Antimicrobial Activity Of Marine *Vibrio* sp., Isolate NI-22

by

Stephen James Towse

Supervisor: Dr. Paul Holloway

A thesis submitted in partial fulfillment

of the Honours Thesis (05.4111/6) Course

Department of Biology

The University of Winnipeg 2005

ABSTRACT

Rapidly emerging strains of bacteria resistant to all but the most advanced antibiotics are a looming public health concern. Many antibiotics that not so long ago were considered first line therapies in the management of certain bacterial infections are no longer effective. Research currently directed towards marine microorganisms presents a vast potential for new compounds able to safely and effectively target resistant species. *Vibrio* sp., isolate NI-22 produced a compound with antibacterial properties against a range of bacterial pathogens such as *Escherichia coli*, *Corynebacterium xerosis*, and *Streptococcus pneumoniae*. Several extraction procedures to adsorb the compound onto Amberlite XAD, Cellulose, Chelex, Sephadex and Polypropylene resins, followed by elution with organic solvents, were tested. Antimicrobial assays were carried out against bacterial isolates to establish a range over which the compound may have therapeutic value. Micronutrients were added to the growth medium and observations made as to their effectiveness for enhancing the zone of bacterial growth inhibition. Marine and terrestrial bacteria have many similarities but the adaptations required by organisms to live in a marine environment have required genotypic changes and therefore new compounds to aid in their survival. The search for new compounds from marine ecosystems is a lengthy and laborious process but many have already been discovered that hold the promise of combating such diseases as Cancer, Alzheimer's and osteoporosis.

ACKNOWLEDGMENTS

I wish to express my sincere thanks and appreciation to Dr. Paul Holloway for being a tremendous project supervisor. His advice and direction during the development of this research was invaluable. Aside from his teaching, research responsibilities and family he was always available for suggestions and comments regarding technical matters and theoretical aspects that may assist in the development of the methodology/protocol.

I would also like to thank my committee members Dr. Anne M. Adkins and Dr. Athar Ata. Their continued support throughout the course of this study and the preparation of this manuscript is greatly appreciated. They gave freely of their time and were extremely patient when bombarded with questions.

Sincere thanks are also extended to the entire technical staff. Without their help answering my many questions the days would have been much longer.

Finally, I wish to thank my wife Karen and my two daughters Georgia and Lauren. The love and support of my family was by far the most important element that allowed me to continue my research and studies.

TABLE OF CONTENTS

Page

ABSTRACT ii

ACKNOWLEDGEMENTS iii

LIST OF FIGURES AND TABLES vii

1. INTRODUCTION 1

2. MATERIALS AND METHODS 6

2.1 Standard Media Preparation 6

2.2 Antimicrobial Assay 6

2.3 Collection and Storage of *Vibrio* sp., Isolate NI-22 7

2.4 Tests to ensure the antimicrobial activity of the isolate 7

2.5 Optimal temperature for the growth of NI-22 8

2.6 Inhibition of Growth of *E. coli* by Amberlite Resins 9

2.7 NI-22 Antibacterial Agent extraction trials with Polypropylene and XAD-7 HP resin 9

2.8 NI22 extraction trials with Sephadex, Chelex, Cellulose and XAD-7 10

2.9 Extraction of NI-22 Antibacterial Agent at low pH 11

2.10 Effect of cations on the observations of antibacterial effect by NI-22 13
2.11 Area of NI-22 inoculation vs. Area of bacterial growth inhibition zone 14
2.12 NI-22 inhibition assays performed against 20 *E. coli* fecal isolates 15
2.13 NI22 inhibition assays performed against eight *Streptococcus pneumoniae* clinical isolates 16
2.14 *Vibrio fischerii* inhibition assay performed against *Escherichia coli* 17

3. RESULTS 18
3.1 Tests to ensure the antimicrobial activity of the isolate 18
3.2 Optimal temperature for the growth of NI-22 18
3.3 Inhibition of Growth of *E. coli* by Amberlite Resins 19
3.4 NI-22 Antibacterial Agent Extraction Trials with Polypropylene and XAD-7 HP resin 19
3.5 NI-22 extraction trials with Sephadex, Chelex, Cellulose and XAD-7 19
3.6 Extraction of NI-22 Antibacterial Agent at low pH 20
3.7 Effect of Cations on the Observations of Antibacterial Effect by NI-22 21
3.8 Size of NI-22 inoculation vs. Size of bacterial growth inhibition zone 24

3.9 NI-22 inhibition assays performed against 20 *E. coli* fecal isolates 24

3.10 NI-22 inhibition assays performed against 8 *Streptococcus pneumoniae* clinical isolates 27

3.11 *Vibrio fischerii* inhibition assay performed against *Escherichia coli* 28

4. DISCUSSION 30

5. CONCLUSIONS 34

6. REFERENCES 35

LIST OF FIGURES AND TABLES

Page

Figure 1: Effect of cations on the antibacterial effect by NI-22 relative to control medium (2% MH agar). Size of the zone of inhibition as a percent increase or decrease from that of the unmodified 2% MH agar. Potassium bromide (KBr) X = 0.08 g/L; Magnesium chloride ($MgCl_2$) X = 5.9 g/L and Calcium chloride X = 1.8 g/L. 23

Figure 2: The effect of increasing the area of NI-22 inoculation on the size of the zone of bacterial growth inhibition. 25

Figure 3: The effect of increasing NI-22 inoculation on the area of the zone of bacterial growth inhibition calculated in mm^2. 25

Table 1: NI-22 inhibition assays performed against *20 *E. coli.* fecal isolates using the standard antimicrobial assay. 26

Table 2: NI-22 inhibition assays performed against eight **S. pneumoniae* clinical isolates. *S. pneumoniae* was inoculated at 3×10^8 cfu/ml on TSA + 5% sheep blood agar (incubation at room temperature without CO_2). 29

INTRODUCTION

A significant increase in the prevalence of bacterial strains virtually resistant to all but the most novel chemical classes of antimicrobials is a growing worldwide health concern. Since the first antibiotic from a marine bacterium was identified and characterized in 1966 (Burkholder *et al.* 1966) several more have also been discovered. Isolation of an antibiotic from a species of *Streptomyces* with activity against an array of Gram-positive organisms, as well as a species of *Bacillus*, which inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) are just two of the novel antimicrobial compounds recently found (Burgess 1998). The ocean is a rich and virtually unfettered environment containing more than 300 000 species of plants and animals all existing in an ecosystem considerably divergent from their terrestrial counterparts (Donia and Hamann 2003). The requirement to survive in conditions of increased salinity, reduced light, high pressure and temperature variations have led to some unusual enzymatic and metabolic adaptations. The search for new natural products from marine microorganisms has already shown promise with the discovery of compounds with possible use as anticancer and cardiovascular agents (Lei and Zhou 2002). With worldwide sales of 45 billion dollars, anti-infectives represent the third largest therapeutic area with commercial sales expected to increase through 2010 (Bush 2004).

For nearly 30 years, pharmaceutical companies have been searching for new antibiotics to counter increasing bacterial resistance. During this period, use of

new chemical derivatives of pre-existing antibiotics has been the only method available, as no novel chemical class of antibiotics has been discovered (Burgess 1999). As a result, bacteria continue to develop new resistance mechanisms to combat the most recent generation of antibiotics. Strains of *E. coli* responsible for infections of the urogenital and gastrointestinal tracts have seen the prevalence of ciprofloxacin resistance steadily increase from 0.5% in 1989 to 1.7% by 1994 (Amyes and Gemmell 1997). Infections involving *C. xerosis* are mainly bacteremia, skin infections (intravenous sites) and pneumonia in immune compromised hosts. Camello *et al.* (2003) observed isolated *Corynebacterium* species that were all susceptible to vancomycin, but most were resistant to the β-lactams. Antibiotic-resistant patterns were not predictable, with multiple antibiotic resistances observed for *C. xerosis*. Increasing patterns of antimicrobial resistance are also being seen for *Streptococcus pneumoniae*, a leading cause of community-acquired pneumoniae, bacterial meningitis, and acute otitis media. Results of the Canadian Respiratory Organism Susceptibility Study (1997 to 2002), report that the proportions of *S. pneumoniae* isolates with the multidrug (resistance to three or more classes of antimicrobials) resistance phenotype increased from 2.7% in 1997 – 1998 to 8.8% in 2001 – 2002 (Zhanel *et al.* 2003). In one study conducted between 1995 and 1998, Carrie *et al.* (2000) looked at the number of prescriptions dispensed for antibiotics in community practice in Manitoba, Canada. Antibiotics such as penicillins, macrolides, and sulfonamides, which previously accounted for 75% of antibiotic use, decreased 19.1% during this period. In contrast, use of newer and/or broad-spectrum agents (ciprofloxacin, cefuroxime and azithromycin/clarithromycin) increased, as

a percentage of total antibiotic use from 14.4% to 19.3%. The repercussions of these newer broad-spectrum agents have implications for the development of antibiotic resistance and data from developed countries suggests their use is on the rise. Even though antibiotic consumption in Canada has decreased, they are still among the most commonly prescribed drugs (Carrie *et al.* 2000; Zhanel 2003). The incidence of bacterial strains/isolates acquiring resistance appears not be slowing down, in fact just the opposite. The discovery of a new class of antibiotics, particularly one with a broad spectrum of use, would be extremely beneficial.

The oceans cover over 70% of the earth's surface and roughly half of all the biodiversity found on the planet is located in the marine environment with 34 of the 36 phyla of life represented (Donia and Hamann 2003). A review of the literature found that nearly half of all marine natural products papers report bioactivity data for new compounds (Faulkner 2000). These have been discovered from marine microorganisms at the rate of approximately 700 per year over the last decade (Blunt *et al.* 2004). Over the last 40 years, marine natural products research has become a multi-disciplined field touching on subjects within biology, chemistry, chemical ecology and pharmacology. During this brief period, bacterial secondary metabolites with pharmacodynamic properties, encompassing such diverse biological activities as antibiotics, antivirals, antimitotic and antineoplastics have already been documented (Kelecom 2002). Other marine organisms, such as blue green algae, seaweeds, horseshoe crabs, marine fungi and sponge metabolites have also yielded substantial natural products such as steroids, cytotoxic and antimicrobial agents

as well as novel and biologically active peptides (Blunt *et al.* 2004). Over 150 metabolites with significant antiviral activity against the HIV have also been isolated (Tziveleka *et al.* 2003). Only a small fraction of potential bioactive secondary metabolites from marine bacteria have been discovered. The genus *Streptomyces* is a good example of the potential number of antimicrobial compounds that are still waiting to be discovered. One of the largest antibiotic-producing genera, it accounts for a large number of antimicrobials reported each year. The research of Watve *et al.* (2001) estimated that the total number of antimicrobial compounds that *Streptomyces* is capable of producing to be in the order of a 100 000.

The *Vibrio* species are a diverse group of bacteria found in abundance in the marine environment and associated with aquatic plants and animals to which they may provide a chemical defense for the host (Engel *et al.* 2002). Research to isolate bioactive compounds from the 74 species of this group has already shown promise with the isolation and identification of many antibiotic compounds (Baranova and Egorov 1989; Kobayashi *et al.* 1994). The objective of the proposed research is to isolate an antibacterial compound from marine *Vibrio* sp., isolate NI-22. Various solid sorbents were used in the development of the extraction protocol. These have been used extensively in research for the extraction of antifungal metabolites from *Bacillus cereus* (Tschen and Tseng 1989), adsorption and purification of teicoplanin, a glycopeptide antibiotic produced by *Actinoplanes teicomyceticus* (Quarta *et al.* 1996; Lee *et al.* 2003) and to increase the production of antibiotics during fermentation (Marshall *et al.*

1990). In the hope of increasing antibiotic synthesis, the growth medium was supplemented with various trace elements.

Materials and Methods

2.1 Standard Media Preparation

All microbiological experiments were carried out using Mueller Hinton broth (Becton Dickinson Sparks, MD) amended with sodium chloride (NaCl, 2% w/v) (VWR Mississauga, ON) and designated 2% MHB. For the preparation of pour plates, 2% MHB was supplemented with 15 g/L agar (VWR Mississauga, ON) and termed 2% MH agar. Media were prepared as per manufacturers directions.

2.2 Antimicrobial Assay

The standard medium for inhibition assays consisted of 25 mL 2% MHB inoculated with *Escherichia coli* (*E. coli*) ATCC 25922. The *E. coli* had previously been grown on 2% MH agar plates, incubated for 24 hours, at 37°C, and placed in 5°C walk-in-fridge until needed. Once inoculated with *E. coli*, the flask (2% MHB) was incubated for 24 hours at 37°C, with shaking at 200 rpm. The culture, 150 µl, was then added to 50 mL of autoclaved, 55°C liquid agar. The liquid agar was then poured into plates and allowed to cool. NI-22 was then applied, using aseptic techniques, in a small circle (4-5 mm in diameter) on top of the agar. The plates were incubated for 24 hours at room temperature (~ 23°C). The addition of the test organism to 2% MH agar plates resulted in hazy growth within the agar following 24-hour incubation. Observing a clearing in the color of the agar surrounding the NI-22 colony identified zones of bacterial growth inhibition. The

clearing of the agar corresponded to a region where the incorporated test organism had not grown.

2.3 Collection and Storage of *Vibrio* sp., Isolate NI-22

NI-22 was collected from the intertidal shore water of the Strait of Georgia on the east coast of Vancouver Island (BC, Canada) in May of 2003. NI-22 was originally isolated on Marine agar (Becton Dickinson Sparks, MD) from a sandy sediment sample. After several subcultures on Marine agar (MA) to purify the isolate, it was shipped to the University of Winnipeg. NI-22 was regrown on MA and cultured in Marine Broth 2216 at room temperature (200 rpm). NI-22 broth culture was stored at –70°C in 50% glycerol. Antibacterial activity was observed during routine screening of all isolates from the 2003 sampling trip against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus pyogenes* (*S. pyogenes*). Only activity/inhibition against *E. coli* was observed.

When required, a small sample of the isolate NI-22 was removed from -70°C storage and aseptically inoculated onto 2% MH agar. The plate was incubated for 24 hours at room temperature (~23°C). Individual colonies were transparent, round with entire margins and slightly raised to flat.

2.4 Tests to ensure the antimicrobial activity of the isolate

NI-22 was tested for growth inhibition against two bacterial strains previously determined to be susceptible, *E. coli* American Type Culture Collection (ATCC) strain 25922 and *Corynebacterium xerosis* (*C. xerosis*) ATCC strain 373. Both

bacterial strains had been previously streaked onto 2% MH agar plates. They were then placed in a 37°C incubator, for 24 hours. Both susceptible strains were cultured in 25 mL 2% MHB, by placing a loop of culture in the broth and incubating the flasks at 37°C on a rotary shaker (~200 rpm) for 24 hours.

Previously prepared 2% MH agar was autoclaved and allowed to equilibrate at 56°C in a water bath. Once the agar had cooled plates were poured containing the respective bacteria (150 µl overnight culture broth in 100 mL agar). NI-22 was then applied, using aseptic techniques, as per standard antimicrobial assay.

2.5 Optimal temperature for the growth of NI-22

Ten 2% MH agar plates were prepared with the addition of *E. coli* ATCC 25922 (previously grown in 25 mL, 2% MHB at 37°C for 24 hours) incorporated into the media (250 µl *E. coli* added to 100 mL agar). NI-22 was aseptically inoculated onto the surface of the medium and five plates were initially incubated for 24 hours at 10°C, 14°C, 23°C, 30°C, and 37°C. The remaining five plates were not inoculated with NI-22 and incubated along with the others at their respective temperatures (control plates for the growth of *E. coli*). Once it was determined that bacterial growth inhibition was evident, the plates were removed from the incubators. For those plates in which growth was occurring at less than optimal temperatures incubation was allowed to continue with observations made at 96 hours and 120 hours.

2.6 Inhibition of Growth of *E. coli* by Amberlite Resins

Amberlite XAD-7 HP resin and Amberlite XAD-16 (Rohm & Haas West Hill, ON), 5 grams each, were individually autoclaved for 20 minutes at 121°C in 125 mL of 2% MHB. Once the flasks cooled excess medium was decanted leaving the resin behind (work undertaken in biological safety cabinet so as not to contaminate the resins). Flasks were then stored in walk-in-fridge (~5°C). Both types of resin, 10 grams each, were also autoclaved dry for 20 minutes at 121°C in small glass media bottles. A small amount of each type of resin was applied to the surface of a standard antimicrobial assay in order to rule out the possibility of the resin inhibiting the growth of the bacteria. Plates were incubated at room temperature (~23°C) for 24 hours.

2.7 NI-22 Antibacterial Agent extraction trials with Polypropylene and XAD-7 HP resin

Polypropylene beads, 1 – 2 mm diameter, (Aldrich) and XAD-7 HP resin, 5 grams each, were autoclaved in 100 mL of 2% MHB for 20 minutes at 121°C. Flasks were inoculated with NI-22 and incubated for 24 hours at room temperature (~23°C) on a rotary shaker (~200 rpm). Decant as much of the broth from the flasks leaving the polypropylene and XAD-7 resin behind. Place 8 mL methanol (Anachemia, Montreal QC) into the flasks and ensure the beads are covered/floating. Decant the methanol and beads into separate 50 ml beakers and intermittently shake/stir at room temperature for 30 minutes. After 30 minutes remove the methanol and discard. Repeat with another 8 mL methanol discarding the solvent after 30 minutes. Add 8 mL ethyl acetate (Anachemia

Montreal, QC) and intermittently shake/stir the solution for 60 minutes. Decant the ethyl acetate and dry down the solvent with air in fumehood. Apply a small amount of the remaining solvent to a paper disk (Becton Dickinson) allowing the solvent to dry between successive applications. As a control, set one blank disk and another disk saturated in ethyl acetate onto the plate. Place the respective disks onto a standard antimicrobial assay. Incubate the plates at room temperature (~23^{o}C) for 24 hours.

2.8 NI22 extraction trials with Sephadex, Chelex, Cellulose and XAD-7

Approximately 1.5 – 2 g, Sephadex (Sigma-Aldrich Mississauga, ON), Chelex - 100 (Bio-Rad Mississauga, ON), cellulose powder fibrous, medium (Sigma-Aldrich St. Louis, MD), and XAD-7HP resin (Rohm & Haas West Hill, ON) were placed into separate 1 Litre flasks containing 100 mL of 2% MHB. The flasks were then autoclaved for 20 minutes at 121^{o}C. Once the broths have cooled filter each supernatant through a 0.45 *u*m pore size filter (VWR Mississauga, ON.). Remove 1 gram of each type of resin and place this into 100 mL of 2% MHB contained in 1 Liter flasks (one flask for each type of resin). Autoclave this mixture for 20 minutes at 121^{o}C and let cool. After the flasks have been inoculated with NI-22 from the streak plate incubate at room temperature (~23^{o}C) for 24 hours on a rotary shaker (200 rpm).

After incubation, to ensure NI-22 was still capable of producing the antibacterial compound, a loopful from each flask was placed onto a standard antimicrobial assay. Decant the broth from each bead/resin flask. The following

procedure was carried out for the extraction of all four types of resin. Wash the resin twice in 5 mL methanol (Anachemia Montreal, QC) for 30 minutes and decant, keeping the solvent (do not combine). Wash once again in ethyl acetate (Anachemia Montreal, QC) for 60 minutes and decant, keeping the solvent. A final wash is performed in chloroform (VWR Mississauga, ON) for 30 minutes; the solvent is decanted and retained. All solvent fractions for all resin types were dried down under air with the small volume remaining applied to six-millimeter (mm) diameter paper disks (Becton Dickinson Sparks, MD). Small aliquots (~20 μl) were applied and allowed to dry between successive applications. All of the paper disks were placed onto 2% MH agar plates inoculated with *E. coli*. A small amount of each type of resin was also applied to these plates. Resin was collected and applied only after all of the solvent extractions had been performed. All of the plates were incubated for 24 hours at room temperature (~23^{o}C).

2.9 Extraction of NI-22 Antibacterial Agent at low pH

All of the following extractions were conducted using 100 mL of autoclaved 2% MHB within a 1 Liter flask. The broth was inoculated with NI-22 from streak plate and incubated for 24 hours at room temperature (~23^{o}C) on a rotary shaker (~200 rpm). After incubation, to ensure NI-22 was still capable of producing the antibacterial compound, a loopful from each flask was placed on Mueller Hinton + 2% NaCl agar plates containing *E. coli*. Add 200 mL of 0.1N HCl (Fisher Scientific Fair Lawn, New Jersey) and shake/mix for 5 – 10 seconds. Sonicate (Will Scientific Inc. Rochester, N. Y.) for 10 minutes at power setting 60 and then centrifuge the mixture for 10 minutes at 2000 rpm. Decant the mixture leaving

any cell/debris behind. Place a small amount of glass wool in the end of a 60 cc polypropylene syringe (Becton Dickinson), in order to prevent the small beads of XAD-7 resin from passing through the tip of the syringe. Five grams of XAD-7 HP resin (Rohm & Haas Co. West Hill, ON) was added to the column and washed with 25 mL of methanol (Anachemia Montreal, QC) and then with 25 mL distilled water. The NI-22 broth was then allowed to slowly filter through the resin. Once the sample had finished flowing through the beads they were rinsed once again with 25 mL distilled water. All of the preceding liquid was discarded. Ethyl Acetate (Anachemia Montreal QC), 25 mL, is then added to the column in an attempt to elute the compound from the resin. The sample was then spun down for 10 minutes at 2000 rpm and the top organic layer containing the ethyl acetate was removed. The solvent was then dried down in the fumehood using air and subsequently reconstituted with 250 µl of methanol (Anachemia Montreal, QC). Paper disks (Becton Dickinson) are then prepared by aliquoting the methanol onto the disks, and allowing them to dry between successive applications. Disks were then applied to 2% MH agar plates containing *E. coli* and incubated for 24 hours at room temperature. A zone of bacterial growth inhibition surrounding the disk was indicative of the presence of the antibacterial compound. Control disks onto which methanol or ethyl acetate were added and dried and no solvents (blank) were also positioned on the plates.

The above extraction procedure was also performed on Mueller Hinton Broth, Marine Broth 2216 (Becton Dickinson Sparks, MD), Heart Infusion Broth, Nutrient Broth (Becton Dickinson Cockeysville, MD), and Trypticase Soy Broth (Becton Dickinson Sparks, MD) all supplemented with 2% NaCl w/v but not

inoculated with NI-22. A control extraction was also performed on distilled water, not including 2% NaCl w/v and uninoculated with NI-22.

Using the above procedure two modifications of the method were attempted. The first involved, substituting 200 mL of 0.1 N HCl for the same volume of distilled water, allowing the extraction protocol to proceed in the range of pH 6.9. The second, reducing the Normality of HCl from 0.1 N to 0.01N (200 mL), thus allowing the extraction protocol to proceed in the range of pH 4.1. Extractions were performed on inoculated (NI-22 #2) and uninoculated 2% MHB flasks.

2.10 Effect of cations on the observations of antibacterial effect by NI-22

Standard antimicrobial assay plates were individually prepared with various chemical constituents added either alone or in combination. These experiments were conducted in order to determine the significance of cations on the antibacterial effect of NI-22 and consequently any variations observed in the zone of bacterial growth inhibition. Control plates containing none of the additional cations and some made without 2% NaCl were also inoculated with NI-22. This was done in order establish a baseline for measurements taken once incubation of the plates was completed and the zones of bacterial growth inhibition were present. The individual chemical constituents found in Marine Broth 2216 were used as the baseline levels. 2% MH agar was prepared containing only one of the following individual constituents either, 5.9 g/L magnesium chloride ($MgCl_2$ - Fisher Scientific Fair Lawn New Jersey), 0.08 g/L potassium bromide (KBr – VWR Mississauga, ON), 0.07 g/L sodium bromide (NaBr – VWR Mississauga,

ON), or 1.8 g/L calcium chloride ($CaCl_2$ – VWR Mississauga, ON). Several of these cations were incorporated together into 2% MH agar including the following combinations (still using the same quantities), $MgCl_2$ + KBr, $MgCl_2$, KBr + $CaCl_2$, and $MgCl_2$, KBr, $CaCl_2$ and 4 g/L magnesium sulfate ($MgSO_4$ – Fisher Scientific Fair Lawn New Jersey).

Further tests were also conducted by varying the amount of MgCl2, KBr, and NaBr added to 2% MH agar from the baseline levels listed above. Each cation was added in one half, twice and four times the quantities (g/L) as listed on the formulation for Marine Broth 2216 (Becton Dickinson Sparks, MD).

Once the plates had been poured and allowed to harden a small inoculation (size of a paper disk) of NI-22 was applied to the surface of the media. Plates were incubated for 24 hours at room temperature. Measurements were taken from the edges of the NI-22 colony to the outside of the zone of bacterial growth inhibition.

2.11 Area of NI-22 inoculation vs. Area of bacterial growth inhibition zone

Increasing the area of inoculation was done to establish whether a relationship existed between the area of the inoculation and the subsequent area of the zone of bacterial growth inhibition. The NI-22 isolate was applied as per the standard antimicrobial assay. The diameter of NI-22 growth (measured after the 24 hour incubation period) was 4.5, 5, 8, 12 and 18 mm. Plates were incubated for 24 hours at room temperature.

2.12 NI-22 inhibition assays performed against 20 *E. coli* fecal isolates

Individual isolates of *E. coli* (20) were inoculated onto 2% MH agar and incubated for 24 hours at room temperature. Once the growth of all isolates was established they were placed in the 5°C walk-in-fridge until further testing. The isolates, #5201, #5715, #5786, #6182, #6470, #6806, #7013, #7077, #7303, #7522, #7726, #7751, #7764, #7927, #8053, #8069, #8297, #8717, #9016, and #9111 had been collected in a previous study from the feces of cattle, swine and chickens from municipal sewage plants. Ten mL of 2% MHB, contained in test tubes, was autoclaved for 10 minutes at 121°C. Once the tubes had cooled down they were inoculated with one of the 20 strains of *E. coli*. One tube was inoculated with the control strain (*E. coli* ATCC 25922) previously grown on 2% MH agar. The tubes were placed in a 37°C incubator for 24 hours with the increase in turbidity of the broth taken as the sign of bacterial growth. Each strain of *E. coli* was added (150 µl) to a bottle, containing 50 mL of 2% MH agar and two large plates were poured. One plate was inoculated with three small circles (approximately the same size as a paper disc, 6 mm - diameter) of NI-22 and the other plate was left to grow as a control. All of the plates were incubated for 24 hours at room temperature. Measurements were taken from the edges of the NI-22 colony to the outside of the zone of bacterial growth inhibition.

2.13 NI-22 inhibition assays performed against 8 *Streptococcus pneumoniae* clinical isolates

Eight *Streptococcus pneumoniae* (*S. pneumoniae*) isolates (#48154, #48957, #49202, #49203, #49710, #50009, #50847, and #50946) were provided from The Canadian Respiratory Organism Susceptibility Study (Zhanel *et al.* 2003). They were initially streaked onto TSA + 5% sheep blood plates (Oxoid Nepean, ON) and grown at 37°C for 18 hours. A small burning tea light candle was placed in the jar with the plates to supplement the environment with 5 – 10% CO_2. Using these as stock cultures additional TSA + 5% sheep blood plates were incubated at 23°C, 30°C, and 37°C for 18 hours in CO_2 jars with a BBL GasPak CO_2 System cartridge (Becton Dickinson, Sparks, MD). Experiments with the same temperature conditions were repeated but the plates were not incubated in the presence of CO_2. In order to test for inhibition 5 mL of Mueller Hinton + 2% broth was inoculated with *E. coli* ATCC 25922 and spread onto one-half of a blood agar plate with a sterile cotton swab. Once dry a small amount of NI-22 #2 was applied to the surface. The other half of the plate was inoculated with NI-22 #2 as a test for bacterial growth. Once prepared these plates were also incubated under all of the same experimental conditions. Control plates were also assessed under these same conditions (temperature and CO_2) to rule out contamination of the blood agar.

A #1 McFarland's standard was prepared in Mueller Hinton broth for each of the eight *S. pneumoniae* strains as well as the *E. coli* ATCC 25922 strain. The standard represents a 3×10^8 cfu/ml of bacteria. This was applied to the surface of TSA + 5% sheep blood plates with sterile cotton tipped applicator swabs and

allowed to dry. To the surface of each strain, NI-22 was applied with a small inoculation loop, resulting in a colony approximately 6 mm in diameter. The plates were then incubated for 18 hours at room temperature without CO_2.

2.14 *Vibrio fischerii* inhibition assay performed against *Escherichia coli*

Vibrio fischerii (*V. fischerii*) was obtained from the Photodyne Corporation. The bacterium was inoculated onto Good Vibrio Media containing, per-litre H_2O; 10.0 grams (g) tryptone, 5.0 g casamino acids (Difco), 25.0 g NaCl, 4.0 g $MgCl_2$ and 1.0 g potassium chloride (KCl) (Winfrey, 1997) using the streak plate method and incubated for 24 hours at room temperature. The plate was then stored at 5^oC until further testing. Standard antimicrobial assay plates were made and *V. fischerii* and NI-22 were inoculated onto the surface of the medium. The plates were incubated for 24 hours at room temperature. Only the presence or absence of a zone of bacterial growth inhibition surrounding the inoculation was noted.

RESULTS

3.1 Tests to ensure the antimicrobial activity of the isolate

The marine *Vibrio* sp., isolate NI-22 was able to inhibit the growth of *E. coli* ATCC 25922 (Gram-negative) and *C. xerosis* ATCC 373 (Gram-positive). The appearance of a zone of bacterial growth inhibition, surrounding the inoculation area, was clearly evident. The inner transparent zone is produced during the growth of NI-22. An outer or more turbid zone was also evident.

3.2 Optimal temperature for the growth of NI-22

After 24 hours, standard assay plates that had been incubated at room temperature (~23^{o}C) and 30^{o}C both exhibited zones of bacterial growth inhibition surrounding the NI-22 colony. The control plates containing *E. coli* contained an opaque lawn of growth. Both plates that had been incubated at 37^{o}C had abundant growth of *E. coli* within the medium but NI-22 did not grow. No growth of *E. coli* or NI-22 was observed on test plates incubated at the lowest temperatures, 10^{o}C and 14^{o}C. Only these plates were placed back in their incubators to grow for a further 72 hours. At the end of this period the plates from the 14^{o}C incubator had growth of *E. coli* and NI-22 with a small zone of bacterial growth inhibition (~ 3 mm). The plates from the 10^{o}C incubator still had no evidence of growth and were placed in the 37^{o}C incubator for 24 hours. When they were removed, *E. coli* and NI-22 had both grown well and a small zone (~3mm) of bacterial growth inhibition was surrounding the isolate.

3.3 Inhibition of Growth of *E. coli* by Amberlite Resins

A small amount of Amberlite XAD-16 and Amberlite XAD-7 HP resin, previously autoclaved in 2% MHB, were both placed on *E. coli* pour plates to determine if the resin inhibited the growth of *E. coli*. The same test was performed on both types of resin that had only been placed in small glass jars before they were autoclaved (nothing else was added). After incubation, the XAD-7 and XAD-16 resins, that were autoclaved in Mueller Hinton + 2% broth, appeared to be surrounded by a small zone of bacterial growth inhibition. The same two resins that were only autoclaved in small glass bottles did not appear to inhibit the growth of *E. coli* within the media.

3.4 NI-22 Antibacterial Agent Extraction Trials with Polypropylene and XAD-7 HP resin

Polypropylene and Amberlite XAD-7 HP resin were placed in the flask during the growth of NI-22 to adsorb the antimicrobial compound and subsequently allowing for its extraction using various solvents. The ethyl acetate fractions obtained from both resins appeared not to inhibit the growth of *E. coli*.

3.5 NI-22 extraction trials with Sephadex, Chelex, Cellulose and XAD-7

All of the fractions (methanol x2, ethyl acetate, and chloroform) that were generated for each of the four types of resins did not cause inhibition during the assay procedure. There were also no zones of bacterial growth inhibition evident on plates containing the four types of resins. Following incubation, large clear

zones of bacterial growth inhibition were evident on those plates inoculated with broth from NI-22 grown in cultures with their respective beads/resins. This was done in order to demonstrate that the beads/resin had not killed the bacteria nor did they inhibit production of the antimicrobial compound.

3.6 Extraction of NI-22 Antibacterial Agent at low pH

The extraction protocol was designed with the expectation of adsorbing the antimicrobial compound onto the XAD-7 resin followed by elution with the organic solvent ethyl acetate. The pH of 2% MHB was taken after NI-22 was incubated for 24 hours and before any procedures had commenced. The pH ranged from a low of 5.73 to a high of 6.81. The pH of 2% MHB, without the addition of NI22, was fairly constant at 7.4 – 7.5. During the extraction procedure once 200 mL of 0.1 N HCl was added to the broth, with or without NI-22 present, the pH decreased to approximately 1.23. Extracts of broth containing NI-22 were inhibitory to *E. coli* with a large zone of bacterial growth inhibition observed. Mueller Hinton + 2% broth without NI-22 was also extracted, as a control for the procedure; it too resulted in bacterial growth inhibition. Several other test extractions, without inoculation with NI-22, were performed using Nutrient Broth, Marine Broth 2216, Heart Infusion Broth and Trypticase Soy Broth all with 2% w/v sodium chloride concentrations. Ethyl acetate extracts of all these media also produced zones of bacterial growth inhibition. Another control extraction (no NI-22) using distilled water in place of Mueller Hinton broth but still using 0.1N HCl in the extraction protocol did not cause inhibition of *E. coli* growth. Paper disks, applied to the surface of the assay plate, saturated

individually with methanol, ethyl acetate and chloroform did not cause inhibition.

The modified extraction process in which distilled water was used in place of 0.1 N HCl resulted in zones of bacterial growth inhibition. This occurred when both types of broth, inoculated and uninoculated were processed.

Reducing the normality of HCL from 0.1 N down to 0.01 N did not prevent the extraction of an inhibitory compound. The extracts of inoculated and uninoculated 2% MHB using 0.01 N HCL also generated zones of *E. coli* growth inhibition using the standard assay.

3.7 Effect of Cations on the Observations of Antibacterial Effect by NI-22

Mueller Hinton agar plates that had not been supplemented with sodium chloride do not maximize the growth inhibition produced by NI-22. Mueller Hinton containing 2% w/v sodium chloride (used during inhibition assays) was used as the control medium for *E. coli* and NI-22 (Figure 1). The greatest inhibition of *E. coli* growth by NI-22 was noted when the medium was supplemented with potassium bromide. Even when the concentration of KBr was increased or decreased it was still as good and in most cases there was an increase in the zone of inhibition over the control (Figure 1). With increasing additions of $MgCl_2$, a steady decrease in the size of the growth inhibition zone was observed. When potassium bromide and magnesium chloride were both added, the zone of inhibition was reduced to approximately two-thirds the size of the control. Sodium bromide was incorporated into agar in the same molarity as

potassium bromide. This test was prepared to see if potassium or sodium was responsible for any changes in the size of the inhibition zone. Over the same four concentrations there appeared to be no difference between potassium bromide and sodium bromide with respect to any changes in the size of the zone of inhibition. Supplementation with $CaCl_2$ or CaCl2 + $MgSO_4$ reduced the zone of growth inhibition. A zone of bacterial growth inhibition was still evident after incubation but the agar was extremely cloudy making accurate measurements difficult. This can be seen in Figure 1 in the reduced zone of inhibition size measurements observed for $CaCl_2$ supplemented 2% MH agar.

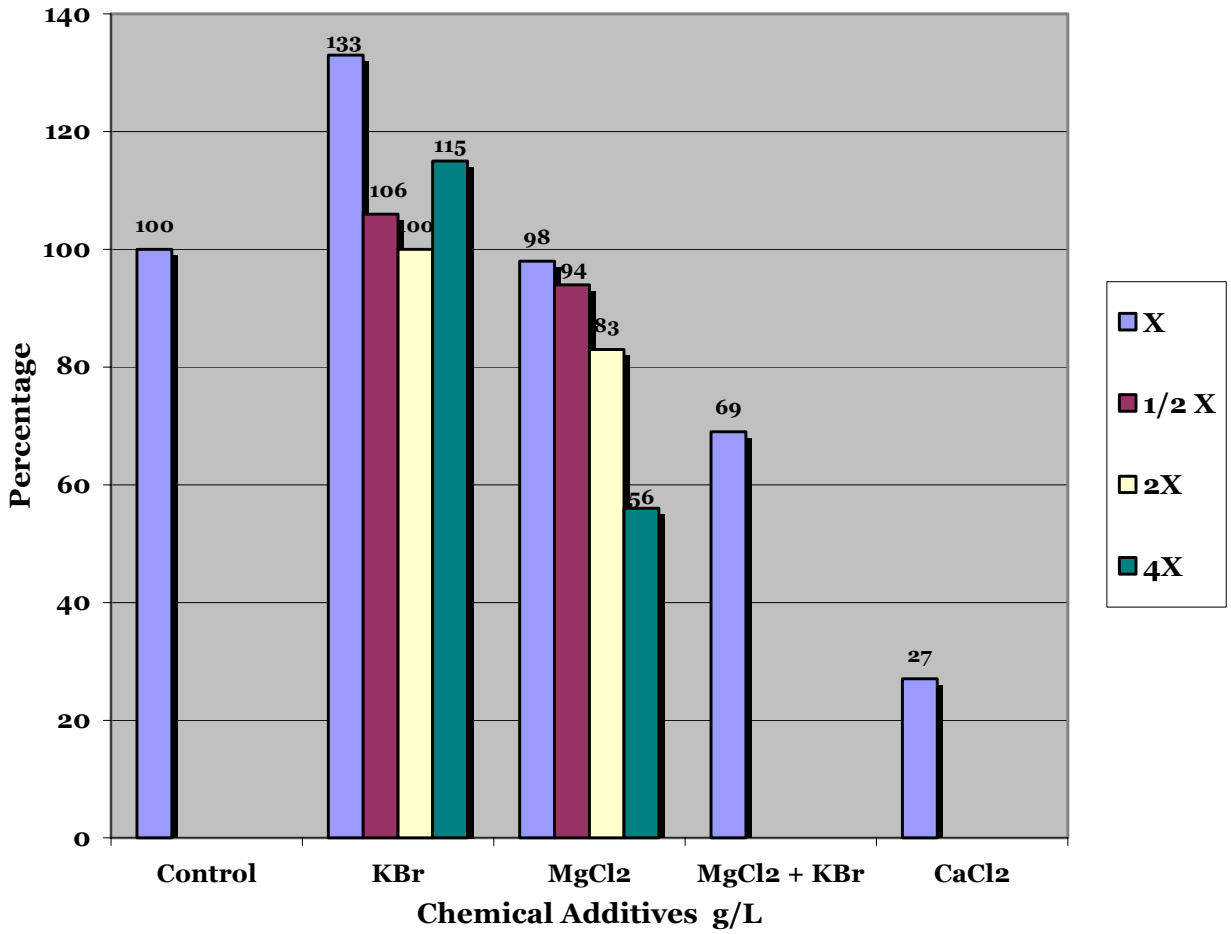

Figure 1: Effect of cations on the antibacterial effect by NI-22 relative to control medium (2% MH agar). Size of the zone of inhibition as a percent increase or decrease from that of the unmodified 2% MH agar. Potassium bromide (KBr) X = 0.08 g/L; Magnesium chloride (MgCl2) X = 5.9 g/L and Calcium chloride X = 1.8 g/L.

3.8 Size of NI-22 inoculation vs. Size of bacterial growth inhibition zone

The size of the NI-22 inoculation when it is grown on the medium (*E. coli* inhibition assay) and the resulting size of the zone of bacterial growth inhibition were tested to see if any correlation existed. Measurement of the NI-22 colony was not recorded until after incubation was completed. The area of the initial inoculation zone was increased gradually by spreading out the bacteria further with the loop. The largest colony was 18 mm in diameter with the smallest just 4.5 mm or 25% as large. Even with this discrepancy the smallest diameter inhibition zones (3.75 mm), belonging to both the 4.5 mm and 5 mm colonies were still 75% as large as the inhibition zones (5 mm) recorded for the 12 mm and 18 mm diameter colonies (Figure 2). Once the area for the zone of inhibition was calculated (Figure 3) for each size colony there was an almost linear increase in the size of the zone of bacterial growth inhibition. This indicates that the antibacterial effect of NI-22 is related to the number of cells in the colony.

3.9 NI-22 inhibition assays performed against 20 *E. coli* fecal isolates

All 20 *E. coli* strains tested against NI-22 were inhibited by the antibacterial compound present in the isolate (Table 1). The average size of the NI-22 colony after incubation was 6 mm. The zone of bacterial growth inhibition ranged from 1 mm to 4 mm with the average size being 1.9 mm.

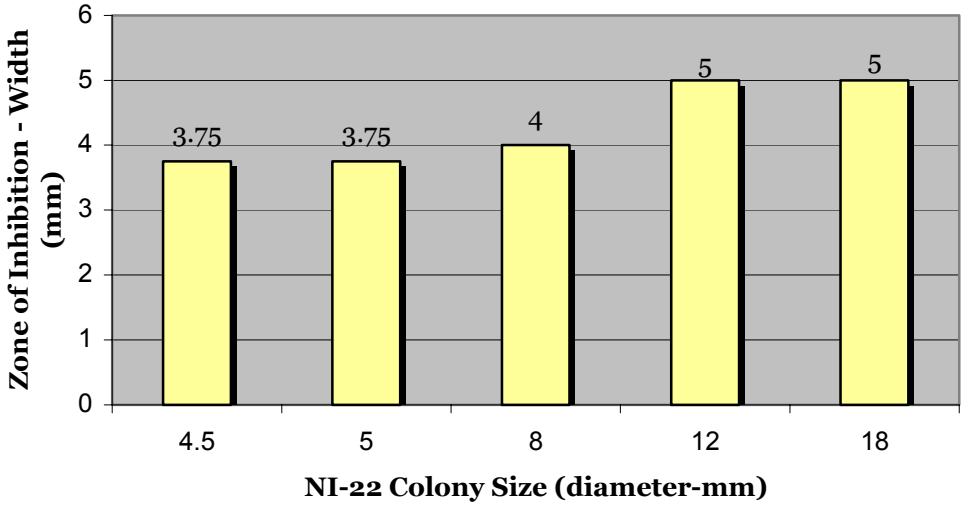

Figure 2: The effect of increasing the area of NI-22 inoculation on the size of the zone of bacterial growth inhibition.

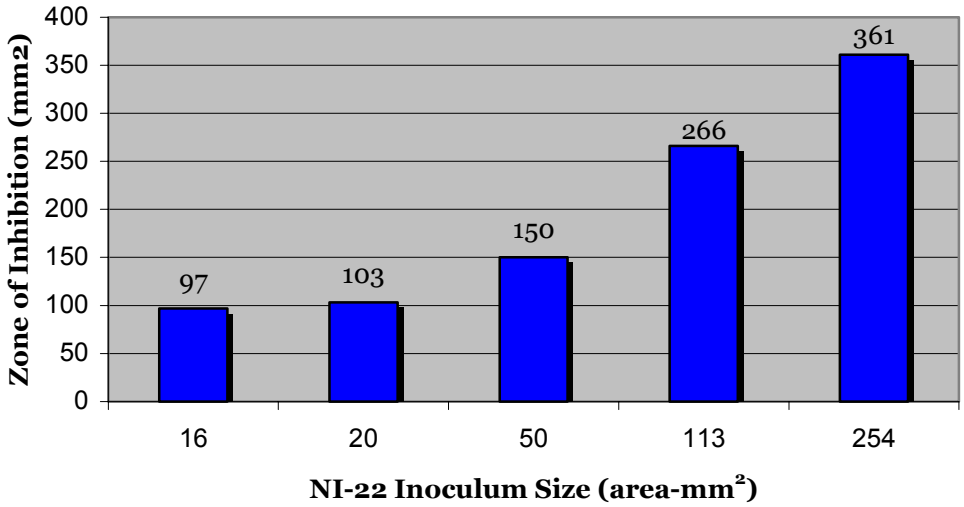

Figure 3: The effect of increasing NI-22 inoculation on the area of the zone of bacterial growth inhibition calculated in mm².

Table 1: NI-22 inhibition assays performed against *20 *E. coli*. fecal isolates using the standard antimicrobial assay.

***E. coli* isolate**	**Inhibition**	**Average Zone diameter**
* 25922	Positive	3.8 mm
9111	Positive	4.0 mm
9016	Positive	1.7 mm
8717	Positive	1.8 mm
8297	Positive	1.0 mm
8069	Positive	2.2 mm
8053	Positive	1.3 mm
7927	Positive	1.8 mm
7764	Positive	1.0 mm
7751	Positive	1.5 mm
7726	Positive	3.0 mm
7522	Positive	1.2 mm
7303	Positive	3.8 mm
7077	Positive	1.8 mm
7013	Positive	1.5 mm
6806	Positive	1.7 mm
6470	Positive	2.0 mm
6182	Positive	1.5 mm
5786	Positive	1.3 mm
5715	Positive	3.8 mm
5201	Positive	1.8 mm

* *E. coli* ATCC strain used as control during experimental inhibition assays.

3.10 NI-22 inhibition assays performed against 8 *Streptococcus pneumoniae* clinical isolates

The eight *S. pneumoniae* isolates grew very well at 30°C and 37°C both with and without CO2. With the exception of #49710 all of the *S. pneumoniae* isolates grew favorably following incubation at 23°C both with and without CO_2. Strain #49710 grew poorly compared to the other seven strains. Under all of the experimental conditions *E. coli* grew very well but NI-22 only grew and produced a zone of bacterial growth inhibition on blood agar plates incubated for 18 hours at 23°C with and without CO_2. Once NI-22 was removed from the 30°C environments (both with and without CO2) and incubated for 24 hours at 23°C it began to grow, but inhibition was not observed. This was not the case for NI-22 on plates removed from the 37°C environments (NI-22 did not grow again). After incubation for 18 hours (without CO_2) a zone of bacterial growth inhibition (Table 2) surrounding NI22 was evident at four of the eight *S. pneumoniae* strains and *E. coli* previously applied to TSA with 5% sheep blood plates at McFarland's standard #1 (3 x 10^8 cfu/ml). The *S. pneumoniae* isolates were #50946, #50847, #48957, and #48154. The remaining four strains appeared to be growing more slowly and were left to incubate, under the same conditions. Following an additional 22 hours (total incubation time approximately 40 hours) three of the remaining four isolates of *S. pneumoniae*, #50009, #49202, and #49203 were also inhibited by NI-22 (a distinctive zone of bacterial growth inhibition was present). The last isolate, #49710, had not grown well enough after 40 hours and was allowed to incubate further. A zone of bacterial growth inhibition was

observed surrounding NI-22 once the plate had incubated for an additional 30 hours (total incubation time approximately 70 hours).

3.11 *Vibrio fischerii* inhibition assay performed against *Escherichia coli*

Following incubation in a standard assay *Vibrio fischerii* did not inhibit the growth of *E. coli* but NI-22 did. This is an indication that the compound produced by our *Vibrio* sp., isolate NI-22 may not be typical of the entire genus.

Table 2: NI-22 inhibition assays performed against eight *_S. pneumoniae_ clinical isolates. _S. pneumoniae_ was inoculated at 3 x 10^8 cfu/ml on TSA + 5% sheep blood agar (incubation at room temperature without CO2).

***S. pneumoniae* isolates**	**Inhibition after 18 - hours**	**Inhibition after 40 - hours**	**Inhibition after 70 - hours**	**Zone of inhibition diameter**
50946	**Positive**	Positive	Positive	1.5 mm
50847	**Positive**	Positive	Positive	2.0 mm
48957	**Positive**	Positive	Positive	1.5 mm
48154	**Positive**	Positive	Positive	1.5 mm
50009	Negative	**Positive**	Positive	1.5 mm
49203	Negative	**Positive**	Positive	1.0 mm
49202	Negative	**Positive**	Positive	1.0 mm
49710	Negative	Negative	**Positive**	1.5 mm

*** *E. coli* ATCC**	**Inhibition after 24 - hours**	**Inhibition after 40 - hours**	**Inhibition after 70 - hours**	**Zone of inhibition diameter**
* 25922	**Positive**	Positive	Positive	2.0 mm

DISCUSSION

The results of the growth inhibition assays against two species of pathogenic bacteria provide some very promising results. The antibacterial effect of NI-22 against 20 *E. coli* fecal isolates and eight *S. pneumoniae* clinical isolates is appealing because it indicates that the bacterium *Vibrio*, is able to target the growth of both Gram-positive and Gram-negative bacteria. The antibacterial property of NI-22 has been tested against three methicillin-resistant *Staphylococcus aureus* (*S. aureus*) and six *E. coli* isolates (personal communication). The *E. coli* were clinical isolates, resistant to at least one of a variety of antibiotics, and one that was multidrug-resistant. The antibacterial compound present in NI-22 inhibited the growth of all six. All three *S. aureus* (two clinical isolates and one ATCC strain) strains were inhibited by NI-22.

Although widely distributed in the aquatic environment, reports of Vibrio strains producing antimicrobial substances are rare in the literature. The group cited in a taxonomic review most frequently as antibiotic producers is the *Alteromonas-Pseudomonas* group (52%) with the *Vibrio* sp. representing only 8.3% (Jorquera *et al.* 2000). As rates of bacterial resistance continue to climb the search for marine compounds with pharmaceutical potential has intensified. The Marine Natural Product Database currently lists approximately 6 000 chemical compounds in 253 separate classes, derived from over 10 000 marine-derived materials (Lei and Zhou 2002). The antibacterial property of the marine

Vibrio sp., isolate NI-22 against both Gram-negative and Gram-positive species may be indicative of a new class of antibiotics something that is urgently needed.

The use of Amberlite XAD resin, Chelex-100 and several other solid adsorbents has been widely reported in the literature for the extraction, isolation, concentration and filtration of a wide variety of compounds (Ashen and Goff 2000). Extraction of aridicins, an antibacterial antibiotic complex (Sitrin *et al.* 1985) and the extraction of new neoverrucosane diterpenoids from the marine gliding bacterium *Saprospira grandis* (ATCC 23116) (Spyere *et al.* 2003) are just two examples of protocols developed using these adsorbent resins. The application of XAD resin has also been employed to concentrate trace organic pollutants (Onodera *et al.* 1993), isolate siderophores from a marine *Vibrio* sp. (Martinez *et al.* 2003) and for the filtration/concentration of mutagenic metabolites from urine (Belisario *et al.* 1985). In many instances, reversed-phase HPLC methods were utilized to further purify solvent extractions (methanol, ethyl acetate, isooctane) and isolate from those fractions the bioactive compounds. The antibacterial effect of NI-22 was not observed once the extraction procedures had attempted to isolate and concentrate the biologically active compound. This may have been a consequence of the organic solvents denaturing the compound during the procedure or that the compound is labile or that the compound is a type of molecule not extracted into the solvents.

The difficulty in culturing marine bacteria while still maintaining production of the active compound has led to the development of specialized media. Even so, once isolated from its natural environment the bacteria may be affected by the loss of specific micronutrients in culture media, changes in the mode of culture

for example from broth to agar, mixing velocity, changes to specific temperature requirements, pH, light or pressure. These may all lead to the quantitative and/or qualitative isolation of a modified marine natural product (Kelecom 2002; Armstrong *et al.* 2001). The rate of penicillin fermentation (gram per cell dry weight) by *Penicillium chrysogenum* decreased over 50% when agitation intensity increased from 600 rpm to 1000 rpm (Marwick *et al.* 1999).

Bacteria express a wide range of biologically active extracellular compounds such as proteins and enzymes. They fulfill many diverse roles such as antibiotic effects, nutrient acquisition and colonization activities (Holmstrom and Kjelleberg 1999). These compounds can also be temperature sensitive, explaining why the antimicrobial effect of NI-22 significantly declined above and below very specific temperatures (i.e. below 14°C and above 30°C). It is also interesting to note that NI-22 was able to grow and express antibacterial activity on blood-containing medium in environments supplemented with 5 – 10% CO_2 at 23°C. Some of the *Pseudoalteromonas* species did not express any antibacterial activity when grown on blood-containing media suggesting that specific nutrient conditions are required for bacteria to express their extracellular biologically active compounds (Holmstrom and Kjelleberg 1999). Confronted with these challenges the problem of maintaining a sustainable supply – i.e., the compound may account for less than 10^{-6}% of the wet weight is another impediment that must be overcome (Donia and Hamann 2003). In order for large-scale production of marine natural products to eventually supply the health care market a reliable supply of the compound must be available or it will remain of novelty value only (Munro 1999). Unfortunately there is no published work on

scale-up procedures as they apply to marine bacterial antibiotic systems (Marwick *et al*. 1999). The research of Bode *et al*. (2002) is utilizing a systematic approach, altering basic cultivation parameters (media composition, aeration, culture vessel, addition of enzyme inhibitors) and observing how these influence the production of secondary metabolites from various microorganisms. Trace elements such as bromide ions added to the growth medium have been shown, in some cases, to increase the amount of antibiotic production over normal medium (Marwick *et al*. 1999). A group of antibiotics produced by *Pseudoalteromonas luteoviolaceus* (*P. luteoviolaceus*) contains small-brominated compounds known to have a strong bactericidal effect (Holmstrom and Kjelleberg 1999). Results obtained in this study indicate that the antibacterial effect of NI-22 was increased when the medium was supplemented with potassium bromide.

The challenges one faces when developing an extraction protocol are as varied as the compounds one may be interested in isolating. The final result, materials and methods and instrumental analysis to determine the stereochemistry of isolated compounds may take years. Previous research within the field is certainly the place from which investigation of new and novel techniques need to arise. Once completed the published article unfortunately makes no mention of the trial and error and duration of the experimental design.

CONCLUSIONS

1. Recovery and isolation of antibacterial compound from NI-22 was unsuccessful during the development of the extraction protocols.

2. NI-22 inhibits the growth of 20 *E. coli* fecal isolates and eight *S. pneumoniae* clinical isolates.

3. The addition of potassium bromide (KBr) to the medium increased the level of antibacterial effect produced by NI-22.

4. Increasing concentrations of magnesium chloride ($MgCl_2$) and calcium chloride ($CaCl_2$) added to the growth medium decreased the level of antibacterial effect produced by NI-22.

REFERENCES

Amyes, S. G. B., and C. G. Gemmell. 1997. Antibiotic resistance. J. Med. Microbiol. 46: 436 – 470.

Armstrong, E., Yan, L., Boyd, K. G., Wright, P. C., J. G. Burgess. 2001. The symbiotic role of marine microbes on living surfaces. Hydrobiologia. 461: 37 – 40.

Ashen, J. B., and L. J. Goff. 2000. Molecular and ecological evidence for species specificity and coevolution in a group of marine algal-bacteria symbioses. Appl. Environ. Microbiol. 66 (7): 3024 – 3030.

Baranova, N. A., and N. S. Egorov. 1989. Production of antibiotic substances by natural variants of the marine bacterium *Vibrio fischeri*. Antibiot. Khimioter. 34 (1): 7 – 10.

Belisario, M. A., Farina, C., and V. Buonocore. 1985. Evaluation of concentration procedures of mutagenic metabolites from urine of diesel particulate-treated rats. Toxicol. Lett. 25 (1): 81 – 88.

Blunt, J. W., Copp, B. R., Munro, M. H. G., Northcote, P. T., and M. R. Prinsep. 2004. Marine natural products. Nat. Prod. Rep. 21 (1): 1 – 49.

Bode, H. B., Bethe, B., Hofs, R., and A. Zeeck. 2002. Big effects from small changes: Possible ways to explore Nature's chemical Diversity. ChemBioChem. 3 (7): 619 – 627.

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A., K. G. Boyd. 1999. Microbial antagonism: a neglected avenue of natural products research. J. Biotechnol. 70 (1-3): 27 – 32.

Burkholder, P. R., Pfister, R. M., and F. H. Leitz. 1966. Production of a Pyrrole antibiotic by a marine bacterium. Appl. Microbiol. 14 (4): 649 – 653.

Bush, K. 2004. Antibacterial drug discovery in the 21st century. Clin Microbiol. Infect. 10 (4): 10 – 17.

Camello, Thereza Christina Ferreira, MATTOS-GUARALDI, Ana Luiza, FORMIGA, Luiz carlos Duarte *et al.* 2003. Nondiphtherial Corynebacterium species isolated from clinical specimens of patients in a university hospital, Rio de Janeiro, Brazil. Braz. J. Microbiol. 34 (1): 39 – 44.

Carrie, A. G., Metge, C. J., and G. G. Zhanel. 2000. Antibiotic use in a Canadian Province, 1995 – 1998. Ann. Pharmacother. 34 (3): 459 – 464.

Donia, M., and M. T. Hamann. 2003. Marine natural products and their potential applications as anti-infective agents. Lancet Infect. Dis. 3 (6): 338 – 348.

Engel, S., Jensen, P. R., and W. Fenical. 2002. Chemical Ecology of marine microbial defense. J. Chem. Ecol. 28 (10): 1971 – 1985.

Faulkner, D. J. 2000. Marine pharmacology. Antoine van Leeuwenhoek. 77 (2): 135 – 145.

Holmstrom, C., and S. Kjelleberg. 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol. Ecol. 30 (4): 285 – 293.

Jorquera, M. A., Riquelme, C. E., Loyola, L. A., L. F. Munoz. 2000. Production of bactericidal substances by a marine vibrio isolated from cultures of the scallop *Argopecten*. Aquac. Int. 7 (6): 433 – 448.

Kelecom, A. 2002. Secondary metabolites from marine microorganisms. An. Acad. Bras. Cienc. 74 (1): 151 – 170.

Kobayashi, M., Aoki, S., Gato, K., Matsunami, K., Kurosu, M., and I. Kitagawa. 1994. Marine natural products. XXXXIV. Trisindoline, a new antibiotic indole trimer, produced by a bacterium of Vibrio sp., separated from the marine sponge Hyrtios altum. Chem. Pharm. Bull. (Tokyo). 42 (12): 2449 – 2451.

Lee, J. C., Park, H. R., Park, D. J., Lee, H. B., Kim, Y. B., and C. J. Kim. Improved production of teicoplanin using adsorbent resin in fermentations. Lett. Appl. Microbiol. 37 (3): 196 – 200.

Lei, J., Zhou, J. 2002. A Marine Natural Product Database. J. Chem. Inf. Comput. Sci. 42 (3): 742 – 748.

Marshall, V. P., McWethy, S. J., Sirotti, J. M., and J. I. Cialdella. The effect of neutral resins on the fermentation production of rubradirin. J. Ind. Microbiol. 5 (5): 283 – 287.

Martinez, J. S., Carter-Franklin, J. N., Mann, E. L., Martin, J. D., Haygood, M. G., and A. Butler. 2003. Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. Proc. Natl. Acad. Sci. USA. 100 (7): 3754 – 3759.

Marwick, J. D., Wright, P. C., and J. G. Burgess. Bioprocess intensification for production of novel marine bacterial antibiotics through bioreactor operation and design. Mar. Biotechnol. 1 (5): 495 – 507.

Munro, M. H. G., Blunt, J. W., Dumdei, E. J., Hickford, S. J. H., Lill, R. E., Li, S., Battershill, C. N., and A. R. Duckworth. 1999. The discovery and development of marine compounds with pharmaceutical potential. J. Biotechnol. 70 (1-3): 15 – 25.

Onodera, S., Nagatsuka, A., Rokuhara, T., Asakura, T., Hirayama, N., and s. Suzuki. 1993. Re-evaluation of solid-pjase adsorption and desorption techniques for isolation of trace organic pollutants from chlorinated water. J. Chromatogr. 642 (1-2): 185-194.

Quarta, C., Borghi, A., Zerilli, L. F., De Pietro M. T., Ferrari P., Trani, A., and G. C. Lancini. 1996. Isolation and structure determination of a novel complex of the teicoplanin family. J. Antibiot. (Tokyo). 49 (7): 644 – 650.

Sitrin, R. D., Chan, G. W., Dingerdissen, J. J., Holl, W., Hoover, J. R., Valenta, K. R., Webb, L., and K. M. Snader. 1985. Aridicins, novel glycopeptide antibiotics. II. Isolation and characterization. J. Antibiot. 38 (5): 561 – 571.

Spyere, A., Rowley, D. C., Jensen, P. R., and William Fenical. 2003. New Neoverrucosane Diterpenoids produced by the marine gliding bacterium *Saprospira grandis*. J. Nat. Prod. 66 (6): 818 – 822.

Tschen, T. S., and C. N. Tseng. 1989. Bacereutin, an antifungal antibiotic isolated from metabolites of Bacillus cereus CHU 130. Proc. Natl. Sci. Counc. Repub. China B. 13 (4): 258 – 261.

Tziveleka, L. A., Vagias, C., and V. Roussis. Natural products with anti-HIV activity from marine organisms. Curr. Top. Med. Chem. 3 (13): 1512 – 1535.

Watve, M. G., Tickoo, R., Jog, M. M., and B. D. Bhole. How many antibiotics are produced by the genus *Streptomyces*?. Arch. Microbiol. 176 (5): 386 – 390.

Winfrey, M. R., Rott, M. A., A. T. Wortman. 1997. Unraveling DNA: Molecular Biology for the Laboratory. New Jersey: Prentice Hall. p. 334.

Zhanel, G. G., Palatnick, L., Nichol, K. A., Bellyou, T., Low, D. L., and D. J. Hoban. 2003. Antimicrobial Resistance in Respiratory Tract Streptococcus pneumoniae Isolates: Results of the Canadian Respiratory Organism Susceptibility Study, 1997 to 2002. Antimicrob. Agents Chemother. 47 (6): 1867 – 1874.