, the use of TCBS agar and a modified MHA, containing 70% seawater (24 ppt), was initially tried for comparison purposes. TCBS agar, a selective culture medium, with 25 μg/mL of OTC has been used by Tendencia and de la Peña (2002) for studying the level of OTC resistant bacteria in shrimp, water and sediments from shrimp farms. The latter medium is a general medium used mostly in susceptibility tests (NCCLS, 2003) but has also been used in FR studies (Herwig et al., 1997). Different OTC concentrations were tested, 25, 50 and 100 μg/mL. Commonly, 25 μg/mL has been in used in studies of the ecology of resistance to tetracyclines in humans since it is assumed that this concentration selects those bacteria possessing resistance genes (Pursell et al., 1996). This or equivalent concentrations have been used in studies of marine environments, taking into account that divalent cations present in seawater, such as Mg^{2+} and Ca^{2+}, commonly used to supplement the media, diminish the potency of tetracyclines.

In this study the selectivity power of the modified MHA containing OTC was much more stringent than that of TCBS medium. This indicates that the antimicrobial activity of OTC was partially inhibited by the TCBS medium, as suggested previously (Tendencia and de la Peña, 2002). Due to the fact that FR was very low even with the lowest OTC concentration in the modified MHA, TCBS medium was chosen for the subsequent study of FR from different marine environments. Oxytetracycline at 50 μg/mL was determined to be the most suitable concentration in TCBS due to the selective power that was not much different from that of 100 μg/mL OTC but allowing more diversity of colonies, similar to that obtained with 25 μg/mL OTC.

The site, type of sample and season of sampling had significant effect on the FR values individually or in combination. Hillsborough Rv. is an urban-influenced site that receives the discharge of Charlottetown city's sewage. Sewage waters usually contain partially metabolized chemical compounds used in human medicine and multiple resistant bacteria which are not fully eliminated during the sewage purification process (Bell et al., 1983; Hirsch et al., 1999; Kümmerer, 2004). Such characteristics were more clearly reflected in the FR of the bacterial flora from sediments (78.9%). The lower FR values of the bacterial flora from the samples obtained in Cap Egmont are in accordance with their isolated location with very limited anthropogenic influence.

The site in Murray Rv. is characterized by the influence of mussel abundance that might mainly affect the FR of the bacterial flora from mussels. Bacterial flora from mussels displayed different levels of FR in comparison with those from adjacent sediments. Bivalves have some degree of selectivity of the bacterial flora they ingest for nutrition purposes (Birkbeck and McHenery, 1982; Kueh and Chan, 1985). Natural

inhibitors involved in the innate host defenses of mussels, such as antimicrobial peptides, may favor OTC-resistant bacteria due to the fact that some bacterial resistance mechanisms confer protection against a wide range of substrates (Nikaido, 1998; Butaye et al., 2003; Van Bambeke et al., 2003), including antimicrobial peptides (Bell and Gouyon, 2003). This effect is noticed in warm season and more markedly in Murray Rv. (FR = 68.1%) and appears to be imperceptible during cold season which coincide with the reported decrease of antibacterial response of mussel antimicrobial peptides at low temperatures (Hernroth, 2003). The effect of antimicrobial peptides may be overshadowed in environments with high levels of anthropogenic pollution, such as Hillsborough Rv.

Cold season reduces the variety of bacterial flora but apparently less reduction occurs for species with OTC-resistance. Seasonal changes in vibrio communities from sediment and water bodies have been noticed (La Rosa et al., 2001; Thompson et al., 2004). Different *Vibrio* species might have different levels of resistance. The variation of the composition of sewage discharges that would be independent from temperature variations in Hillsborough Rv. and the effect of the antimicrobial peptides that might favor resistant bacteria in the warm season in Murray Rv. may prevent the trend of having higher FR during cold season in the bacterial flora of sediments and mussels from those respective sites.

It is also important to consider that some degree of natural resistance is commonly present in environments without anthropogenic pollution (Jones, 1986; Magee and Quinn, 1991; Boon and Catanach, 1999; Ash et al., 2002). Jones (1986) considered that among the factors that contribute to the elevated occurrence of antibiotic resistance

in natural aquatic environments is the species composition of the bacterial communities and the resistance associated with growth in low nutrient environments. Regarding the particular case of OTC, the presence of this antibiotic is not a necessary causal condition for the emergence of high frequency of resistance (Kapetanaki et al., 1995; Kerry et al., 1995). Thus, the resistance mechanisms found in bacteria at these sites are likely the consequence of inhibitors other than OTC.

Different methods were used in this study to identify marine bacterial isolates including phenotypic characterization and genomic information. Individually, each of the methods has their limitations but the combination of methods, described as a polyphasic approach, is recommended for identification of bacterial isolates (Vandamme, 1996, 2003; Stackebrandt, 2002). The conventional method gave a taxonomic general frame of the isolates based on basic phenotypic characteristics. Automated identification using the Biolog system was also used in this study. This method has been applied for some years mainly for bacteria of clinical importance and also for some marine environmental bacteria and vibrios (Ivanova et al., 1998; Makemson et al, 1998; Austin et al., 1995; Noble and Gow, 1998; Vandenberghe et al., 1999, 2003). In this study it was found that the bacterial growth and identification gave optimal results with the addition of 1.5% NaCl in the inoculating fluid and incubation of the microplates at 22°C. However, there were several isolates that could not be identified because of the lack of growth in the GN microplates or because the degree of similarity (SIM) was too low. It is difficult to understand the reason for the lack of growth in any of the 95 carbon substrates in several GN microplates since the same salinity and incubation temperature was used in the BUG medium where growth was always achieved. Also, the limited environmental marine

bacteria in the Biolog database prevented identification of some bacterial isolates. However the metabolic information obtained from the substrate utilization in the GN microplates was helpful for a more complete phenotypic characterization. The genotypic method used was the analysis of the 16S rDNA sequence similarity. Species are considered to include strains with approximately 70% or greater DNA-DNA relatedness (Wayne et al., 1987), which has been found to be equivalent to 97%, or more, similarity of the 16S rDNA (Stackebrandt and Goebel, 1994). This method complemented the phenotypic characterization, obtained from the Biolog metabolic pattern, to provide a final identification. The inaccuracy in the species identification for several isolates is not uncommon since the genomic information obtained in the 16S rDNA sequence is considered to lack resolving power at the species level (Roselló-Mora and Amman, 2001). It was found in this study that bacteria that had 97%, or more, 16S rDNA sequence similarity with the isolates do not necessarily share the same species, as noticed in other reports. It has been considered that the circumscription for species of the *Vibrionaceae* is at least 99.2% 16S rDNA sequence similarity (Kita-Tsukamoto et al., 1993).

Among the isolates obtained from sediments and mussels of three different representative sites of PEI, two genera, *Vibrio* and *Shewanella,* were mainly identified using the combination of the genotypic and phenotypic methods. These genera are commonly found in marine environments (Venkateswaran et al., 1989; Hariharan et al., 1995; Maugeri et al., 2000; Croci et al., 2001; Hervio-Heath, 2002; Ivanova et al., 2003a,b,c, 2004a; Thompson 2003a,b; Toffin et al., 2004; Yoon et al., 2004). Belonging to each of these genera the species *V. splendidus* and *S. colwelliana* were identified with

reasonable of confidence. The former species appear to be more commonly found in PEI marine environments as noticed previously (Hariharan et al., 1995).

The final objective was to describe the antimicrobial susceptibility of bacterial isolates from sediments and mussels against 19 antibiotics belonging to different families used in human and veterinary medicine. The genera *Vibrio* and *Shewanella* are frequently non susceptible to streptomycin (89.5% resistant, 5.3% intermediate) and only of intermediate susceptibility to erythromycin (44.7%). These bacterial genera also showed low percentage (< 20%) of non susceptible isolates to ampicillin, amoxicillin/clavulanic acid, carbenicillin, cephalothin, oxolinic acid, gentamicin and amikacin, and full susceptibility to cefotaxime, enrofloxacin, ciprofloxacin, flumequine, tetracycline, oxytetracycline, ormetoprim-sulfadimethoxine, trimethoprim-sulfamethoxazole, florfenicol and chloramphenicol. The level of non susceptibility to streptomycin is high and constant regardless of the season and genus of bacteria. There are no reports on the susceptibility to aminoglycosides of *Shewanella* environmental isolates, but the high frequencies of streptomycin resistant vibrios are not uncommon. Giles et al. (1991) found high percentage of streptomycin resistant vibrios isolated from fish in Atlantic Canada. Ottaviani et al. (2001) found more than 90% streptomycin resistant vibrios from fresh and frozen shellfish. Also, high percentages of streptomycin resistant vibrios not associated with the use of antibiotics have been reported in Spanish and Japanese fish farms (Babelona et al., 1998; Sørum, 1999). However, streptomycin resistant *V. harveyi* has been associated with the use of antibiotics in shrimp hatcheries of Indonesia (Teo et al., 2002). *Vibrio* and *Shewanella* species likely possess intrinsic mechanisms of non

susceptibility to this antibiotic. Intrinsic resistance mechanisms are mostly permanent with high frequency levels among the bacterial population (Hayes and Wolf, 1990).

In case of other antibiotics, the percentages of non susceptibility varied according to the season and the genus of the isolates. Mostly, higher percentages of non susceptibility were observed in those isolates recovered during the warm season among *Shewanella* isolates whereas among *Vibrio* isolates the percentage of non susceptible isolates were similar in both seasons and generally higher than in the case of *Shewanella* isolates.*Vibrio* isolates commonly showed multiple-non susceptibility to 4 antibiotics, whereas *Shewanella* isolates were mainly non susceptible to a single antibiotic. The resistance of different *Vibrio* strains to different antibiotics including aminoglycosides is usually not associated with the presence of plasmids (Giles et al., 1995; Li et al., 1999; Ripabelli et al., 2003). Resistance genes encoded in chromosomes are associated with multiple resistance efflux mechanisms whereas specific efflux transporters are associated with genetic mobile elements (Butaye et al., 2003; Kumar and Schweizer, 2005). Sometimes resistance in chromosomes occurs due to the insertion of gene cassettes coding for resistance derived from superintegrons (Rowe-Magnus et al., 2001). Resistance genes encoded in chromosomes which confer more stable resistance might explain the constant percentage of non susceptible isolates to some antibiotics during warm and cold seasons and the multiple non susceptibility of *Vibrio* isolates in this study. In contrast, plasmid encoded mechanisms in *Shewanella* isolates might explain the increased non susceptibility in warm season and the general non susceptibility to only one antibiotic. It has been suggested that *Shewanella algae* represents a reservoir of resistance plasmids against quinolones (Poirel et al., 2005). Plasmid conjugation and

transformation is facilitated by increased nutrients and high density of bacterial cells (Bale et al., 1988; Paul et al., 1991; Davidson, 1999; Sobecky, 2002) which occur in warm seasons in aquatic environments.

The absence of non susceptible bacteria against tetracyclines suggests that although 50 μg/mL OTC exert some degree of selectivity among the marine bacterial flora recovered in TCBS medium, the selected flora would not be clinically resistant. Therefore it can be concluded that the subpopulation screened in TCBS containing 50 μg/mL OTC possess a low level resistance against OTC. The E test demonstrated that the activity of OTC is significantly decreased in TCBS medium in comparison to MHA 1.5% NaCl and that *Shewanella* tolerates higher concentrations of OTC than *Vibrio* isolates in both media but more efficiently in TCBS medium. Activation of resistance mechanisms by the presence of bile (Prouty et al., 2004; Noriega et al., 2005) might occur in *Shewanella* in a more efficient way than in *Vibrio* species.

Taking into consideration all these observations, it would be advisable, for FR studies using TCBS with similar OTC concentrations as used in this study, to use bacterial flora from sediments since it appears to be a good indicator of the degree of OTC-low level resistance associated with anthropogenic pollution in marine environments. Mussels, in contrast, appear to behave as a dynamic microenvironment with a selective influence in the bacteria filtered that would modify the proportion of OTC-low level resistant bacteria in relation to what occurs outside them. Cold temperatures appear to favor bacteria that have OTC-low level resistance and natural OTC-low level resistance can be found in aquatic environments without major anthropogenic influence. In addition, standard breakpoints for susceptibility test of

marine bacteria are required since existing breakpoints were established for terrestrial animals (NCCLS, 2002). It is also desirable to search for better conditions for growth of marine bacteria in Biolog GN microplates and to develop a database that includes more environmental bacteria. The main genera able to grow in TCBS from PEI marine environments, *Vibrio* and *Shewanella*, have different susceptibility patterns to several antibiotics, affected in different ways by the sampling season. Considering their different antibiotic susceptibilities and the differentiable characteristics of their colonies, it is advisable to evaluate them separately. If a more specific study of FR against OTC is attempted *V. splendidus* is preferable considering its wide distribution in marine environments.

Further research is required to confirm the effect of antimicrobial peptides in the proportion of resistant bacteria in mussels. Also, molecular studies would be necessary to confirm if the resistance displayed against several antibiotics in *Vibrio* and *Shewanella* isolates is encoded in chromosomal and plasmid genes, respectively. The study of the origin of constant and high resistance to streptomycin in *Shewanella* and *Vibrio* isolates is necessary in order to determine if there is any selective pressures unique to PEI marine environments.

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APPENDIX A

A.1. Colony counts of sediment samples from Hillsborough Rv. cultured in MHA and TCBS with and without different concentrations of OTC

Sample	Culture Media	0 µg/mL of OTC						25 µg/mL of OTC						50 µg/mL of OTC						100 µg/mL of OTC					
		10^{-1}		10^{-2}		10^{-3}		10^{-1}		10^{-2}		10^{-3}		10^{-1}		10^{-2}		10^{-3}		10^{-1}		10^{-2}		10^{-3}	
1	MHA			173	150	13	10			2	0	0	0			0	1	0	0			0	0	0	0
	TCBS	127	114	18	7			88	74	7	16			37	42	5	5			19	35	10	6		
2	MHA			139	109	6	7			1	0	0	0			0	0	0	0			0	0	0	0
	TCBS	64	72	8	0			43	30	2	0			45	27	2	1			10	5	2	3		
3	MHA			101	106	2	8			0	2	1	0			0	0	0	0			0	0	0	0
	TCBS	86	194	22	14			89	61	8	10			52	41	5	10			34	17	10	6		
4	MHA			77	74	3	1			1	0	0	0			0	0	0	0			0	0	0	0
	TCBS	137	115	13	3			83	48	2	1			48	17	7	7			18	11	3	4		
5	MHA			319	412	11	33			9	11	0	0			3	0	0	0			1	0	0	0
	TCBS	138	82	22	14			76	60	21	16			47	53	10	5			30	45	88*	9		

*: considered to be outlier

A.2. Colony counts of sediment samples from Hillsborough Rv. obtained during warm season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-3}		10^{-4}		10^{-3}		10^{-4}	
1	44	37	2	1	34	21	3	2
2	94	75	2	4	75	65	2	3
3	110	132	19	12	136	144	13	49
4	50	47	9	4	26	37	5	3
5	25	21	1	3	21	9	2	1
6	4	11	2	0	0	-	0	-

A.3. Colony counts of mussel samples from Hillsborough Rv. obtained during warm season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	$10^{-3.3}$		$10^{-4.3}$		$10^{-3.3}$		$10^{-4.3}$	
1	189	309	0	0	54	3	0	0
2	297	216	0	2	37	2	1	0
3	226	172	2	0	5	38	0	0
4	202	252	2	0	23	20	0	0
5	257	325	0	0	2	17	0	1
6	#	#	38	59	53	99	7	3

: High number of colonies unable to be counted

A.4. Colony counts of sediment samples from Hillsborough Rv. obtained during cold season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-1}		10^{-2}		10^{-1}		10^{-2}	
1	126	241	30	8	104	77	32	2
2	50	33	5	0	12	5	2	0
3	167	113	39	25	44	65	14	7
4	85	59	24	26	58	33	26	0
5	142	168	41	41	67	157	8	0
6	181	32	46	33	144	6	12	7

A.5. Colony counts of mussel samples from Hillsborough Rv. obtained during cold season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-1}		10^{-2}		10^{-1}		10^{-2}	
1	7	1	0	1	2	0	0	0
2	0	0	0	0	0	0	0	0
3	57	32	22	9	37	57	7	7
4	5	10	0	1	0	0	0	1
5	0	1	1	0	0	0	0	0
6	5	10	1	0	0	0	0	0

: High number of colonies unable to be counted

A.6. Colony counts of sediment samples from Cap. Egmont obtained during warm season and cultured in MHA and TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-3}		10^{-4}		10^{-3}		10^{-4}	
1	14	18	0	4	1	1	0	0
2	25	20	1	0	0	1	0	0
3	4	6	0	0	1	1	0	1*
4	20	10	4	0	1	0	0	0
5	9	6	1	4	2	4	0	0
6	19	12	1	0	1	3	0	0

*: considered to be outlier

A.7. Colony counts of mussel samples from Cap. Egmont obtained during warm season and cultured in MHA and TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-4}		10^{-5}		10^{-4}		10^{-5}	
1	90	71	5	9	59	46	-	-
2	37	27	0	1	3	8	0	0
3	61	36	3	22	5	5	-	-
4	820	872	21	22	-	120	2	1
5	211	42	0	0	-	8	0	0
6	27	48	0	0	0	-	0	0

A.8. Colony counts of sediment samples from Cap. Egmont obtained during cold season and cultured in MHA and TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-1}		10^{-2}		10^{-1}		10^{-2}	
1	2	26	2	0	14	0	0	0
2	10	0	0	1	3	0	0	0
3	360*	19	0	21*	1	2	409*	2
4	35	2	0	3	0	0	0	0
5	-	18	2	1	0	0	1	2
6	78	0	1	8	3	0	4	4

*: considered to be outlier

A.9. Colony counts of mussel samples from Cap. Egmont obtained during cold season and cultured in MHA and TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-1}		10^{-2}		10^{-1}		10^{-2}	
1	0	0	0	0	0	12	0	0
2	22	0	0	2	13	1	1	0
3	0	9	3	0	9	1	0	0
4	0	19	0	0	1	3	0	0
5	10	0	1	0	4	0	0	1
6	110	1	14	0	12	0	1	-

A.10. Colony counts of sediment samples from Murray Rv. obtained during warm season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-3}		10^{-4}		10^{-3}		10^{-4}	
1	78	131	5	8	13	4	3	2
2	69	56	13	2	-	13	0	0
3	608	624	54	33	53	91	3	3
4	59	63	4	7	2	3	0	0
5	91	64	6	6	8	4	0	0
6	161	176	20	41	20	17	1	1

A.11. Colony counts of mussel samples from Murray Rv. obtained during warm season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-4}		10^{-5}		10^{-4}		10^{-5}	
1	42	73	0	0	3	4	2	1
2	21	24	2	4	16	20	1	1
3	15	16	1	0	3	7	1	0
4	6	16	0	1	0	3	0	2
5	2	3	4	4	9	7	1	14*
6	5	14	2	0	9	4	1	4

A.12. Colony counts of sediment samples from Murray Rv. obtained during cold season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-1}		10^{-2}		10^{-1}		10^{-2}	
1	64	42	6	10	47	23	5	2
2	251	250	28	46	239	220	34	21
3	70	52	4	10	42	40	2	7
4	160	150	7	8	55	92	1	3
5	235	262	27	36	53	142	22	4
6	248	289	25	23	120	178	21	7

A.13. Colony counts of mussel samples from Murray Rv. obtained during cold season and cultured in TCBS with and without OTC

Sample number	0 µg/ml				50 µg/ml			
	10^{-2}		10^{-3}		10^{-2}		10^{-3}	
1	9	10	0	0	5	2	0	0
2	5	1	0	0	0	0	0	0
3	10	5	1	3	5	10	0	0
4	6	10	1	0	4	9	1	0
5	3	1	1	0	1	7	0	0
6	6	3	1	0	5	7	0	1

APPENDIX B

B.1. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Hillsborough Rv during warm season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
01SHRW	Sediment OTC 0	Yellow	Circular	5-8	Short and thick rods		-	+	+	A/A	S	+	*Vibrio*
02SHRW	Sediment OTC 0	Cream	Circular/ undulate	5-8	Small curved rods		-	+	+	K/A	S	-	*Vibrio*
03SHRW	Sediment OTC 0	Light green	Circular	3-7	Very short rods		-	+	+	K/A	S	+	*Vibrio*
04SHRW	Sediment OTC 0	Green (black center)	Circular	2-3	Short and thick rods		-	+	$+^{w}$	K/K H_2S	S	-	*Shewanella*[+]
05SHRW	Sediment OTC 0	Green (dark center)	Circular		Short and thick rods		-	$+^{w}$	-	K/A H_2S	R	-	*Shewanella*
06SHRW	Sediment OTC 0	Yellow	Punctiform	1	Short and thick rods		-	+	+	A/A^{W}	S	-	*Vibrio*
07SHRW	Sediment OTC 50	Very light green	Circular		Small rods	+	-	+	+	K/A	S	+	*Vibrio*
08SHRW	Sediment OTC 50	Dark green	Circular		Small curved rods	+	-	+	+	A/A	S	-	*Vibrio*
09SHRW	Sediment OTC 50	Green (almost translucent) (black center)	Circular		Long rods (sometimes in chains)	-	-	+	$+^{w}$	K/K H_2S	S	-	*Shewanella*
10SHRW	Sediment OTC 50	Green (tiny black center)	Circular		Long rods (sometimes in chains)	+	-	+	$+^{w}$	K/K H_2S	S	-	*Shewanella*[+]

11SHRW	Sediment OTC 50	Cream (green center)	Circular		Short and thick rods		-	+	+	A/A	S	+	*Vibrio*
01MHRW	Mussel OTC 0	Cream (yellow center)	Circular		Long rods		-	+	+	A/A	S	+	*Vibrio*
02MHRW	Mussel OTC 0	Yellow (green after)	Circular		Short and thick rods		-	+	+	A/A	S	+	*Vibrio*
03MHRW	Mussel OTC 0	Cream	Circular		Short rods		-	+	+	A/A	S	+	*Vibrio*
04MHRW	Mussel OTC 0	Cream	Circular		Short and thin rods		-	+	+	K/A	S	+	*Vibrio*
05MHRW	Mussel OTC 0	Yellow	Irregular	6-7	Short and thick rods		-	+	+	A/A	S	+	*Vibrio*
06MHRW	Mussel OTC 50	Yellow	Circular	1-3	Short and thick rods	+	-	+	+	A/A	S	+	*Vibrio*
07MHRW	Mussel OTC 50	Green (dark center)	Circular	2-3	Short and thick rods	+	-	$+^{W}$	+	K/K	S	-	*Pseudomonas*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; N: growth in absence of NaCL; G: Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak; S: susceptible; R: resistant; $^{+}$: *S. putrefasciencis* according to RapID NF Plus System

B.2. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Cap Egmont during warm season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
01SCEW	Sediment OTC 0	Green (light, slight dark center)	Circular, entire	5-7	Very short rods	-	-	+	+	A/A^{w}	S	-	*Vibrio*
02SCEW	Sediment OTC 0	Green (dark, slight dark center)	Circular, entire	3-4	Medium rods		-	+	+	K/K	R	-	*Pseudomonas*
03SCEW	Sediment OTC 0	Yellow (cream center)	Circular, undulate	7	Short and thick rods			+	+	A/A	S		*Vibrio*
04SCEW	Sediment OTC 0	Yellow	Irregular	8	Short and thick rods, slightly curved	-	-	+	+	A/A	S	+	*Vibrio*
05SCEW	Sediment OTC 0	Cream (slight dark center)	Circular, entire	4	Short and thick rods, slightly curved	+	-	+	+	K/A	S	+	*Vibrio*
06SCEW	Sediment OTC 0	Yellow	Circular, Undulate	12	Short and thick rods, slightly curved	+	-	+	+	A/A	S	+	*Vibrio*
07SCEW	Sediment OTC 50	Green (dark)	Circular, entire	3	Long rods (in chains)	-	-	+	+	K/K	S	-	*Pseudomonas*
01MCEW	Mussel OTC 0	Yellow (clearer edges)	Circular, entire	6-10	Short and thick rods	-	-	+	+	A/A	S	+	*Vibrio*
02MCEW	Mussel OTC 0	Green (slight dark center)	Circular, entire	2-5	Short rods	-	-	+	+	A/A^{w}	S	-	*Vibrio*
03MCEW	Mussel	Yellow (slight	Circular,	2-4	Short and	-	-	+	+	A/A^{w}	S	-	*Vibrio*

	OTC 0	dark center)	entire		thick rods								
04MCEW	Mussel OTC 0	Yellow	Undulate	6-8	Short and thick rods, slightly curved	+	-	+	+	A/A	S	+	*Vibrio*
05MCEW	Mussel OTC 0	Green (slight dark center)	Circular, entire	4-6	Medium rods	-	-	+	-	A/A	S	-	*Vibrio*
06MCEW	Mussel OTC 0	Green (dark)	Circular, undulate	2-3	Medium rods	-	-	+	+	A/A^{W}	R	-	*Aeromonas*
07MCEW	Mussel OTC 50	Yellow-cream	Circular-spindle	6-10	Short and thick rods, slightly curved	-	-	+	+	A/A	S	+	*Vibrio*
08MCEW	Mussel OTC 50	Green (slight dark center)	Circular, erose	1-3	Medium rods	-	-	+	+	A/A^{w}	R	-	*Aeromonas*
09MCEW	Mussel OTC 50	Green (dark)	Circular, entire	4	Long rods	-	-	+	+	K/K H_2S	R	-	*Shewanella*
10MCEW	Mussel OTC 50	Green (slight dark center)	Circular, undulate	6	Short and thick rods	-	-	+	+	A/A	S	-	*Vibrio*
11MCEW	Mussel OTC 50	Green (translucent edge)	Circular, entire	2-3	Short and thick rods	+	-	+	+	A/A	S		*Vibrio*
12MCEW	Mussel OTC 50	Green (translucent edge)	Circular, undulate	5	Short and thick rods	-	-	+	+	A/A^{w}	R	-	*Aeromonas*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; N: growth in the absence of NaCl; G : Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak; S: susceptible; R: resistant

B.3. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Murray Rv during warm season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
1SMRW	Sediment OTC 0	Yellow	Circular, undulate Flat	4-6	Medium rods	+	-	+	+	A/A	S	+	*Vibrio*
2SMRW	Sediment OTC 0	Light green (bright, almost cream)	Circular, entire Convex	3-6	Thick and short rods	+	-	+	+	K/A	S	-	*Vibrio*
3SMRW	Sediment OTC 0	Black (translucent edges)	circular	3-5		-	-	+	+	K/A^{w} H_2S	S	-	*Shewanella*$^{+}$
4SMRW	Sediment OTC 0	Yellow	Circular	2-6	Medium rods	+	-	+	+	A/A	S	-	*Vibrio*
5SMRW	Sediment OTC 0	Green (yellowish)	Circular (dots)	4-5									Lost
6SMRW	Sediment OTC 0	Black (green yellow edges)	Circular		Large rods	-	-	+	+	K/A H_2S	S	-	*Shewanella*
7SMRW	Sediment OTC 50	Green (black center)	Circular	3	Large rods	-	-	+	+	K/A H_2S	S	-	*Shewanella*$^{+}$
8SMRW	Sediment OTC 50	Green (lighter edge)	Circular-entire Flat	3	Short rods	+	-	+	+	A/A^{w}	S	-	*Vibrio*
9SMRW	Sediment OTC 50	Green (bright)	Circular convex	3	Large and thin rods	+	-	+	+	K/K H_2S	R	-	*Shewanella*$^{+}$
1MMRW	Mussel OTC 0	Yellow	Circular		Short and thick rods		-	+	+	A/A	S	-	*Vibrio*
2MMRW	Mussel OTC 0	Yellow	Circular	5	Medium rods	+	-	+	+	A/A		-	*Vibrio*
3MMRW	Mussel OTC 0	Yellow	Circular Flat	8	Short and thick rods	+	-	+	+	A/A	S	+	*Vibrio*

4MMRW	Mussel OTC 0	Green (bright)	Circular, convex	3	Short and thick rods	-	-	+	+	A/A	S	-	*Vibrio*
5MMRW	Mussel OTC 0	Yellow greenish (bright)	Irregular, pulvinate	3	Short and thick rods	-	-	+	+	A/A	S	-	*Vibrio*
6MMRW	Mussel OTC 0	Green	Circular	5	Short and thick rods	-	-	+	+	A/A	S	-	*Vibrio*
7MMRW	Mussel OTC 50	Light green (lines up to light)	Circular	3	Very short rods	-	-	+	-	A/A	S	-	*Vibrio*
8MMRW	Mussel OTC 50	Dark green (bright)	Circular, convex	3	Short and very thick rods	+	-	+	+	K/A	S	+	*Vibrio*
9MMRW	Mussel OTC 50	Light green (dots)	Circular	6	Medium rods	-		+	-	A/A	S	-	*Vibrio*
10MMRW	Mussel OTC 50	Light green (bright)	Circular, convex	6	Short and thick rods	+	-	+	+	K/K	S	+	*Pseudomonas*
11MMRW	Mussel OTC 50	Light green (wrinkles)	Circular, convex	5	Short rods	+	-	+	+	A/A	S	-	*Vibrio*
12MMRW	Mussel OTC 0	Light green	Circular, flat	6	Short and thick rods	-	-	+	+	A/A	S	-	*Vibrio*
13aMMRW	Mussel OTC 0	Light green			Short and thick rods		-	+	+	K/A	S	+	*Vibrio*
13bMRW	Mussel OTC 0	Green			Short and thick rods		-	+	+	K/A	S	+	*Vibrio*
14MMRW	Mussel OTC 50	Dark green	Circular	4-7	Short and thick rods	+	-	+	+	A/A	S	+	*Vibrio*
15aMMRW	Mussel OTC 0				Very short rods		-	+	+	A/A	S	-	*Vibrio*
15bMMRW	Mussel OTC 0				Large rods		-	+	+	K/K	R	-	*Pseudomonas*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; N: growth in the absence of NaCl; G: Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak; S: susceptible; R: resistant; $^{+}$: *S. putrefasciencis* according to RapID NF Plus System

B.4. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Hillsborough Rv during cold season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
1SHRC	Sediment OTC 50	Light green, brilliant	Circular, convex, entire	1-3	Short rods	-	-	+	+	K/K H_2S	S	-	*Shewanella*
2SHRC	Sediment OTC 50	Dark green, opaque (dark spot)	Circular, flat, entire	1-3	Long rods	-	-	+	+	K/K	S	-	*Pseudomonas*
3SHRC	Sediment OTC 50	Dark green (black spot)	Circular, flat, entire	1-3	Long rods, chains	-	-	+	+	K/K H_2S	S	-	*Shewanella*
4SHRC	Sediment OTC 50	Yellow (cream), brilliant	Circular, convex, entire	1.5-2.5	Very short and thick rods	+	-	+	+	A/A	S	-	*Vibrio*
5SHRC	Sediment OTC 50	Dark green (dark spot)	Circular, flat, entire	2.5-3	Medium rods	-	-	+	+	K/K	S	-	*Pseudomonas*
6SHRC	Sediment OTC 50	Dark green (black spot)	Circular, flat, entire	2.5-5	Medium rods	-	-	+	+	K/K H_2S	S	-	*Shewanella*
7SHRC	Sediment OTC 50	Black (translucent edges)	Circular, flat, entire	4	Medium rods	-	-	+	+	A/A^W H_2S	S	-	*Shewanella*
8SHRC	Sediment OTC 50	Cream (greenish), brilliant	Circular, convex, entire	2.5	Short and thick rods	+	-	+	+	K/A	S	+	*VIbrio*
9SHRC	Sediment OTC 0	Intense yellow, brilliant	Circular, convex, entire	2-5	Rods	-	-	-	+	A/A	R	-	*Aeromonas*
10SHRC	Sediment OTC 0	Yellow (darker center,	Circular, umbonate,	4-5	Rods	-	-	-	+	A/A		-	*Vibrio*

		translucent edges)	undulate										
11SHRC	Sediment OTC 50	Dark green	Circular, flat, entire	5-6	Short rods	-	-	+	+	K/K	S	-	*Pseudomonas*
1MHRC	Mussel OTC 50	Cream-white (greenish), brilliant	Circular, convex, entire	3	Very short and thick rods	-	-	+	+	K/A	S	+	*Vibrio*
2MHRC	Mussel OTC 0	Dark green, opaque	Circular, flat, entire	5-6	Medium rods	-	-	+	+	K/K	S	-	*Pseudomonas*
3MHRC	Mussel OTC 0	Cream-white (greenish)	Circular, convex, entire	6-8	Short and thin rods	+	-	+	+	K/A	S	+	*Vibrio*
4MHRC	Mussel OTC 50	Green	Circular, convex, entire	1	Medium-large rods	+	-	+	+	A/A	S	+	*Vibrio*
5MHRC	Mussel OTC 0	Yellow	Circular, convex, entire	1-7	Short and thick rods	+	-	+	+	A/A	S	+	*Vibrio*

1: TCBS plates (with or without oxytetracycline) and type of sample origin ; N: growth in the absence of NaCl; G : Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak; S: susceptible; R: resistant

B.5. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Cap Egmont during cold season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive Genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
1SCEC	Sediment OTC 50	Dark green (dark spot), opaque	Circular, flat, entire	2-3	Short and thick rods	-	-	+	+	K/K H_2S	S	-	*Shewanella*
2SCEC	Sediment OTC 50	Light green	Circular, convex, entire	1-3		-	-			A/A	R^I		*Aeromonas*
3SCEC	Sediment OTC 50	Light green (whitish), brilliant	Circular, pulvinate, entire	1.5-2.5	Very short and thick rods	+	-	+	+	K/A	S	+	*Vibrio*
1MCEC	Mussel OTC 50	Dark green (black spot), opaque	Circular, flat, entire	3	Short and thick rods	-	-	+	+	K/K H_2S	S	-	*Shewanella*
2MCEC	Mussel OTC 50	Dark green, brilliant	Circular, flat, entire	1-3	Long and thin rods	-	-	+	+	K/K	S	-	*Pseudomonas*
3MCEC	Mussel OTC 50	Light green (whitish edges)	Circular, pulvinate, entire	2.5-3	Short and curved rods	+	-	+	+	K/A	S	+	*Vibrio*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; N: growth in the absence of NaCl; G : Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak

B.6. Phenotypic characterization and presumptive identification of the representative isolates of sediments and mussels obtained from Murray Rv during cold season

Isolate		Colony characteristics			Bacterial shape	Tests							Presumptive Genus
Code	Origin[1]	Color	Shape	Size (mm)		N	G	O	C	TSI	O129	H	
1SMRC	Sediment OTC 50	Dark green (dark spot), opaque	Circular, flat, entire	2-5	Large and thin rods	-	-	+	+	K/K	S	-	*Pseudomonas*
2SMRC	Sediment OTC 50	Green	Circular, flat, entire	2-6	Very short and thick rods	-	-	+	+[w]	K/K	S	-	*Pseudomonas*
3SMRC	Sediment OTC 50	Green (black spot)	Circular, flat, entire	2-3.5	Medium rods	-	-	+	+	K/K H_2S	S	-	*Shewanella*
4SMRC	Sediment OTC 50	Black, translucent-greenish edges	Circular, flat, entire	2-5	Medium and thin rods, chains	-	-	+	+	K/K	S	-	*Pseudomonas*
5SMRC	Sediment OTC 50	Yellow, translucent edges	Circular, flat, entire	3-5	Short and thick rods	-	-	+	+	A/A	R^I	-	*Aeromonas*
6SMRC	Sediment OTC 50	Green-yellowish (granular)	Circular, convex, entire	3	Short rods	-	-	+	+	A/A	R^I	-	*Aeromonas*
7SMRC	Sediment OTC 50	Cream-whitish	Circular, convex, entire	2-5	Short and thick rods	-	-	+	+	A/A	S	+	*Vibrio*
1MMRC	Mussel OTC 50	Green (dark center)	Circular, flat, entire	4	Short and thick rods	-	-	+	+	K/K	S	-	*Pseudomonas*
2MMRC	Mussel OTC 50	Black, translucent-greenish edges, brilliant	Circular, flat, entire	5-6	Long and thin rods, chains	-	-	+	+	A/A H_2S	S	-	*Shewanella*

3MMRC	Mussel OTC 50	Light green, brilliant	Circular, flat, entire	2	Medium rods	-	-	+	+	A/A	S	-	*Vibrio*
4MMRC	Mussel OTC 50	Green-yellowish, brilliant	Circular, convex, entire	2.5	Medium rods	-	-	+	+	A/A	R^I	-	*Aeromonas*
5MMRC	Mussel OTC 50	Yellow, brilliant	Circular, flat, entire	5	Short rods	+	-	+	+	A/A	S	-	*Vibrio*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; N: growth in the absence of NaCl; G : Gram stain, confirmed with KOH reaction; O : oxidase; C : catalase; H : hemolytic; ^{W}: weak; S: susceptible; R: resistant; R^I: intermediate resistant

B.7. Biolog identification of the representative isolates of sediments and mussels obtained from Hillsboroough Rv. during warm season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SHRW	Sediment 0 OTC	72					No growth
2SHRW	Sediment 0 OTC	24	-	0.32	6.92	*Aeromonas veronii/sobria* DNA group 8	No ID
3SHRW	Sediment 0 OTC	24	97	0.71	4.06	*Vibrio tubiashi*	*Vibrio tubiashi*
4SHRW	Sediment 0 OTC	24	100	0.66	5.22	*Burkholderia glumae*	*Burkholderia glumae*
5SHRW	Sediment 0 OTC	24	97	0.57	6.41	*Shewanella putrefaciens* A	*Shewanella putrefaciens* A
6SHRW	Sediment 0 OTC						No growth
7SHRW	Sediment 50 OTC	24	89	0.62	4.64	*Vibrio splendidus*	*Vibrio splendidus*
8SHRW	Sediment 50 OTC						No growth
9SHRW	Sediment 50 OTC	24	75	0.59	3.22	*Burkholdera glumae*	*Burkholdera glumae*
10SHRW	Sediment 50 OTC	24	100	0.56	6.94	*Birkholderia glumae*	*Birkholderia glumae*
11SHRW	Sediment 50 OTC						No growth
1MHRW	Mussel 0 OTC						No growth
2MHRW	Mussel 0 OTC	24	97	0.59	6.12	*Vibrio splendidus*	*Vibrio splendidus*

3MHRW	Mussel 0 OTC	24	100	0.84	2.36	*Vibrio splendidus*	*Vibrio splendidus*
4MHRW	Mussel 0 OTC		-	0.41	8.75	*Vibrio splendidus*	No ID
5MHRW	Mussel 0 OTC	24	76	0.61	3.00	*Aminnobacter aminovorans*	*Aminnobacter aminovorans*
6MHRW	Mussel 50 OTC	24	100	0.82	2.74	*Vibrio splendidus*	*Vibrio splendidus*
7MHRW	Mussel 50 OTC		-	0.10	14.34	*Flavobacterium tirrenicum* (*Chryseobacterium*)	No ID
1SHRW	Sediment 0 OTC	72				No growth	-
2SHRW	Sediment 0 OTC	24	-	0.32	6.92	*Aeromonas veronii/sobria* DNA group 8	No ID
3SHRW	Sediment 0 OTC	24	97	0.71	4.06	*Vibrio tubiashi*	*Vibrio tubiashi*
4SHRW	Sediment 0 OTC	24	100	0.66	5.22	*Burkholderia glumae*	*Burkholderia glumae*
5SHRW	Sediment 0 OTC	24	97	0.57	6.41	*Shewanella putrefaciens* A	*Shewanella putrefaciens* A
6SHRW	Sediment 0 OTC						No growth

1: TCBS plates (with or without oxytetracycline) and type of sample origin

B.8. Biolog identification of the representative isolates of sediments and mussels obtained from Cap Egmont during warm season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SCEW	Sediment 0 OTC	72					No growth
2SCEW	Sediment 0 OTC	24	-	0.39	4.28	*Burkholderia glumae*	No ID
3SCEW	Sediment 0 OTC	24	87	0.65	3.77	*Vibrio aestuarianus*	*Vibrio aestuarianus*
4SCEW	Sediment 0 OTC	24	-	0.26	4.47	*Vibrio aestuarianus*	No ID
5SCEW	Sediment 0 OTC	24	100	0.64	5.53	*Vibrio carcariae*	*Vibrio carcariae*
6SCEW	Sediment 0 OTC	24	97	0.70	4.22	*Vibrio carcariae*	*Vibrio carcariae*
7SCEW	Sediment 50 OTC	72					No growth
1MCEW	Mussel 0 OTC	72					No growth
2MCEW	Mussel 0 OTC	72					No growth
3MCEW	Mussel 0 OTC	72					No growth
4MCEW	Mussel 0 OTC	24	-	0.43	3.13	*Vibrio splendidus*	*Vibrio*
5MCEW	Mussel 0 OTC	72					No growth
6MCEW	Mussel 0 OTC	72					No growth

7MCEW	50 OTC Mussel	72	No growth
8MCEW	50 OTC Mussel	72	No growth
9MCEW	50 OTC Mussel	72	No growth
10MCEW	50 OTC Mussel	72	No growth
11MCEW	50 OTC Mussel	72	No growth
12MCEW	50 OTC Mussel	72	No growth

1: TCBS plates (with or without oxytetracycline) and type of sample origin

B.9. Biolog identification of the representative isolates of sediments and mussels obtained from Murray Rv during warm season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SMRW	Sediment 0 OTC	48	72	0.52	4.13	*Vibrio tubiashi*	*Vibrio tubiashi*
2SMRW	Sediment 0 OTC	72					No growth
3SMRW	Sediment 0 OTC	72	96	0.83	2.02	*Vibrio tubiashi*	*Vibrio tubiashi* [§]
4SMRW	Sediment 0 OTC	48	-	0.14	3.90	*Vibrio tubiashi*	No ID
5SMRW	Sediment 0 OTC						Lost
6SMRW	Sediment 0 OTC	48	98	0.55	6.87	*Vibrio tubiashi*	*Vibrio tubiashi*
7SMRW	Sediment 50 OTC						No growth
8SMRW	Sediment 50 OTC	48	99	0.56	6.76	*Burkholderia glumae*	*Burkholderia glumae*
9SMRW	Sediment 50 OTC	48	87	0.55	5.77	CDC group II-E subgroup A	CDC group II-E subgroup A
1MMRW	Mussel 0 OTC						No growth
2MMRW	Mussel 0 OTC	24	99	0.87	1.90	*Vibrio tubiashi*	*Vibrio tubiashi*
3MMRW	Mussel 0 OTC	24 48	89 100	0.50 0.54	6.12 7.95	*Vibrio aestuarianus* *Vibrio splendidus*	*Vibrio aestuarianus* *Vibrio splendidus*
4MMRW	Mussel 0 OTC						No growth

5MMRW	Mussel 0 OTC						No growth
6MMRW	Mussel 0 OTC						No growth
7MMRW	Mussel 50 OTC						No growth
8MMRW	Mussel 50 OTC	24	-	0.43	8.76	*Vibrio alginolyticus*	No ID
9MMRW	Mussel 50 OTC						No growth
10MMRW	Mussel 50 OTC	48	100	0.72	4.22	*Vibrio splendidus*	*Vibrio splendidus*
11MMRW	Mussel 50 OTC						No growth
12MMRW	Mussel 0 OTC						No growth
13aMMRW	Mussel 0 OTC	48	98	0.75	3.52	*Vibrio splendidus*	*Vibrio splendidus*
13bMMRW	Mussel 0 OTC	24	-	0.44	4.74	*Aeromonas hydrophila* DNA group 1	No ID
14MMRW	Mussel 50 OTC	72	97	0.63	5.47	*Aeromonas veronii* DNA group 10	*Aeromonas veronii* DNA group 10
15aMMRW	Mussel 0 OTC	24	-	0.38	3.03	*Vibrio tubiashi*	*Vibrio tubiashi*

1: TCBS plates (with or without oxytetracycline) and type of sample origin ; [§]: Biolog ID with too few positives matches

B.10. Biolog identification of the representative isolates of sediments and mussels obtained from Hillsborough Rv during cold season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SHRC	Sediment 50 OTC	24	72	0.59	2.77	*Burkholderia glumae*	*Burkholderia glumae*[§]
2SHRC	Sediment 50 OTC	72					No growth
3SHRC	Sediment 50 OTC	24	99	0.68	4.83	*Burkholderia glumae*	*Burkholderia glumae*[§]
4SHRC	Sediment 50 OTC	24	-	0.18	10.45	*V. mediterranei*	No ID
5SHRC	Sediment 50 OTC	24	83	0.59	4.49	*Burkholderia glumae*	*Burkholderia glumae*[§]
6SHRC	Sediment 50 OTC	24	-	0.24	4.62	CDC group II-E subgroup B	No ID
7SHRC	Sediment 50 OTC	24	100	0.64	5.52	*Burkholderia glumae*	*Burkholderia glumae*
8SHRC	Sediment 50 OTC	48	98	0.53	7.26	*Vibrio vulnificus*	*Vibrio vulnificus*
9SHRC	Sediment 0 OTC	24	71	0.60	2.31	*Vibrio tubiashi*	*Vibrio tubiashi*
10SHRC	Sediment 0 OTC	48*	100	0.77	3.43	*Vibrio tubiashi*	*Vibrio tubiashi*
11SHRC	Sediment 50 OTC	24	-	0.28	12.68	*Shewanella puterfaciens* B	No ID
1MHRC	Mussel 50 OTC	24	-	0.24	9.50	*Vibrio splendidus*	No ID
2MHRC	Mussel 0 OTC	24	-	0.20	14.78	*Shewanella putrefaciens*	No ID

3MHRC	Mussel 0 OTC	24	-	0.24	9.54	*V. splendidus*	No ID
4MHRC	Mussel 50 OTC	24	100	0.67	5.04	*Vibrio proteolyticus*	*Vibrio proteolyticus*
5MHRC	Mussel 0 OTC	24	100	0.56	6.83	*Vibrio alginolyticus*	*Vibrio alginolyticus*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; [$]: Biolog ID with too few positives matches

B.11. Biolog identification of the representative isolates of sediments and mussels obtained from Cap Egmont during cold season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SCEC	Sediment 50 OTC	72	68	0.54	3.00	*Vibrio tubiashi*	*Vibrio tubiashi*[§]
2SCEC	Sediment 50 OTC	72	-	0.23	11.37	*Vibrio mediterranei*	No ID
3SCEC	Sediment 50 OTC	72	91	0.60	5.16	*Vibrio vulnificus*	*Vibrio vulnificus*
1MCEC	Mussel 50 OTC	72					No growth
2MCEC	Mussel 50 OTC	24	69	0.59	2.24	*Vibrio tubiashi*	*Vibrio tubiashi*
3MCEC	Mussel 50 OTC	24	100	0.65	5.40	*Vibrio splendidus*	*Vibrio splendidus*
		72	97	0.64	5.25	*Vibrio vulnificus*	*Vibrio vulnificus*

1: TCBS plates (with or without oxytetracycline) and type of sample origin; [§]: Biolog ID with too few positives matches

B.12. Biolog identification of the representative isolates of sediments and mussels obtained from Murray Rv during cold season

Isolate Code	Origin[1]	Incubation Time (h)	Biolog top-rated ID choice				Biolog ID
			PROB	SIM	DIST	Species	
1SMRC	Sediment 50 OTC	72					No growth
2SMRC	Sediment 50 OTC	72					No growth
3SMRC	Sediment 50 OTC	24	75	0.60	3.03	*Burkholderia glumae*	*Burkholderia glumae*[§]
4SMRC	Sediment 50 OTC	24	79	0.60	3.64	*Vibrio tubiashi*	*Vibrio tubiashi*
5SMRC	Sediment 50 OTC	24	-	0.23	6.70	*Vibrio mediterranei*	No ID
6SMRC	Sediment 50 OTC	24	-	0.28	12.68	*Vibrio mediterranei*	No ID
7SMRC	Sediment 50 OTC	24	92	0.50	7.16	*Vibrio alginolyticus*	*Vibrio alginolyticus*
1MMRC	Mussel 50 OTC	72					No growth
2MMRC	Mussel 50 OTC	24	-	0.43	2.55	*Burkholderia glumae*	No ID
3MMRC	Mussel 50 OTC	24	98	0.68	4.66	*Vibrio harveyi*	*Vibrio harveyi*
4MMRC	Mussel 50 OTC	24	-	0.31	10.00	*Vibrio tubiashi*	No ID
5MMRC	Mussel 50 OTC	24	-	0.38	10.27	*Aeromonas hydrophila* DNA group 1	No ID

1: TCBS plates (with or without oxytetracycline) and type of sample origin; [§]: Biolog ID with too few positives matches

B.13. Sequences of the 16S rDNA of bacterial isolates from representative marine environments of PEI

Isolate code	16S rDNA sequences
7SHRW	ANNGCTNCATGCAGTCGAGCGGAACGCACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGG ACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCG CATAATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTA GCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA GCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAA GGGGGTANNGTTAATAGCNCTATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAG CCGCCGCCGGTAATNCAANNNNC
8SHRW	GGNTCGTGCTTTGAGGGGACCNCNTNACATCCTTNNGGTGNTTNAATGGGCGTCGAGCGGCGGACGGGT GAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAAT GCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGT TGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGTGT GTAGTTAATAGCTGCNCATCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCCCCNG GCGGTAATNCAAANNNNNA
9SHRW	ACGGCTCCATGNNGTTGTNGAGNANGTTNNTGCTTGCTCTACNCTGTCGAGCGGCGGACGGGTGANTCC TGCCTNTGGNTNTGCCCACTCNAGGGGGATNACTNTTGGNNACGACTGCTAATACCGCATACGCCCTAC GGGGGAAANGAGGGNACCTTCNNGCCTTTCTCNATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGA GGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGA CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC CATGCCGCGTGTGTGAANAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGCAGTTGTTT AATAAACAACTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCCGCNGGTA ATNCAANNNN
10SHRW	GANTGCTACCTGCNGTCGAGCGGTACCAANGGAGCTTGCTCCTGAGGTGACTAGCGGCGGACGGNTGAG TAATGCCTAGGTNTCTGCNCANTCNAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCC TACNGGGGAAAGGAGGAGACCTTCCAGCCTTCCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGG TGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTG AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCACCGAGGAGGAAAGCTCAAGC GTTAATACGTTTGGGTGTGACGTTCTCNCANAANAACACCGGCTAACTTCGTGCCANCNGCCNCNGTAA TNCAANGC

6MHRW	GGCTGNCTTNCNGNTCAGCNGTCGACCNNCNGTCCTTAGGGTGCGTTNTGGGCGTCNAGCGGCGGACGG GTGAGTAATGCCTANGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATA ATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTA GTTGGTGAGGTAATGGCTCACCAAGGCGACATATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATATNCGAAAGCC TGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGG GGTAGTGTTAATAGCGCTATCTCTTGACGTTAGCACAGAAGAAGCACCGGCTAACTCCGTGCCACCGCC GCCGGTAATNCAAANNAG
7MHRW	GCAGTCGGCGGTACAGGATTNCTTGCTATTTGCTGCGAGCGGCGGACGGGTGAGTAATGCCTAGGGATC TGCCCAGTCGAGAGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGG AGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCT CACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGT GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACGGTTAATAACCGTT AGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCGCCCGCCGGTAATNCAAANN NNTC
7MCEW	CTCCTGCAGTCGAGCGGAACACANTAACAATCCTTCGGGTGCGTTATGGGCGTCGAGCGGCGGACGGGT GAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAAT GCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGT TGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA TGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGTGT GTAGTTAATAGCTGCNCNTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCCGCCG CGGTAATNCAAANNNNNNNC
8MCEW	TCCTGCAGTGCCGAAACACACATTGACCCTTCGGGTGTTTGTTGGGCGTCGAGCGGCGGACGGGTGAGT AATGCCTGGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAACGTCT ACGGACCAAAGAGGGGGACCTTCGGGCCTCTCGCTTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGT GAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGAACTG AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGCNTTNAA GTTAATAGCTTNATNGTTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCCGCCGCGG TAATNCAAANNNN
9MCEW	GCNCCNTNNAGTCNAGCGGTACATTTCNAACTTGCTTTTGAAATGACGANCGGCGGACGGGTGATTAAT GCCTAGGGAACTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACG GGGGAAAGGAGGGGCTCTTCGGACCTTTCGCGATTGGATGTACCTAGGTGGGATTAGCTTGTTGGTGAG GTAANAGCTCACCAAGGCGACGATCCCTANCTGTTCTGAGAGGATGATCAGCCATCTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCNAAAGCCTGATGCACCCA

	TGCCGCGNGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTAACGGTTAA TACCCGTTAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTTCGTGCCAGCCGCCCGCGGTAAT NCAANNNNG
11MCEW	GTGCNANCATGCAGTCGAGCGACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTA ACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTT GGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCT AGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCC TGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAA ACGTGTAAGTAACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCCG CCGGCGGTAATNCAANNNNNN
8SMRW	GCCGTGNNTGTCNCCGGTACAGGATTAGCTTGCTATTTGCTGACCAGCGNCNGACNGGTGAGTNATGNC TNTCNNNCTGCCCNGTCGANGNGGATAACNGTTGNAAACGACTGCTNNTACCGCATACGCCCTACGGNN NNNAGGAGGGGACCTTCNGGCCTTTCTCGATTGGATGAACCTCCGTGGNATTATCTANTTGGTGAGGTA ATGGCTCNCCAANGCGACGATCCCTATNTGTTCTGANANGATGATCNNCCACACTGGNACNGAGACACN GCCCATGACTCCTANGGGCANGNGCCACCTGNGGAATATTGNACAATGGGCNANAGCCTGATGCATCCA TGCCGCGTGTGTGAAGAAGGNCTTCTNNTTGTAAANCACTTTCANTATGGANNANAGGTTAACNGTTAN NANCCGTTANCTGTGACNTTACCTACCGAAGAANGACAGGCTAACTTCGTGCCAGCCGCCGCCGGGTAA TNCAAANNNAN
9SMRW	CGGTANANGNTTTANCTTGCTAATTTGCTGACCAGCGGCGGACGGGTGAGTAATGCCTANGGNACTGCC CTGTNTAGGGGNATAACAGTTGGAAACGACTGNNAATACCGCATACTCCCTACGTGGGTTAAAAGAGGG TAACCTTCGGGCCTTCTGCGAATGGATGTACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACC ANGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTG AAGAAGGCCTTCGGGTTGTAAAGCACTTTCANCGAGGAGGAAAGCTTAAGCGTTAATAGCGTTTANGTG TGACGTTACTCGCAGAAGAAGGACCGNCTAACTTCGTGCCAGCCGCCGCGGTAATTCACTTATCC
7MMRW	CTGCAGTNAGCGAAACACACATTGACCCTTCGGGTGATTTGTTGGGCGTCGAGCGGCGGACGGGTGAGT AATGCCTGGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAACGTCT ACGGACCAAAGAGGGGGACCTTCGGGCCTCTCGCTTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGT GAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGA GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCA GCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTCGTGAGGAAGGCATTANAG TTAATAGCTTTAGTGTTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCNGCCGCGGG TAATNCAANNNC
8MMRW	CTGTTGCGAGCGANCNCTTACTGACCTTCGGGGAACGNNAAGGGCGTCGAGCGGCGGACGGGTGAGTA ATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTA CGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTG

	AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCANCCACACTGGAACTGAG ACACGGTCCACACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG CCATGCCGCGTGTGTGAAGAACGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGT TAATAGCTGCATTANTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCCGCCNGCGGT AANNCAANCAAGNCN
14MMRW	CACGGNTAACCGCCNNNNNTTCANCNAGCGGTNNATGAAGTANCTTGCTACTTCGCTGACNAGCGGCGG ACGGGTGAGTANTGCCTGGGAATATGCCTTGATGTGGGGGATAACTATTGGAAACNATAGCTAATACCG CATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATTAGCCCAGGTGGGATTA GCTAGTTGGTGGGGTAAAGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACA CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAA CCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCAGTGAGGAA GGTGNTAGTGTTAATAGCACTATCATTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAG CCGCCGCGGTAAATNCACGCGNNNC
1SHRC	CTGCCGTGNNNTNGNGCGGTANNGGATTANCTTGNTGTTNGCTGACCAGCGNNNGACNGGNGAGTNAT GNCTNTCCNNCTGCCCTNTCGNNGNGGATAACNGTTGNNNACGACTGCTNNTACNGCATACGCCCTNCG GNNNNNANGAGGGGACCTTCNGGCCTTTCTCNATTGCCTGAACCTCCGTGGNATTANCTANTTGGTCGA GGNNATGGCTCACCAANGNNACNATCCCTATNTGTTCTGACTANNATGATCGNCCACACTGGNACNGAA ANACNGCCCATGCTCCTACGGGCANGNACCACCTGNGGAATATTGCACAATGNGNNAAAGCCNGATGC ATCCTTGCCGCGTGTGTGCAAGGAAGGNCTTCTNNCTTGTAAANCANTTTCANNNTGNNNNANNGGTTA ACNGTTANNANNCNTTAACTNNNANNNTACCNACNGGAAGAAANACAGGCTAACTTCGTGCCAGCCGC CGCGGTAATNCAANNNANN
2SHRC	GCTCCTGCANGTGAGCGGTACNGGAATTAGCTTGCTATTCGCTGACGAGCGGCGGACGGGTGAGTAATG CCTAGGGATCTGCCCAGTCGAGGGGGATAACANTTGGAAACGACTGCTAATACCGCATACGCCCTACGG GGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGG TAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCA TGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACGGTTAA TAACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCCGCGGTAATN CAATTCANNN
3SHRC	GCTCCTGCATGTCAGCGGTACAGGATTAGCTTGCTATTCGCTGACGAGCGGCGGACGGGTGAGTAATGC CTAGGGATCTGCCCAGTCGAGGGGGATAACANTTGGAAACGACTGCTAATACCGCATACGCCCTACGGG GGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGT AATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACAC GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCAT GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACGGTTAAT AACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCCNCCGGTAATN CAAATTCANNG

5SHRC	GTCGTCNNGCGGTACCGGATTTCTTGCTATTCGCTGACGAGCGGCGGACGGGTGAGTAATGCCTAGGGA TCTGCCCAGTCGAGGGGGATAACANTTGGAAACGACTGCTAATACCGCNTACGCCCTACGGGGGAAAG GAGGGGACCTTCGGGCCTTTCGCGATTGGANGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAANGNC TCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATCGATCANCCACACTGGGACTGANACACNGCCC CATACTCCTACGGGAGGCAGCAGTGGGGAATATTGCNCANTGGGCNAAAGCCTGATGCCAGCCATGNC GCGTGTGTGAANAACGCCTTCGGGTTGNAAAGCNCTTTCAGTAGGGAGGAAAGGTTAACGGTTAATNAC CGTTAGCTGTGACGTTACCTACANAAGAAGGACCGGCTAACTTCGTGCCNNCCCCCGCCGGTAANNCAN AACACCNCC
6SHRC	GGTATCACGCCTCCCATNCCNNTCGAGCGGTACNGNNAGAAAGCTTGCTTTCTTTGCTGACCAGCGGCG GACGGGTGAGTAATGCCTAGGGATCTGCCCANTCGNGGGGGNATAACNNTTGGAAACGACTGCTAATA CCGCNTACGCCCTACGGGGGNAAAGGAGGGGACCTTCNGGCCTTTCGCGATTGGATGAACCTAGGTGGN ATTAGCTAGTTGGTAAGGTAATGGCTTACCANGGCGACGATCCCTNNCTGTTCTGAGAGGATGATCACC CCCACTGGGACTGANACACGNNCCCAGTACTCCTACGGCAGGCAGCCTGTGGGNAATATAGCATAATGG GGNCAAACCCTGNATGCACCCATGCCNCCGTGTGTGAAAAACGGCCTTCGGGNTGTAAAGCCACTTTCN GTAGGGGAGGTAAAGGTNNTGNTTTAATACCCANATTACTGTGACNCTTNCCTACNGAAGANAGGACCC GGTTTANCTTCCGTGCCAGCNNACCGGCGGTAAANNCNACNGCCNC
7SHRC	GTTACGGCCCCCGTCACTAAGNTNTTCANGANTTAGCTTGCTATTNNCTACGAGCGGCGGACGGGTGAG TAATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACANTTGGAAACGACTGCTAATACCGCATACGCCC TACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGG TGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACG GTTAATAACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCNGCGG TAATNCAAANNNANC
8SHRC	ACCATGCAGTCGACGGANCNACCTNACAATCCTTCGGGTGCGTTAATGGGCGTCNAGCGGCGGACGGGT GAGTANTGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGAGAAACGATGGCTAATACCGCATAA TGCCTACGGNCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAG TTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGA ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGANAGCCTG ATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGGG TANCGTTNATAGCGCTATCTCTTGACGTTACAACAGAAGAAGCACCGGCTAACTCCGTGCCAGCCGCCN GCGGGTAATNCACACAA
11SHRC	NANTCGAGCGGAAACAGNGGGGNGCTTGCTTTTTTNGCTGTCNAGCGGCNGACGGGTGAGTAATGCCTA TATATCTGCCTAGTCGTGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACNCCCTACGGGGGA AAGGAGGGGACCTTCGGGCCTTTNGCGATTANATGAGTCTAGGTGGGATTAGCTAGTAGGTGAGGTAAT GGCTCACCTAGGCGACNATCCCTAGCTGTTCTGAGAGGATGATCANCCACNNTGGGANTGACACACGGC CCANACTNCTACGGGAGGCAGCAGNGGGGAATATTGCACANTGGGCGAAAGCCTGANGCCCCCATGCC

	GCGTGTGTAAAAAANGCCNTCGGGTTGNAAAGCNCTNTCNGCGANGGANGAANGGTTGTGTGTNAATA ACNNCATAGCTGTGGATGTTACTCNCAAAANNAGCACCNGNTAACTTNCGTGCCATCCCGCCGCAGGTA ATACAANCNNCGCCC
1MHRC	GCNACCATGCAGCGAGCGGAACACACCATTGANTCTTCGGANGATTTGTTGGGCGTCGAGCGGCGGACG GGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCAT AATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCT AGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTG GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCC TGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGG TGTGTAGTTAATAGCTGCGCATCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCACCCC NNNGGGTNAATCAAANNNNNN
4MHRC	TCTGTNNCGGNCNCCTNCANTCCTTNGGGTGCGTTAATGGGCGTCNAGCGGCGGACGGGTGAGTAATGC CTATNGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGG GCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGNTGAG GTAATGACTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGAC ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCC ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGTGTGTAGTTA ATAGCTGCANCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCACCCCCNNGGGTTAA TCAAANNNNNNN
1SCEC	NANGCNGCGGNACANNATAAANTTGCTTTCTTTGCTGACGAGCGGCGGACGGGTGAGTAATGCCTANGG ATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAA GGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGGTAAGGTAATGG CTTACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGC GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGTGGTTTAATACGCC ATTACTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCCCCNNGGTAATACAAA NNNNNNNNNNN
2SCEC	CNNCGNTTANNGNTNGCNTNCAGTCGAGCGGTACNTTTCTAGCTTGCTAGAAGATGACGAGCGGCGGAC GGGTGAGTAATGCTTGGGAATATGCCTNTACGTGGGGGACAACAGTTGGAAACGACTGCTAATACCGCA TAATGTCTTCGGACCAAAGGAGGGGACGCTTCGGCACCTTTCGCGTATTGATTAGCCCAAGTGGGATTA GCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATTGATCAGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAA AGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGA AGAGTTAAGGGTTAATACCCCTTATCTTTGACGTTAGCAACAGAAGAAGGACCGGCTAACTCCTGCACC CCCCCNNNNNANAAATNAAAAAAANNNNNNNN
3SCEC	CGTNNANNGCTACCATGCAGTCGAGCGGAACGACACTAACAANCCTTCGGGTGCGTTAATGGGCGTCGA GCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCT

	AATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGT GGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGGAGAGGATGAT CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT GGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTT GTGAGGAAGGGTGTANNGTTAATAGCNNNATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTC CGTGCCACCCCCCCCNGGGNNAANANNAAAANNNNNNN
1MCEC	ANNGCTCCCATGCATGCGAGCGGTACAGGAATTAGCTTGCTATTTGCTGACGAGCGGCGGACGGGTGAG TAATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAATTGGAAACGACTGCTAATACCGCATACGCCC TACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGG TGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACG GTTAATAACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCNNCGG TNAATNCAAATTCANN
3MCEC	GACAGGCTACCATGCAAGTCGACGGAACGCACTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGG CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATA CCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGA TTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCC ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG AAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAG GAAGGGNGTNNNGTTAATAGCNNNATCTCTTGACGTTAGCAACAGAAGAACACCGGCTAACTCCGTGCC ACCCCNGCCGGTAATNCAANNNNNNNC
3SMRC	TCCCGCTGCTNGTGNGNNTCGNCGGTACCGGAATTAGCTTGNTNTTTGCTNNCCNGCGNCCGACTGGTG NGTGATGNNTNTCCNNNTGCCCTGTCGNTGNGGATAACTNTTGNNNACNNNTGCTNNTACNGCATNCNC CCTNTNGNNNTNNNGNGGGGANCTTNCGGCCTTTCTNNATNGCCTTNACNTCCGTNGNATTATCTNATTG GTCGACGGTCCTGGCATCTGGANCGCNCACNGATNCCTNTNTGTTNTGACANNATGATCGTCCACANNG NNACNGAAANACNGCCCATGCTCCTANNGGCTNNGCCACCTNNGGNNTATTGTACANTGGGNATTGAG CCNGATNCATCCTTGCCNCGTGTGTGCATGGANGNNCTTCTTTCTTNTAACNCANTTTCATNNTGNNTNA NGNGTTAACNGTNANNATNCTTNAACTNNNANTNTACCNACCNNAAGAAANACAGGCTAACTTCGTGC CAGCCCCCNCCNGTAAATTCAANTNCANNN
5SMRC	GGNTACAGCTACCATGCAAGTCGAGCGGTACATTTCTAGCTTGCTAGAAGATGACGAGCGGCGGACGGG TGAGTAATGCTTGGGAATATGCCTTTACGTGGGGGACAACAGTTGGAAACGACTGCTAATACCGCATAA TGTCTTCGGACCAAAGGAGGGGACGCTTCGGCACCTTTCGCGTATTGATTAGCCCAAGTGGGATTAGCT AGTTGGTAAGGTAATGGCTTACCAAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCC TGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAGAG TTAAGGGTTAATACCCCTTATCTTTGACGTTAGCAACAGAAGAAGGACCGGCTAACTCCGTGCCAGCNC

	CCGCGGTAATACAAANNNNC
1MMRC	GGNTACAGCTACCATGCAGTCGAGCGGTACAGGAATTAGCTTGCTATTNGCTGACGAGCGGCGGACGGG TGAGTAATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATAC GCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAG TTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGG ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTG ATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTT AACGGTTAATAACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCGCC CGCCGGTAATNCAAANNNG
2MMRC	GAGNGGCTCCTGCNTGTCGAGCGGTACAGGATTAGCTTGCTATTNGCTGACGAGCGGCGGACGGGTGAG TAATGCCTAGGGATCTGCCCAGTCGAGGGGGATAACAATTGGAAACGACTGCTAATACCGCATACGCCC TACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCTAGGTGGGATTAGCTAGTTGG TGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC AGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAACG GTTAATAACCGTTAGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTTCGTGCCAGCCCCNNCGG TAATNCAAATTCANG
4MMRC	TTCTTCTTGGANAGATNCGAGCGGCGGACGGGTGAGTNTGCTTGGGAATATGCCTTTACGTGGGGGACA ACAAGTTGGAAACGACTGCTAATACCAGCATAATGTCTTCGGACCAAAGGAGGGGACGCTTCGGCACCT TTCGCGTATTGATTATCCCAAGTGGGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGACGATCCC TAGCTGGTTTGAGAGGATGATCAGCCNNACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG GGTTGTAAAGCACTTTCAGTTGTGAGGAAGAGTTAAGGGTTAATACCCCTTATCTTTGACGTTAGCAACA GAAGAANGACCGGCTAACTCCGTGCCAGCCGCCGCGGTAATNCAAGNGN
5MMRC	TGCCCTTCGGGTGTTTGTTGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCTGATG TGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTTCGGGCCAAAGAGGGGGACCTTCG GGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGAC GATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCANACTCCTACGGGA GGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGG CCTTCGGGTTGTAAAGTACTTTCAGCAGTGAGGAAGGTTCATACGTTAATAGCGTATGGATTTGACGTTA GCTGCAGAAGAAGCACCGGCTAACTCCGTGCCACNGCCGGGGGTAATACAANTN

B.14. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Hillsborough Rv. during warm season, recovered in TCBS plates with 50 µg/mL OTC

Isolate code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
7SHRW	Emb AJ874361.1	*Vibrio splendidus*	99
8SHRW	Emb AJ874367.1	*Vibrio splendidus*	99
9SHRW	Gb AY159791.1	*Shewanella* sp. B07	93
10SHRW	Gb AY579751.1	*Shewanella* sp. HAW-EB4	95
11SHRW*			
6MHRW	Emb AJ560649.1 VSP560649	*Vibrio pomeroyi*	98
	Emb AJ874361	*Vibrio splendidus*	97
7MHRW	Gb AY326275.1	*Shewanella* sp. LT17	99

* Isolates that could not be recovered for sequencing

B.15. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Cap Egmont during warm season, recovered in TCBS plates with 50 µg/mL OTC

Isolate Code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
7SCEW*			
7MCEW	Gb AY620964.1	*Vibrio tasmaniensis* strain 562	98
	Gb AY155585.1	*Vibrio supersteus* strain G3-29	98
8MCEW	Gb DQ090766.1	*Vibrio halioticoli*	98
9MCEW	Gb AY170366.1	*Shewanella waksmanii*	97
10MCEW*			
11MCEW	Gb AF468443.1	Artic sea ice bacterium ARK9973	99
	Gb AY669172.1	Marine sediment bacterium OC-12 isolate	99
12MCEW*			

* Isolates that could not be recovered for sequencing

B.16. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Murray Rv. during warm season, recovered in TCBS plates with 50 μg/mL OTC

Isolate Code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
7SMRW*			
8SMRW	Gb AY190533.1	*Shewanella gaetbuli*	82
9SMRW	Gb AF500080.1	*Shewanella affinis* KMM 3586	95
	Gb AY653177.1	*Shewanella colwelliana*	95
7MMRW	Gb DQ090766.1	*Vibrio halioticoli*	99
8MMRW	Gb AY967728.1	*Vibrio harveyi*	98
	Gb AF537959.1	*Vibrio alginolyticus* strain VM122	98
9MMRW*			
10MMRW*			
11MMRW*			
14MMRW	Gb AY837115.1	*Vibrio* sp. 14G04	98

* Isolates that could not be recovered for sequencing

B.17. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Hillsborough Rv. during cold season, recovered in TCBS plates with 50 µg/mL OTC

Isolate Code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
1SHRC	Gb AY326275.1	*Shewanella* sp. LT17	78
2SHRC	Gb AY326275.1	*Shewanella* sp. LT17	99
3SHRC	Gb AY326275.1	*Shewanella* sp. LT17	99
4SHRC*			
5SHRC	Gb AY326275.1	*Shewanella* sp. LT17	94
6SHRC	Emb AJ344473.1 SFR344473	*Shewanella frigidimarina*	93
	Gb AF173966.1 AF173966	*Shewanella baltica*	93
7SHRC	Gb AY326275.1	*Shewanella* sp.	98
8SHRC	Emb AJ674361.1	*Vibrio splendidus*	98
11SHRC	Gb AF003549.1 AF003549	*Shewanella woodyi*	93
1MHRC	Emb AJ874367.1	*Vibrio splendidus*	99
4MHRC	Gb AY620966.1	*Vibrio tasmaniensis* strain 373.11	97

* Isolates that could not be recovered for sequencing

B.18. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Cap Egmont during cold season, recovered in TCBS plates with 50 µg/mL OTC

Isolate code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
1SCEC	Gb AF173966.1 AF173966	*Shewanella baltica*	99
2SCEC	Emb AJ318946.1	*Pseudoalteromonas* sp.	99
3SCEC	Emb AJ874363.1	*Vibrio splendidus*	98
1MCEC	Gb AY326275.1	*Shewanella* sp. LT17	99
2MCEC*			
3MCEC	Emb AJ874363.1	*Vibrio splendidus*	98

* Isolates that could not be recovered for sequencing

B.19. Molecular identification by sequencing the 16S rDNA of the representative isolates of sediments and mussels obtained from Murray Rv. during cold season, recovered in TCBS plates with 50 µg/mL OTC

Isolate code	Results which sequences produced significant alignments		
	NCBI Number	Species	Identities (%)
1SMRC	AY326275.1	*Shewanella* sp. LT17	99
2SMRC	AY326275.1	*Shewanella* sp. LT17	99
3SMRC	Gb AF295592.1	*Shewanella* sp. ACEM-9	92
4SMRC		*Shewanella colwelliana*	97
5SMRC	Emb AJ318946.1 PSP318946	*Pseudoalteromonas* sp. OFCI-21	99
6SMRC	AJ318946.1	*Pseudoalteromonas* sp.	99
7SMRC	AB195982.1	*Vibrio campbellii*	98
1MMRC	Gb AY326275.1	*Shewanella* sp. LT17	99
2MMRC	Gb AY326275.1	*Shewanella* sp. LT17	99
3MMRC*			
4MMRC	Emb AJ318946.1 PSP318946	*Pseudoalteromonas* sp. OFCI-21	97
5MMRC	Emb AJ440009.1 VSP440009	*Vibrio gallicus*	98

* Isolates that could not be recovered for sequencing

APPENDIX C

C.1. Interpretative breakpoints used for disk diffusion sensitivity tests against 19 antibiotics

Group	Antibiotic	Disk Content (μg)	Zone diameter (mm)		
			susceptible	intermediate	Resistant
Beta-lactams	AMC*	20/10	≥ 18	14 - 17	≤ 13
	AMP	10	≥ 18	14 - 17	≤ 13
	CAR	100	≥ 18	14 - 17	≤ 13
	CTX	30	≥ 18	14 - 17	≤ 13
	KF*	30	≥ 18	15 - 17	≤ 14
Quinolones	ENR*	5	≥ 23	17 - 22	≤ 16
	OA	2	≥ 23	17 - 22	≤ 16
	CIP	5	≥ 23	17 - 22	≤ 16
	UB	30	≥ 23	17 - 22	≤ 16
Aminoglycoside	AK*	30	≥ 17	15 - 16	≤ 14
	CN*	10	≥ 15	13 - 14	≤ 12
	S	10	≥ 17	15 - 16	≤ 14
Tetracyclines	OT*	30	≥ 19	15 - 18	≤ 14
	TE	30	≥ 19	15 - 18	≤ 14
Potentiated Sulfonamides	SOR	1.25/23.75	≥ 16	11 - 15	≤ 10
	SXT*	1.25/23.75	≥ 16	11 - 15	≤ 10
Phenicols	FFC	30	≥ 18	13 - 17	≤ 12
	C*	30	≥ 18	13 - 17	≤ 12
Macrolide	E*	15	≥ 23	14 - 22	≤ 13

AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin; *: antibiotics whose interpretative breakpoints are established by the NCCLS (2002)

C.2. Diameter zones obtained after disk diffusion sensitivity tests of bacterial reference strains suggested by the NCCLS in MHA media with and without NaCl supplementation

Antibiotic Group	Ant.	Inhibition zones (mm) of reference strains								
		As. salmonicida subs. *salmonicida* ATCC 33658						*E. coli* ATCC 25922		
		QC range	MHA	MHA	MHA	MHA	MHA*	QC range	MHA	MHA*
Beta-lactams	AMP	35-44	38	36	38	34	40	13-22	14	15
	AMC		40	Nd	40	28	36		21	20
	CAR		48	Nd	43	40	48		21	23
	CTX		48	Nd	49	42	54		34	36
	KF		29	29	24	28	35		18	23
Quinolones	ENR	37-49	44	38	38	35	49	-	35	36
	OA	33-45	37	38	37	36	41	28-40	26	30
	CIP		48	Nd	47	33	45		39	41
	UB		48	Nd	45	41	52		30	37
Aminoglycosides	CN	22-32	22	22	24	22	23	23-34	24	18
	AK		25	Nd	26	25	26		22	19
	S		12	Nd	17	12	12		16	10
Tetracyclines	OT	28-38	38	35	37	33	40	25-35	30	31
	TE		37	34	36	34	40		29	30
Potentiated Sulfonamides	SOR	21-35	27	25	22	29	28	13-22	15	15
	SXT	24-39	30	30	31	34	34	26-36	Nd	30
Phenicols	FFC	34-47	40	35	37	34	41	20-32	21	21
	C		36	35	37	35	40		23	26
Macrolide	E	19-31	22	21	21	18	20	13-20	13	10

Ant.: antibiotics; QC: quality control; MHA: Mueller Hinton agar; MHA*: Mueller Hinton agar supplemented with 1.5% NaCl ; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin; Nd: not determined.

C.3. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Hillsborough Rv. during warm season

Ant.	Inhibition zones (mm)					
	7SHRW *Vibrio**	8SHRW *Vibrio**	9SHRW *Shew.**	10SHRW *Shew.**	6MHRW *Vibrio**	7MHRW *Shew.**
AMP	19	22	24	10	17	36
AMC	25	28	29	24	21	40
CAR	27	34	32	15	21	42
CTX	40	36	44	41	35	44
KF	20	21	6	9	10	27
ENR	33	30	34	30	26	40
OA	19	19	31	21	20	33
CIP	35	31	34	30	29	36
UB	34	29	40	31	30	44
CN	15	14	19	16	16	16
AK	16	17	20	19	18	18
S	10	10	10	8	9	6
OT	32	38	28	26	33	36
TE	32	33	25	22	34	34
SOR	30	34	28	29	25	31
SXT	29	32	29	27	26	41
FFC	36	32	39	32	25	37
C	27	29	40	32	29	35
E	22	19	25	20	20	32

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *: most probable genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;

C.4. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Cap Egmont during warm season

Ant.	Inhibition zones (mm)				
	10MCEW *Vibrio**	11MCEW §	12MCEW *Shew.**	13MCEW *Vibrio**	14MCEW §
AMP	10	25	12	26	39
AMC	23	25	23	27	40
CAR	12	30	30	30	48
CTX	38	24	32	27	26
KF	21	26	6	24	41
ENR	29	30	37	28	30
OA	19	29	33	27	6
CIP	30	32	26	29	33
UB	29	34	40	32	18
CN	18	15	12	14	13
AK	19	16	15	15	25
S	11	9	6	9	12
OT	34	27	23	28	35
TE	33	28	25	29	35
SOR	27	44	28	38	40
SXT	30	32	30	35	42
FFC	35	30	30	30	30
C	33	35	33	37	30
E	21	20	20	20	24

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *: most probable genus; §: undefined genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;

C.5. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Murray Rv. during warm season

Ant	Inhibition zones (mm)						
	8SMRW §	9SMRW *Shew.**	7MMRW *Vibrio**	8MMRW *Vibrio**	9MMRW *Vibrio**	11MMRW *Vibrio**	14MMRW *Vibrio**
AMP	44	25	23	6	25	26	31
AMC	48	30	25	22	28	28	31
CAR	47	32	26	6	29	27	36
CTX	44	42	26	26	27	25	43
KF	30	6	25	16	26	24	27
ENR	40	32	30	29	35	30	28
OA	35	35	28	24	6	27	20
CIP	41	32	33	31	38	31	30
UB	43	26	36	33	20	31	30
CN	20	20	23	17	14	15	17
AK	22	19	25	18	15	15	18
S	6	10	18	10	10	9	15
OT	36	26	31	30	27	29	30
TE	35	28	31	30	25	30	30
SOR	33	28	30	25	30	40	32
SXT	38	25	30	26	35	30	31
FFC	39	35	32	31	30	30	31
C	40	37	35	33	35	32	38
E	33	25	21	18	20	20	25

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *: most probable genus; §: undefined genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;

C.6. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Hillsborough Rv. during cold season

Ant.	Inhibition zones (mm)									
	1SHRC *Shew.**	2SHRC *Shew.**	3SHRC *Shew.**	5SHRC *Shew.**	6SHRC *Shew.**	7SHRC *Shew.**	8SHRC *Vibrio**	11SHRC *Shew.**	1MHRC *Vibrio**	4MHRC *Vibrio**
AMP	39	37	39	40	32	41	26	6	8	12
AMC	40	38	40	42	38	42	26	6	23	23
CAR	44	40	41	44	38	44	30	9	10	15
CTX	45	42	42	45	44	46	36	25	37	36
KF	29	26	27	28	13	29	19	6	15	20
ENR	40	38	39	40	48	42	28	38	30	29
OA	35	33	33	34	43	36	21	36	23	22
CIP	36	37	36	38	43	40	27	30	30	30
UB	43	40	38	41	52	48	31	40	32	30
CN	19	18	18	21	18	19	12	15	18	19
AK	20	19	20	22	19	22	14	17	18	22
S	10	6	8	9	6	6	9	10	11	13
OT	36	33	34	35	40	36	27	28	31	30
TE	36	32	34	34	39	35	25	26	29	28
SOR	24	32	34	28	38	32	29	24	28	32
SXT	32	37	37	37	44	36	29	33	27	32
FFC	39	38	36	39	48	40	28	34	34	31
C	38	37	37	39	46	40	28	35	30	30
E	34	32	31	34	30	34	19	24	23	20

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *: most probable genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;

C.7. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Cap Egmont during cold season

Ant.	Inhibition zones (mm)				
	1SCEC *Shew.**	2SCEC *Psalt.**.	3SCEC *Vibrio**	1MCEC *Shew.**	3MCEC *Vibrio**
AMP	40	34	28	43	31
AMC	45	32	27	38	32
CAR	46	36	30	48	34
CTX	48	32	29	40	36
KF	22	25	21	32	23
ENR	47	32	24	40	27
OA	41	28	19	36	20
CIP	48	30	24	37	29
UB	27	36	24	44	25
CN	19	12	16	21	18
AK	22	12	19	22	19
S	8	6	12	8	12
OT	38	32	28	34	30
TE	34	31	28	35	30
SOR	34	29	27	40	30
SXT	40	25	28	40	30
FFC	44	37	32	40	30
C	46	32	28	40	31
E	32	29	19	32	19

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *Psalt.*: *Pseudoalteromonas*; *: most probable genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;

C.8. Diameter zones obtained after disk diffusion sensitivity tests of bacterial isolates obtained from Murray Rv during cold season

Ant.	Inhibition zones (mm)											
	1SMRC *Shew.**	2SMRC *Shew.**	3SMRC *Shew.**	4SMRC *Shew.**	5SMRC *Psalt.**.	6SMRC *Psalt.**.	7SMRC *Vibrio**	1MMRC *Shew.**	2MMRC *Shew.**	3MMRC *Vibrio**	4MMRC *Psalt.**.	5MMRC *Vibrio**
AMP	42	44	41	40	36	34	28	41	41	25	36	28
AMC	42	44	40	42	36	34	26	45	44	28	30	27
CAR	46	48	42	46	39	36	29	47	44	25	36	30
CTX	46	46	44	46	30	31	34	44	45	26	27	31
KF	29	29	29	27	27	26	20	31	27	25	28	25
ENR	40	48	40	40	56	34	34	44	38	30	38	24
OA	34	39	34	34	50	37	31	34	34	29	35	24
CIP	37	45	36	39	46	34	37	42	36	32	38	27
UB	43	52	44	43	60	51	38	47	43	34	48	28
CN	20	18	18	19	12	28	19	22	19	13	12	11
AK	20	20	19	21	11	12	20	22	20	14	14	12
S	8	7	6	6	6	8	14	6	6	8	6	8
OT	34	37	34	35	37	34	30	37	32	29	38	29
TE	32	37	33	33	34	30	28	37	31	29	36	28
SOR	29	36	33	35	35	36	27	32	29	31	34	27
SXT	37	36	35	33	34	36	28	36	32	32	35	28
FFC	38	43	40	38	38	41	30	42	37	30	41	31
C	36	42	38	38	40	36	28	40	40	30	37	34
E	28	37	35	34	29	32	19	35	29	23	31	17

Ant.: antibiotics; QC: quality control; *As. salm.*: *Aeromonas salmonicida* subs. *salmonicida* ATCC 33658; *Shew.*: *Shewanella*; *Psalt.*: *Pseudoalteromonas*; *: most probable genus; AMP: ampicillin; AMC: amoxicillin/clavulanic acid; CAR: carbenicillin; CTX: cefotaxime; KF: cephalothin; ENR: enrofloxacin; OA: oxolinic acid; CIP: ciprofloxacin; UB: flumequine; CN: gentamicin; AK: amikacin; S: streptomycin; OT: oxytetracycline; TE: tetracycline; TE: tetracycline; SOR: Ormetoprim-sulfadimethoxine; SXT: Trimethoprim-Sulfamethoxazole; FFC: Florfenicol; C: chloramphenicol; E: erythromycin;