ISOLATION AND CHARACTERIZATION OF ANTIVIRAL MARINE MICROORGANISMS

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ABSTRACT

For an Island community, the ocean is a rich habitat abundant with life. It is a valuable and versatile source of food, revenue, recreation, and is of great economic importance. The ocean also acts as a source of many biologically active agents, which are assuming greater importance as they are discovered. The number of uses and users of the ocean will continue to increase in the coming decades. We have previously reported the presence of marine organisms that can inactivate human viruses, which are common in ocean waters receiving domestic sewage. In this study, we aimed to isolate and identify these marine anti-viral agents (MAVAs), determine their active principle(s), and to characterize the biological, chemical, and physical parameters that would enable their optimal culture *in-vitro*.

In these experiments, we isolated marine bacteria that have significant antiviral activity against the human enterovirus, poliovirus type 1 (Sabin LSc2ab), from several sites around the island of Oahu. Antiviral activity in coastal marine waters was removed by: a) prior removal of bacteria by membrane filtration (0.45µm filter), or b) the addition of certain antibiotics used in previous studies (streptomycin, penicillin). MAVA activity was restored to filtered coastal waters upon seeding a small inoculum of natural

marine coastal water and allowing the MAVAs to grow. These results indicate that natural populations of marine bacteria are responsible for antiviral activity. The bacterial isolate was provisionally identified by amplification and nucleotide sequencing of the 16s rRNA gene. The sequence was compared to others in the public domain using BLAST searches and confirmed as a *Marinomonas* sp. The potential applications of MAVAs are diverse and include, the biological control of viruses polluting the ocean ecosystem, control of disease transmission from sewage polluted waters, and chemotherapy for viral illnesses in humans and other animals.

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CHAPTER ONE

Introduction

I. Introduction and Rationale

The ocean is a source of economically and biologically important flora and fauna. It is used for natural and farmed food (aquaculture species), for recreational activities and as a natural avenue for waste disposal. In a time when standard methods of disease control such as antibiotics are becoming increasingly ineffective, there is a need for new treatments and management strategies for common illnesses. Pharmacological and therapeutically important products are being sought from marine sources (Bernan et al., 1997; Carte, 1996; Rinehart, 1992; Tzivelekaet al., 2003). Hawaii represents a geographic location with immense opportunities for the discovery of biologically active products (Fujioka and Loh, 1996).

In Hawaii the ocean is subject to point source and non-point source pollution by sewage outfalls and terrestrial runoff, respectively. These pollutants contain a variety of microorganisms as well as viruses that may still be infectious. This means then, that there are active viruses in the seawater we use for recreational and other purposes; this is not a novel discovery (Akin et al., 1976). Since human viruses have existed in the ocean for such a long time (Gerba and Schaiberger, 1975, Loh et al.,1979), it is reasonable that there may be marine bacteria that have the ability to inactivate them (Bernan et al., 1997).

Through previous studies, we reported the presence of novel marine antiviral agents (MAVAs) in Hawaiian coastal waters, the first of such reports for the Pacific Ocean (Fujioka et al., 1980, 1981). Data on the properties of MAVA strongly suggest that some microorganisms have the ability to inactivate human pathogenic enteroviruses such as poliovirus types 1,2, and 3, Coxsackievirus B4 and B5, and echovirus types 7 and 27.

We have isolated several bacteria with antiviral activity against poliovirus 1 (Sabin LSc2ab) from coastal waters on the windward side of Oahu. Through 16S rDNA sequencing, this microorganism has been provisionally identified as a *Marinomonas sp*. Potential uses of MAVAs include;

1. The biological control of human enteropathogenic virus contamination, and thus disease transmission in sewage-polluted waters. This would be particularly important to communities such as those found in Hawaii, where coastal waters have high fishing and recreational burdens, and to regions where loss of these marine resources and amneties would have a devastating effect on the lifestyle and economy of the people.

2. The chemotherapeutic treatment of viral diseases of humans and animals. To be of practical use, it is imperative that MAVAs be isolated in pure culture, identified, characterized, and their spectrum and mechanisms of antiviral activity be clearly established. Future research will benefit from identifying and purifying the active principle(s) and chemically characterizing them.
3. The biological control of viral diseases of marine animals. There are few practical ways to prevent viral transmission to marine mammals. Viral transmission is especially troublesome when marine organisms are kept in captivity for various uses (farming) or raised under aquarium or coastal pen conditions. The seeding of MAVAs to these special environments has the possibility to control viral disease transmission. In summary, there are very limited measures that can be taken for the effective control, prevention, and therapy of viral infections and the diseases they cause. MAVAs are a potential solution to this problem and need to be further explored.

II. The Origin of Antiviral Activities in Ocean Waters

It has been previously reported that some components in natural seawater are harmful to viruses, especially human enteric viruses (Akin et al., 1976; Denis et al., 1977; Gunderson et al., 1967; Lo et al., 1976; Lycke et al, 1965; Matossian et al., 1967; Mitchell et al., 1971; Shuval et al., 1977; Loh et al., 1979; Toranzo et al., 1982; Gerba and Schaiberger, 1975). Elements of natural seawater, such as temperature, heavy metals, high salts, toxins, particulates, by-products of marine microorganisms, as well as the direct action of microorganisms are thought to be responsible for the antiviral activity of marine water.

The first documented marine bacterium responsible for the antiviral activity of seawater was obtained from the North Sea. The microorganism was identified as a *Vibrio marinus* sp. (Magnusson et al. 1966, 1967). However, this bacterium quickly lost its antiviral properties after it was purified and cultured under laboratory conditions. Many other investigators have reported comparable antiviral activity from marine microorganisms e.g. from the Red Sea, the Mediterranean, the Baltic Sea, Atlantic Ocean, Gulf of Mexico and the Sea of Japan (Denis et al., 1977; Gunderson et al., 1967; Lo et al., 1976; Lycke et al., 1965; Magnusson et al., 1966; Matossian and Garabedian, 1967; Shuval et al., 1970; Yoshimizu et al., 1989).

The first of antiviral activity in the tropical waters of the Pacific Ocean came from our laboratory (Fujioka et al., 1980). The bacteria recovered were able to maintain activity against enteric viruses even after isolation, purification and maintenance under laboratory conditions (Fujioka and Loh, 1981). Based upon phenotypic characteristics, such as gram stain, colony morphology, color, and gliding motility, the two isolates were both provisionally identified as a marine *Flexibacter* sp.

These studies, however, were conducted with seawater samples obtained from sites located on the west (or leeward) side of Oahu. In the experiments described here, our objective was to determine whether MAVAs inhabit the waters off the windward side of Oahu. This will determine if these types of antiviral agents are ubiquitous in waters around the island.

III. Goals and Objectives

The objectives of the work described here were:

1. To isolate and identify from Hawaiian marine waters, marine antiviral agents (MAVAs) and determine their activities against important viral pathogens (e.g. polioviruses, coxsackieviruses, hepatitis A virus, rotaviruses, etc.)
2. To optimize *in vitro* growth conditions for MAVAs.
3. To identify isolated MAVAs based on 16S ribosomal DNA nucleotide sequence methods.

IV. Research Methods

A. Virus Agents

The number and types of viruses to be tested in this project were limited principally to those whose source is sewage and which can be transmitted in ocean waters and cause serious disease in their respective permissive animal host. The viral pathogens are poliovirus type 1 (Sabin strain LSc2ab), and coxsackievirus B2 (isolate).

B. Isolation and Identification of MAVAs

Methods developed by this laboratory (Fujioka et al., 1980) were used to isolate and identify MAVAs. Briefly, to test for antiviral activity, seawater (surface) samples (500ml) from various sites were collected and inoculated with a known quantity of a human enteric virus. The samples were incubated at 27°C, the mean temperature of surface waters around Hawaii, and aliquots removed daily (for 4 days) and assayed in cell culture for residual infectious virus. Any sample exhibiting a 90% (T90) or greater loss in virus titer as compared to a control (PBS) within three days, were considered positive for the presence of MAVAs. Once a seawater sample is found to be positive for the presence of MAVAs, bacteria were cultivated on selected culture media and the suspected MAVA colonies were picked, streaked for purification, and retested for antiviral activities. Colonies that displayed significant antiviral activity were further characterized by employing standard bacteriological and molecular methods (Gunderson et al., 1967, Suzuki et al., 1995).

C. Media Selection

During preliminary phases of this study, the following three kinds of selective growth media were used:

1. Marine agar (2216 DIFCO) - an enriched growth medium to select for those heterotrophic bacteria that grow in seawater.
2. Anderson agar – a growth media made up of the seawater in which the microorganisms were originally present and extracts to minimally enrich the media. This medium was found to be most appropriate and was used selectively in previous studies (Yoon, D.W. pers. comm.)
3. TCBS (Thiosulfate-Citrate-Bile Salts-Sucrose 0650 DIFCO) agar – a highly selective growth medium for members the genus *Vibrio* sp. as used in previous studies (Fujioka et al., 1981)

We also tested each bacterial isolate for susceptibility to several antibiotics (Penicillin, Streptomycin, Ampicillin, Tetramycin) as cited in previous studies (Lau et al., 1978). This combination of an antibiotic response profile will facilitate the isolation of MAVAs in pure culture.

D. Identification of Marine Bacterial Isolates

To facilitate identification of MAVAs, we employed standard molecular methods such as PCR and SDS-PAGE. Universal primers (for 16S rDNA amplification) designed for gram-negative bacteria were used and results were compared against an existing worldwide genetic database (GenBank) (http://www.ncbi.nlm.nih.gov).

Previous experiments conducted by our laboratory involving the sampling of seawater taken from 3 sites, a marine site, a brackish water site and a freshwater site, indicated that marine antiviral agents were present only in marine and brackish water samples (Yoon, D.W. pers. comm.). MAVAs were absent in the freshwater sample, so therefore served as a comparative control. Filtration of marine and brackish water samples through 0.45μm filters removed all MAVA activity (Fujioka et al., 1981). Seeding of filtered seawater with a small volume of unfiltered marine water (1:1000 dilution) confirmed the presence of MAVAs within a few days. We believe this technique is an appropriate way to reduce the background bacterial and other populations, and thus this technique was adopted for these studies.

E. Cell lines

The screening of seawater samples for antiviral activity against enteroviruses required the use of cell culture methods to grow and quantitatively assay the remaining infectivity of each enterovirus using the plaque assay method. This approach is accurate and desirable although it is slow, expensive and allows only a limited number of water samples to be screened. It is imperative to use a cell culture line that is susceptible to a spectrum of enteric viruses, as well as one that does not require excessive maintenance and expense. The following viable cell lines were initially used: Vero cells (African green monkey kidney ATCC CCL 81) LLC-MK2 (Rhesus monkey kidney ATCC CCL7) and BGMK cells (Buffalo green monkey kidney). However, this was later limited to the exclusive use of BGMK cells after they exhibited the optimal viral plaque formation for both polio and coxsackie viruses.

V. Literature Search on Viruses Used

A. Polio virus

Poliovirus belongs to the virus family Picornaviridae, which is one of the largest families of viruses and includes many important pathogenic as well as non-pathogenic human and animal viruses. The Picornavirus family includes the subgrouping of enteroviruses of which at least 72 types of human enteroviruses exist presently. These viruses are small RNA viruses that have no envelope and because of such, are referred to as "naked" icosahedral viruses. The capsids of these viruses are resistant to many known chemical agents (detergents) and to environmental stresses. The viruses are transmitted by the fecal-oral route and are able to withstand the harsh acidity of the mammalian gastrointestinal tract. The positive strand of viral RNA is surrounded by an icosahedral capsid of protein and is about 30nm in diameter. This capsid includes 12 pentameric verticies that are made up of four viral proteins (VP1-VP4). The capsids are stable at temperatures up to 50°C, and stable in the presence of detergents. The positive stranded RNA genome of the virus consists of about 7500 bases including a poly-A tail at the 3′ end which has been found to enhance infectivity, and a protein of 22-24 amino acids in length (viral protein G) attached to the 5′ end, which may play a role in packaging the viral genome into the viral capsid and initiating RNA synthesis. Interestingly, it has been found that the naked viral RNA is able to effectively infect a cell without the viral capsid (Fields, 1985), and thus does not exhibit host-primate tropism specificity as required by the complete virus.

The viral genome encodes a polyprotein that is cleaved to produce the structural proteins of the virus. Poliovirus produces several proteases some of which are able to degrade a cap-binding protein found in eukaryotic ribosomes, which allows for blocking translation of cellular mRNA. The viral genome also encodes an RNA-dependant viral RNA polymerase.

a) Replication

Once the virus binds to a specific receptor (CD155) on a susceptible primate host cell, VP4 one of the 5 viral proteins, is released, reconformation of the virus occurs and the viral genome able to penetrate the host cell membrane. Once inside the host cell, the genome binds to the ribosomes, which recognize a unique RNA loop in the genome sequence. This binding along with the production of a viral protease cleaves the cap-binding protein and prevents cellular mRNA binding. A poly-protein is then generated within 15 minutes of infection. After cleavage of the long polyprotein by encoded proteinases, viral RNA polymerases are available to replicate a negative stranded mRNA genome and templates can then be synthesized. The amount of viral RNA in each cell increases rapidly (about 400,000 copies per cell). This amount of viral mRNA can easily out-compete cellular mRNA for the components needed for protein synthesis. This allows CPE to occur within the host cell. Viral structural proteins are made by cleavage of the viral polyprotein by encoded proteases. Assembly of capsid and RNA genome takes to form an infectious virus. Five protein subunits associate into the pentamers that will eventually make up the viral capsid. After the new viral genome is inserted into this new viral procapsid, the entire infectious virus is then released from the cell by lysis.

Poliovirus is perhaps the most studied enterovirus due to the vast outbreak of poliomyelitis in the early 1900's and is still prevalent in some countries today. There are 3 serotypes of the virus. Poliovirus serotype 1 (Sabin LSc2ab) an attenuated laboratory strain was used in this study.

B. Coxsackie Virus

Coxsackie viruses were discovered in 1948 when two virologists (Dalldorf and Sickles) were studying parapoliomyelitis viruses in young children in a New York hospital. Two viruses were found in the stools of children who exhibited signs of poliomyelitis. These two viruses, when isolated in newborn mice were found to be unique because they were not neutralized by antisera against polioviruses. In recognition of the small village in the state of New York where the children lived, the viruses were termed "Coxsackie" viruses.

Coxsackie viruses are small enteroviruses of the family Picornaviridae and make up only six serotypes out of about 213 total serotypes of other picornaviruses (Bendinelli, 1998).

These viruses were first identified for their pathogenicity in mice and hamsters and were then divided into two groups (Coxsackie A viruses and Coxsackie B viruses) based on their differences in tissue pathology, such as neurological disease, pancreatitis and fatal acute myocarditis.

The viruses are about 28nm in diameter, are icosahedrally symmetrical, and have a single positive strand RNA genome. The viral capsid consists of 60 copies of 4 viral proteins. The RNA genome is quite similar to that of poliovirus in form and function.

Coxsackie B (CVB) viruses are resistant to low pH levels, this allows them to pass through the acidic gastric environment and finally replicate in the mucosal cells of the small intestine. Viral RNA genomes have been found latent in cells (heart tissue) where no intact virus or viral proteins are detected. Coxsackie viruses are also somewhat heat stable as they can survive for about 30 minutes at 50°C. The single stranded RNA molecule is about 7,400 nucleotides long. The four capsid proteins are generated from the polyprotein products by proteolytic cleavages, which are virus encoded.

a) Replication

Coxsackie viruses have been grown in monkey, hamster, pig, human amnion, HeLa, Vero, and BGMK cell culture types (Bendinelli, 1998). A protein called viral attachment protein mediates viral attachment to the cell surface. Once the virus has attached successfully, it penetrates the cell membrane and migrates through the cytoplasm until a vesicle is reached. Once inside the cell, the virus is disassembled and replication enters the eclipse phase where no infective virus particles are released. Protein and RNA synthesis then occur and assembly of new virus particles takes place.

How the virus ruptures the cell surface during release is unclear however, usually the membrane is dramatically damaged.

b) Pathogenesis

CVB infections are characterized by a variety of human diseases, which are not specific to geographical location. However, in temperate climates infections seem to spread in late summer to early fall, whereas in tropical climates infections appear throughout the year. Young children seem to be most susceptible to disease. Viral disease is characterized by fever, pleural inflammation, severe headache, sore throat and unilateral chest pain. Abdominal tenderness associated with pancreatitis and hepatitis may occur. Most patients readily recover from these and other cold-like symptoms however, some infections are increasingly debilitating wherein the patient will be acutely ill for 2-3 weeks followed by 1-2 months of fatigue. In this condition it is important that the patient not demonstrate excessive physical exertion until complete recovery is achieved, otherwise heart disease is likely.

Because of these severe symptoms of disease, there is great interest in solving many of the pathogenic problems of the virus. Study reveals that cytotoxic T cells, lymphokines, complement, cytokines, and autoimmune antibodies target myocardial cells, causing necrosis. However one argument to this theory was brought to light in a study wherein patients who developed severe disease were given immunosuppressive therapy, which did not increase patient survival (Ku, 1998). Also, the infectious virus is absent from the tissue at the time of diagnosis making the determination of etiology difficult, there is also a low incidence of disease (1:3000) with a high incidence of infection.

A Coxsackie B2 virus (clinical isolate) was used for this study.

C. Epidemiology of Poliovirus and Coxackie Virus

CVB is commonly detected via the classic approach, tissue culture. Using methods such as polymerase chain reaction would be ideal however, there is no current PCR approach capable of identifying and differentiating all serotypes. PCR is easily used to determine if an enterovirus is present in a sample, but the serotype of the virus will remain unknown and it does not determine whether the virus is infectious. Virus detection from clinical samples is somewhat more difficult than identifying virus from cell culture as detection methods must be a great deal more specific and sensitive. An ELISA has been developed for CVB specific IgM, as well as for anti-poliovirus antibodies. These types of tests are usually very accurate and are confirmed by cell culture methods, e.g. almost 90% of culture confirmed CVB infections (Tracy et al., 1997). These immunologically based tests are relatively rapid and easy to perform. In this way, ELISA is a useful tool for epidemiological investigations and outbreaks for both types of viruses.

The task of obtaining epidemiological information is further complicated due to the fact that enterovirus infections are very common. It is estimated that over 20 million enterovirus infections occur yearly in the United States alone (Cabelli et al., 1982). Of these 20 million infections it is believed that one quarter to one half are due to infection with CVB. The relatively few outbreaks of illness resulting from poliovirus infections occurring in the U.S. is due to the active vaccination programs formatted for all persons born within the U.S. before schooling. Prevalence of specific serotypes of Coxsackie virus can vary based on the time of year as well as geographical location. Certain populations such as newborn children seem to develop the disease at higher rates. Factors such as physical stress are also associated with disease severity. Although these factors seem to be the trend, it is difficult to determine the at-risk population.

D. Viral Transmission

Polio and Coxsackie viruses exist in the environment. Viruses are found frequently in sewage and in sewage contaminated water. Viruses have also been isolated from stool samples, rectal swabs, CSF, throat swabs, nasal secretions and other body fluids with the exception of urine.

Viral shedding in stools occurs for up to 70 days and also can occur in respiratory secretions. Transmission also may occur via droplet spread as well as oral to oral route but this is more common with Coxsackie virus A infections. Coxsackie viruses can cause nosocomial infections and are commonly spread within a family setting as well as within institutions. The primary cause of nosocomial infection probably involves contamination after patient contact or by environmental contamination of the nasogastric tube. Swimming in sewage-polluted waters is also a source of transmission for both viruses. The most important preventative measures in this case are good hand washing after patient contact, and cohort isolation in the case of an outbreak.

E. Treatment

Coxsackie viruses are common causes of meningial symptoms but are rarely fatal. No antiviral therapy is available as yet for these types of enterovirus infections, and treatment is solely supportive. Chronic enterovirus infections have been treated with gamma immunoglobulin and in some cases this has seemed to control to the infection on a small scale. The wide use of gamma immunoglobulin in a clinical setting has not been evaluated, probably due to cost and complications. No vaccines exist against CVB viruses. Vaccination has been tested in animal models and has proven useful against the development of myocarditis and acute infection. Most people born in the U.S. receive vaccinations against poliovirus. This is reinforced by the World Health Organization's goal to globally eradicate the virus. Antisera is readily available against poliovirus though severe disease rarely occurs.

CHAPTER TWO

Materials and Methods

I. Sample Sites

Previous experiments involved screening for MAVA from waters on the west coast of Oahu (Fujioka and Loh, 1996). Freshwater sites were tested (Manoa stream), as were brackish water sites (Ala Wai canal). Several coastal sites were tested including Ala Moana beach, Pearl Harbor, Kuhio Beach and Magic island. All seawater sites displayed some MAVA activity. These sites were selected because their geographical location provide for frequent use for recreation as well as some sewage contamination via point source and preeminently non-point source permeation. The sites were also in close proximity to the most heavily populated areas of the island, which contributes to their popularity.

In the experiments described in this project, however, seawater samples were taken from the windward shores of Oahu (figure 1). It is necessary to add data from these sites so that a general survey of the waters surrounding the entire coastline of Oahu can be screened for MAVA activity. Data from these sites will indicate whether MAVA exist in waters on the windward side of the island.

Three sites were chosen along the windward coast of Oahu;

1. Hukilau Bay (Laie, HI) is primarily used moderately for recreation by swimmers and fishermen. There is a sewage treatment plant nearby (Laie/HRI Waste Water Treatment Plant (WWTP)) but sewage is treated by UV disinfection and the plant does not have an ocean outfall, but is used for irrigation.
2. Kahana Bay (Kahana Valley, HI) is very shallow and receives freshwater from the Kahana valley river, which drains the entire valley. The bay includes a small pier that is infrequently used for launching watercraft. The area also includes a frequently used camping area.
3. He'eia Pier (Kaneohe, HI) is used heavily for fishing, and many recreational fishing boats use the facility daily. A sewage pumping station is nearby (Ahuimanu) and this has an ocean outfall. The surrounding Kaneohe Bay has been the subject of much environmental scrutiny in the last 50 years. In 1951, Kaneohe Marine Corps Air Station built the first sewage collection station for the growing community. This system was detrimental to the ecology of the bay because it discharged practically untreated sewage into the bay until 1972. The City and County of Honolulu then built a municipal treatment plant for the Kaneohe area, which used a settling and chlorinating process to treat sewage before discharging it to the bay (Mokapu Peninsula Kailua, HI).

An average of 34.34 gallons per second was being discharged daily by 1972. It was then that a significant change was detected in the bay's ecology. This change was evident in decreasing diversity and displacement of corals and algae. Benthic organisms such as sea cucumbers and feather-duster worms began to dominate the population. Because of these ecological changes, the formation of the citizens group "Kaneohe bay in Crisis" took place (Banner and Baily, 1970).

Through the efforts of this group, and the Kaneohe community, a diversion for the sewage outfall was constructed. As of 1988, the small WWTP at Ahuimanu continued to discharge treated sewage into Kaneohe bay, with plans to connect to the nearby ocean outfall of Mokapu Peninsula in Kailua (Cox and Fan, 1973). Although progress has been made, there are still periodic breakdowns that can discharge sewage and thus, viruses into the bay (Tanimura, 1988).

II. Sample Collection

All coastal surface seawater samples were collected in sterile containers between February 2004 and April 2005, and were processed within 2-6 hours. Sampling conditions for each sample site were noted along with temperature, turbidity, salinity, weather, human activity (which was usually minimal), depth (0.5 meters) and distance from shore (2-3meters) where minimal), depth (0.5 meters) and distance from shore (2-3meters) where sample was taken. Samples from all collections were assayed in healthy BGMK cell cultures. Seawater samples were taken from the following sites: Hukilau bay, Kahana bay, and He'eia pier along the windward coast of Oahu, Hawaii (figure 1).

A 500ml sample of seawater at the surface was collected from each previously discussed sampling site and filtered through a sterile Gelman 0.45μm Metricel® filter twice, then followed by filtration through a sterile Gelman 0.22μm filter (figure 2). Five ml of the unfiltered water sample was then added to the 500ml of filtrate to provide a 100X dilution. This creates a dilute marine environment, which includes less microbial background. While previous experiments used only raw seawater (Fujioka et al., 1980, 1981, Fujioka and Loh, 1996) we used filtered seawater seeded with raw seawater to indicate whether seeding a filtered seawater sample would still produce MAVA activity. Purified poliovirus 1 (Sabin strain LSc2ab) of a known virus titer was added to the dilute seawater sample. A known titer of virus was also added to phosphate buffered saline (PBS) under the same conditions and used as a control. Each sample along with a seawater control, were stirred slowly for four days at room temperature (rt). Sub-samples were removed for testing at time 0, and days 1, 2, 3, and 4 and a penicillin/streptomycin antibiotic solution (10,000 U/ 10mg) was added upon removal to prevent further growth of other contaminating bacteria (figure 3) as done in previous experiments (Fujioka et al., 1981, Yoon, D.W. pers. comm.). These aliquots were further diluted from the original sample to 10^{-5} and each was assayed for virus by plaque assay on BGMK cell monolayers. After cytopathic effect (CPE) was observed, the cells were stained with a crystal violet formalin mixture (0.1%/10%). Plaques were counted and the PFU/ml determined (figure 4).

III. Virus Purification

Purified poliovirus 1 was prepared via standard picornavirus purification methods (Mahy and Kangro, 1996). Briefly, viral particles were released from infected cells via a freeze-thaw procedure (3 times), followed by centrifugation at 10,000rpm for 20 minutes to remove large cellular debris. PEG-6000 and NaCl was added to the supernatant, which was then incubated on ice. This mixture was then solubilized by stirring for one hour followed by overnight refrigeration to precipitate the virus. This method protects the virus particles against chemical and physical denaturation. The precipitate was collected the following day, and resuspended in TES buffer and aspirated through a 22-guage needle. PEG was then removed by centrifugation (10,000rpm for 4 minutes), at which point the virus is enriched, but not completely purified.

To further purify and band the virus, a cesium chloride gradient (5-40%) was prepared in a Beckman SW41 ultracentrifuge tube. The virus sample containing detergent 1% NP40 to free virus particles from any remaining membranes, was then layered on top of the gradient and centrifuged at 80,000xg at 4°C for 4 hours.

Virus bands visible (blue bands 2/3 down the gradient) after centrifugation were collected, pooled, and re-ultracentrifuged for 2 hours at 40,000 RPM. The virus pellet was resuspended in 5ml PBS buffer, and placed in an ultracentrifuge tube on top of a 60% sucrose cushion. CsCl was then removed by centrifugation of the virus mixture for 3 hours at 35,000 RPM. The virus was collected from the sucrose cushion using an 18 gauge needle, and titered in cell culture.

IV. Membrane Filtration

The concentration of fecal indicator bacteria was measured in each sample. The membrane filtration technique is widely used to monitoring natural waters for the presence of fecal indicator bacteria. From each sample site, 500ml of seawater was collected in sterile sample bottles and brought directly to the laboratory for processing. Ten ml of the sample was diluted in a buffer solution (34.0g KH_2PO_4, 1.25ml PBS(81.1g$MgCl_2$/1L ddH_2O)/ 500ml sterile dH20, pH7.2)yielding a 1:10 dilution (10^{-1}), after which it was serially diluted in a decimal series to (10^{-2}).

Sterile Gelman 0.45μm Metricel® (47mm) filters were used for all membrane filtration procedures. A UV sterilized filtration unit was prepared and connected to a vacuum source. All seawater dilutions (10^{-1}, 10^{-2} x25 ml) were filtered through this apparatus and the resulting membrane filters were placed on either mEM (m-Endo Medium 0333 DIFCO), which is selective for *enterococci*, or M-FC (Fecal Coliform 0677 DIFCO) medium, which encourages growth of fecal coliforms. Along with these dilutions, 25, 50 and 100ml of raw seawater from each site was also filtered in the same manner and the filters were incubated on the same media.

The fecal coliform media (M-FC agar) selects for fecal coliforms using an enriched lactose medium as well as an incubation temperature of 44.5°C. Because stable incubation temperature is essential, media and filter discs were sealed in snap tight petri dishes and whorl top® plastic disposable bags and submerged in a water bath. After a 24 hours, colonies of fecal coliforms distinguishable by their deep blue color were counted to yield the number of colonies 100/ml sample (EPA water quality standards).

The mE medium (containing nalidixic acid) is recommended for culturing *enterococci* in fresh and marine waters. Each filter from the filtering procedure described abovewas transferred to a culture plate containing medium, inverted, and incubated at 41°C for 48 hours. After incubation, colonies of *enterococci* appear red in color, and for further confirmation a substrate test was performed. Those filters displaying red colonies were transferred to EIA medium (Esculin Iron Agar 0488 DIFCO) and incubated for 20 minutes at 41°C. Confirmed *enterococcus* colonies developed a black precipitate where the bacteria on the filter contacted the medium. The concentration of colonies of *enterococci* was calculated by counting to yield the number of colonies 100/ml sample (EPA water quality standards).

V. Bacterial isolation and purification

From each seawater sample, a sub-sample was prepared that did not contain antibiotic solution (time 0 through day 4). Each sub-sample was diluted from the original stock to 10^{-5}. Each dilution was then streaked on Anderson agar plates (15mm). Colonies that grew from these dilutions were isolated and purified. Once a pure culture was achieved (streaking for isolation and growing out colonies in pure culture), each isolate was transferred to Anderson's broth media and enumerated using standard bacterial population counting methods (diluting and plating). Each isolate was then seeded into filtered seawater (0.22μm) to a known concentration and poliovirus virus of a known titer was added. These, along with a seawater control, were again stirred slowly for four days at room temperature. Sub-samples were measured for residual virus titer by testing in cell culture at time 0 hours, and times 1, 2, 3, and 4 days post inoculation. Penicillin/streptomycin antibiotic solution was added for the cell culture assay. These sub-samples were also further diluted from the original sample to 10^{-5} and assayed on BGMK cell monolayers. After CPE was observed, the cell cultures were stained with crystal violet. Plaques were counted and the number of PFU/ml were calculated to determine if anyantiviral activity was expressed.

Once a significant decrease in virus titer was observed (T90) by a pure microorganism, the bacterium was characterized by morphology, gram staining, catalase and oxidase production, and antibiotic susceptibility (Kirby-Bauer method). Each isolate that demonstrated MAVA activity was genetically characterized on the basis of its 16S rDNA sequence.

VI. 16s rDNA sequencing

DNA was extracted from bacterial isolates using a standard DNA extraction procedure (Dneasy Tissue Kit).

A fragment of the 16S rDNA gene was amplified using PCR using eubacterium specific primers 27f (5'AGAGTTTGATCCTGGCTCAG3') and 1522r (5'AAGGAGGTTATCCANCCRCA-3') (Suzuki and Giovannoni, 1995). The PCR conditions were the same for all reactions. Briefly, a final volume of 100μl (primers/template, 0.2mM dntp, 2.5u T*aq*) was used in an eppendorf mastercycler thermal cycler programmed with a hot start follwed by 35 cycles of denaturation at 96°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 3 min (Suzuki and Giovannoni, 1995)

The PCR products were purified (Qiagen PCR purification kit). DNA sequencing templates were prepared, and sequencing was performed in a commercial facility. Genetic comparisons wereedited and assembeled manually. The unknown marine bacterium was then provisionally identified by using comparisons made with GenBank.

VII. Antibiotic susceptibility testing

A bacterial isolate was grown in TSA broth for 48 hours. Fresh plates containing Mueller-Hinton agar were inoculated with the isolate by swabbing the surface with a cotton swab dipped into the bacterial culture. The surface of the agar was covered evenly by swabbing in three directions and then allowed to dry for 5 minutes.

Antibiotic discs (tetracycline 30mg, Ampicillin 10mg, and penicillin 10mg) were dispensed on to the media using an aseptic technique. Plates were inverted and incubated at 25°C for 18 hours. After incubation, the zone of inhibition was measured and recorded.

VIII. Characterization By API®20 NE

API®20 NE is a standardized micro-method used to identify non-enteric gram negative bacteria. Since isolate HE-1 was a gram-negative rod, we used this system for further characterization of the organism. The API®20 NE system uses 8 conventional tests and 12 assimilation tests to characterize an unknown organism (bioMerieux REF 20 050). The conventional test wells were inoculated with a bacterial suspension which reconstituted the test media. After incubation, color changes indicating the metabolism of the organism are noted for each test. The test wells were inoculated with a bacterial suspension (0.5ml) in a minimal medium and growth was observed if the strain was able to metabolize the single carbon source presented. Reactions in the API®20 NE test kit were recorded according to the manufacturers instructions. This method is not usually used for marine samples, but does provide some background information on the nature of non-enteric bacterial samples.

VIII. Gram Staining and Other Identification Tests

Gram staining was performed by standard methods for each pure isolate. Catalase and oxidase production were tested by adding reagent from DIFCO® Catalase reagent droppers to a smear of each pure isolate on a clean microscope slide and oxidase production was also tested by using

DIFCO® oxidase reagent droppers in a similar manner. Colony characteristics such as culture growth and morphology were also recorded.

IX. Millipore Amicon® Ultra-15 to Test for Active Microbial Products

In order to determine if the bacterium itself was responsible for virucidal activity, a Millipore Amicon® Ultra-15 device was used to concentrate all material in a bacterial suspension above the 10k molecular weight cut-off. Eight ml of a bacterial susupension of HE-1 isolate in Anderson's broth was added to the Millipore Amicon® Ultra-15 tube and centrifuged at 5k RPM for 25 minutes. The resulting supernatant is said to be "cell free" and will only contain those molecules smaller than 10k MW. The supernatant was added to filtered seawater along with a virus aliquot of a known titer, incubated with stirring for four days, and infectivity was assayed in cell culture. If any antiviral microbial products exist, virus survival would theoretically decrease.

X. Assay for the Effects of The Microbial Isolate on Coxsackie B2 Virus Infectivity

Isolates demonstrating antiviral activity against Poliovirus 1 were also tested against Coxsackie B2 virus. The qualifying isolate was seeded into filtered seawater (0.22μm) at a known concentration and Coxsackie B2 virus of a known titer was added. This, along with a seawater control, and a control containing PBS buffer and Coxsackie B2 virus, were stirred slowly for four days at room temperature. Sub-samples (5ml) were removed for testing on cell culture at time 0 hours, and 2, and 4 days post inoculation.

Penicillin/streptomycin antibiotic solution was added for cell culture assay. Ten fold dilutions were then made (10^{-1} 10^{-5}) and assayed on BGMK cell monolayers. When CPE was observed, infected cells were stained with crystal violet, plaques were counted, and PFU/ml determined.

CHAPTER THREE

Results and Discussion

I. Testing of Three Sample Sites for the Presence of MAVA

Seawater samples from all three sites were found to inactivate poliovirus type 1 at different rates (figure 7). Compared with the PBS control, all samples demonstrated various degrees of reduction of virus titer by day four (96 hours). Antiviral assay results from seawater samples from He'eia pier were compiled and geometrically averaged. Seawater samples from this site showed the highest reduction of viral infectivity with a viral titer of 5.8X103PFU/ml at 96 hours, which was significantly lower than that of the control with a viral titer of $5.8X10^{5}$PFU/ml at 96 hours. The mechanism responsible for two-log reduction of poliovirus infectivity remains to be elucidated. The present evidence makes this sampling site a good candidate for further study as well as one which may uncover many other MAVA isolates.

The results from the antiviral assay of seawater samples taken at Kahana bay were similarly compiled, geometrically averaged, and shown to have MAVA properties with a final viral titer of $2.9X10^{4}$PFU/ml at 96 hours, a

1.6 log reduction from the PBS control. This also indicated that there is a potential for finding and isolating MAVA's from this site.

The results from the third site, Hukilau Bay also revealed a smaller reduction in virus titer of $2.8X10^4$PFU/ml, a reduction of just 1.5 logs from the control.

On the basis of the preceding data, it can be concluded that screening for MAVA can be productive at all three sites. Because the highest antiviral activity appears to be associated with bacterial isolates sampled at He'eia pier, it is apparent that bacterial isolates that demonstrate antiviral properties may most likely be found there.

The autoclaving of seawater samples before the addition of active virus prevented the antiviral effects of the sample as reported in previous studies (Fujioka and Loh, 1996).

II. Testing of Three Sample Sites for the Presence of Fecal Indicator Bacteria

Questions have arisen involving whether or not viruses released into coastal marine waters can survive long enough and in high enough concentrations to cause illness in those people using these sites for recreational purposes (Girones et al. 1989). It has been found that increased risk for illness, particularly gastroenteritis, occurs more frequently in bathers than non-bathers (Fleisher et al., 1993). The infectious agents responsible for these illnesses are suspected to be viral, among them the enteroviruses. It is proven that these viruses are discharged in human feces which have been released via sewage contamination to recreational water used by swimmers (Cabelli et al., 1982). Results reported in previous studies suggests that bathing and swimming in sewage polluted waters results in an increased risk of incidences of gastroenteritis, respiratory ailments, as well as ear, eye, and skin ailments (Cabelli et al., 1982).

It has also been shown that the fecal indicator bacteria *Enterococcus* spp., showed the greatest correlation to gastrointestinal symptoms (Cabelli et al., 1982). Also, the frequency of gastrointestinal illness had a high degree of association with the distance from known sources of sewage contamination. Studies have shown that even low *enterococci* counts (10/100ml) were associated with significant disease rates of about 10 in 1,000 persons who displayed gastroenteritis symptoms (Cabelli et al., 1982). This indicates that swimming in even minimally sewage-polluted seawater (point-source) can provide a significant route of transmission for gastroenteritis agents.

US EPA Water quality standards for recreational waters (Fresh water and Marine waters) state that no coastal waters for recreational use should contain more than 35 CFU/100ml sample for *enterococci*, and 200 CFU/100ml sample for fecal coliforms (US EPA, 1986). The Hawaii state recommendations are more stringent, stating that coastal marine waters should not exceed 7 CFU/100ml for *enterococci* and 200 CFU/100ml for fecal coliform.

Results from screening for fecal indicator bacteria from all three sampling sites by standard membrane filtration methods are depicted in figure 13. The geometric mean of all samples from three seawater sampling sites showed that fecal coliform indicator counts were below the 200 CFU/100ml EPA standards for fecal coliform.

Samples from Kahana Bay and He'eia pier sites met the EPA standard for *enterococci* (35 CFU/100ml) but did not meet the Hawaii standard for *enterococci* (7 CFU/100ml) with counts at 11.2 and 14.9 CFU/100ml, respectively (figure 9). It is suspected that non-point source pollution contributes to such excessive counts at the Kahana Bay site. It was previously described that the bay provides an outlet for major streams in that area. It can also be inferred that in addition to non-point source contamination, He'eia Pier is affected by some point source infiltration (Ahuimanu WWTP). Again, this makes this particular site a good candidate for MAVA screening.

III. Marine Bacterial Isolation and Purification

Of the six isolates tested, not all demonstrated an antiviral effect on the infectivity of the virus. It was shown that four of the six isolates (HU-1, HU-2, KA-1, KA-3) all resulted in negligible effect on the virus titer (figure 8). The log reduction of all four of these isolates was higher at 90 hours than that of the control. Two individual isolates (HE-1 and KA-2), however, did have an antiviral effect on virus survival. The most significant being that of isolate HE-1, demonstrating a reduction in virus titer of 4 logs (99.99%) from the control at 96 hours. This purified isolate met the criteria for MAVA activity (inactivation of T90 at 96 hours) and its properties were further analyzed.

IV. Identification Via 16S rDNA Sequencing

On the baseis of 16S rDNA sequencing, HE-1 was found to be a *Marinomonas* sp., the first in the database that was isolated from Pacific waters. The organism was also determined to be a catalase positive, oxidase positive, gram negative rod, and formed concentric raised colonies with smooth margins. Colonies are yellowish in color and transparent. Cell morphology was examined by phase-contrast light microscopy of 24-hour old cultures grown on Anderson's agar plates.

VII. Antibiotic Susceptibility Testing

The control plate demonstrated normal growth, presenting an uninterrupted lawn of bacteria that was undisturbed. At eighteen hours post incubation, the zone of inhibition was visible in the plates containing Tetracycline (30mg) antibiotic disks and Ampicillin (10mg) antibiotic disks and measured at 1.7mm each. This confirms that the isolate is susceptible to both tetracycline and ampicillin.

VIII. MAVA Identification Using API®20 NE

Using the API®20 NE kit it was found that isolate HE-1 was positive for both β–glucosidase as well as β-galactosidase production. These results did not lead to a conclusive identification of the isolate according to the identification table provided.

VII. Millipore Amicon® Ultra-15 and Active Microbial Products

In order to determine if the antiviral properties of isolate HE-1 were attributable to the microorganism itself, or some cellular component, or metabolite produced by the bacterium, the supernatant recovered from a centrifuged and filtered aliquot of the isolate was tested. Inoculation of this supernatant with a known titer of poliovirus type 1 produced no significant effect on virus infectivity. At 96 hours post-inoculation the supernatant displayed a difference of only 0.06 logs from the control (figure 10). This demonstrated that the antiviral activity produced by the isolate must require some kind of intimate contact with the entire microorganism.

VIII. Assay for the Effects of Isolate HE-1 on Coxsackie B2 Virus Infectivity

The antiviral activity of the isolate on Coxsackie B2 virus infectivity was next examined. At 96 hours post-inoculation, the isolate reduced the virus titer by just 0.5 logs as compared with the titer of the control virus at 96 hours (figure 11). In this case, the isolate did not meet the qualification for MAVA activity (T90 at 96 hours).

IX. *Marinomonas* sp.

As previously indicated, the closest species identification of isolate HE-1 with the BLAST database revealed a *Marinomonas* sp. (97% confidence) that was isolated from the Black Sea (Ivanova et al. 2005). The genus *Marinomonas* was created to accommodate two misclassified marine species in 1984 (Ivanova et al. 2005). *M. mediterranea* obtained from the Mediterranean Sea in 2005 was found to have antibacterial properties against both gram positive and gram negative microorganisms (Lucas-Elio et al., 2004). The *M. mediterranea* produced a soluable protein, called marinocne that was responsible for the antibacterial activity.

Many anti-viral compounds have originated from the ocean or from organisms that reside in it. Antivirals such as Ara-A, and Ara-C for example, are all semi-synthetic compounds based on nucleosides isolated from a marine sponge (Donia and Hamann, 2003). It is likely that the true producers of these compounds are either of microbial origin or because of microbial interaction with the organism. This is plausible since large amounts of microorganisms are associated with the tissues of these marine organisms. Since several types of marine microorganisms may potentially have a role in antiviral treatment, it is imperative to continue this line of investigation. However, this goal will be difficult because very few marine bacteria are able to be cultivated under standard laboratory conditions.

CHAPTER FOUR

Summary and Conclusions

The results of these studies have met the overall goals and objectives of this project as shown by:

1. The isolation and identification of a marine antiviral agent or MAVA, from marine waters in the Hawaiian environment, which can be grown in pure culture. The MAVAs have of antiviral activities against poliovirus type-1 (Sabin LSc2ab), and coxsackie B2 virus (isolate).
2. The identified microorganism has been activity maintained in the laboratory and its antiviral properties remained effective even after the 6^{th} cultural passage.
3. Based upon 16S ribosomal DNA nucleotide sequences the MAVA has been provisionally identified as belonging to the *Marinomonas* sp., and represents the first of its kind to be documented in Hawaiian waters.

Possible applications for these anti-viral marine microorganisms include but are not limited to;

1. Control of disease transmission from sewage polluted waters

It has been reported that transmission of viruses to humans can occur through recreational use of sewage-polluted waters (Cabelli et al., 1982). Because pristine coastal areas are of great economic importance to our tourism industry here in Hawaii, any harmful effects occurring to natural use of this resource could be reason for economic devastation. If a resource such as the MAVA was applied directly for sewage treatment before discharge into the ocean, this would represent a natural biological control of viral contaminants in the ocean environment.

2. The active principle(s) of the MAVAs after isolation and identification have the potential to be used in the therapy and prophylaxis of viral illnesses in humans. If an antiviral compound, or the mechanism by which this microorganism inactivates virus could be revealed, we might have the means to treat in a safe and un-invasive manner those diseases of viral etiology. This type of therapy is becoming increasingly important as the need arises for alternatives to present treatments.

3. Preservation of island economics and environment

   A solution such as MAVA could prove ideal and cost-effective to the shellfish industry, which is especially sensitive to infectious agents. MAVA's could also prove to preserve the marine environment as used for a source of recreation by keeping our coastal waters free from dangerous pathogens released into the open ocean via sewage contaminants. MAVA's would also be important in preventing the intake of human viruses by filter-feeding sea animals such as mussels and clams and again reduce the risk of viral transmission to humans.

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APPENDIX

Figures

Figure 1

Map of Oahu Island Including Sampling Sites

SOEST: Map of Oahu, Hawaii showing streams and tributaries. School of Ocean and Earth Technology, UHM, Honolulu, Hawaii 96822

Figure 2

Sample Collection and Filtration

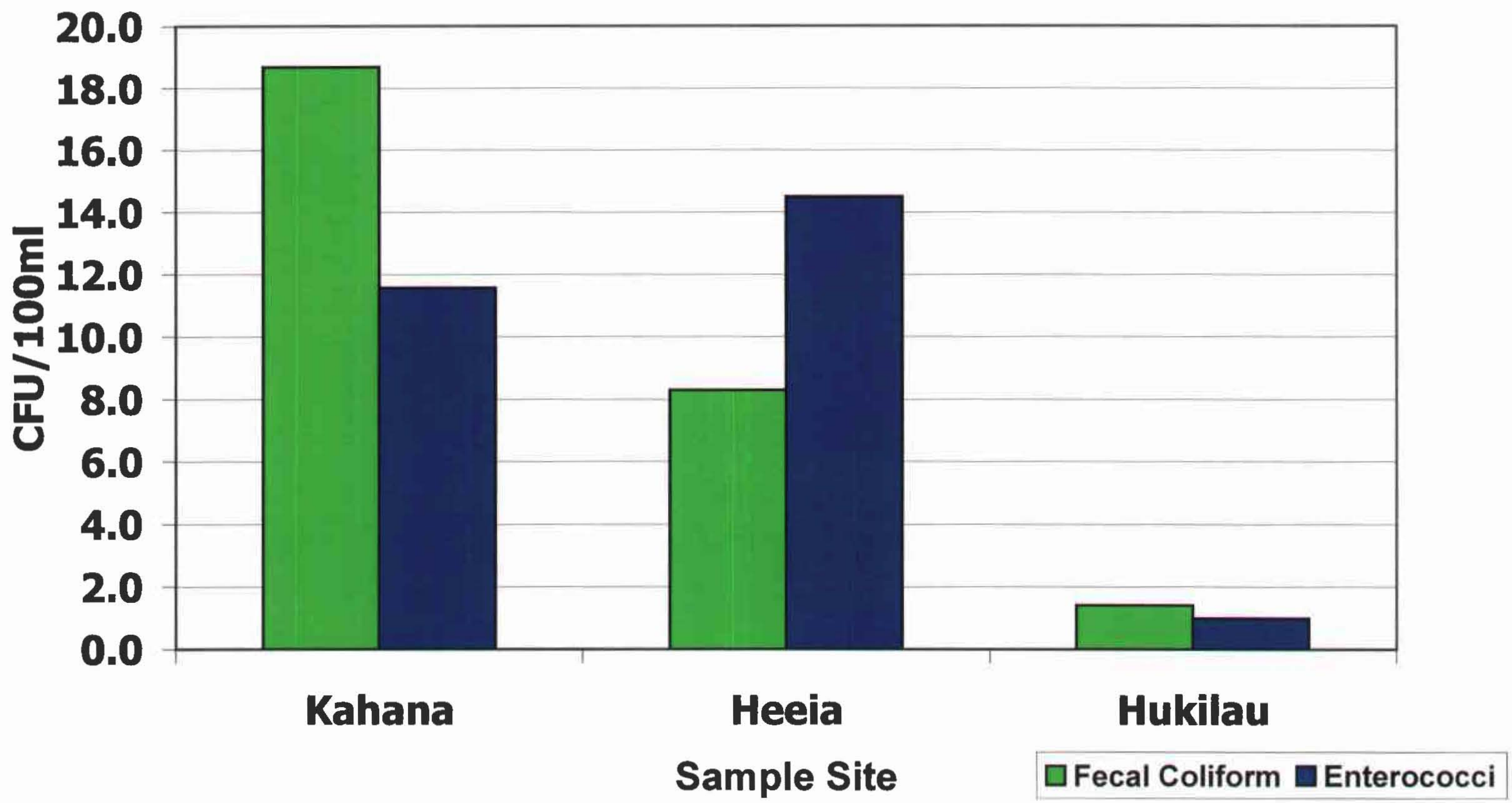


Figure 3

Assay for MAVA in Seawater from Sample Sites

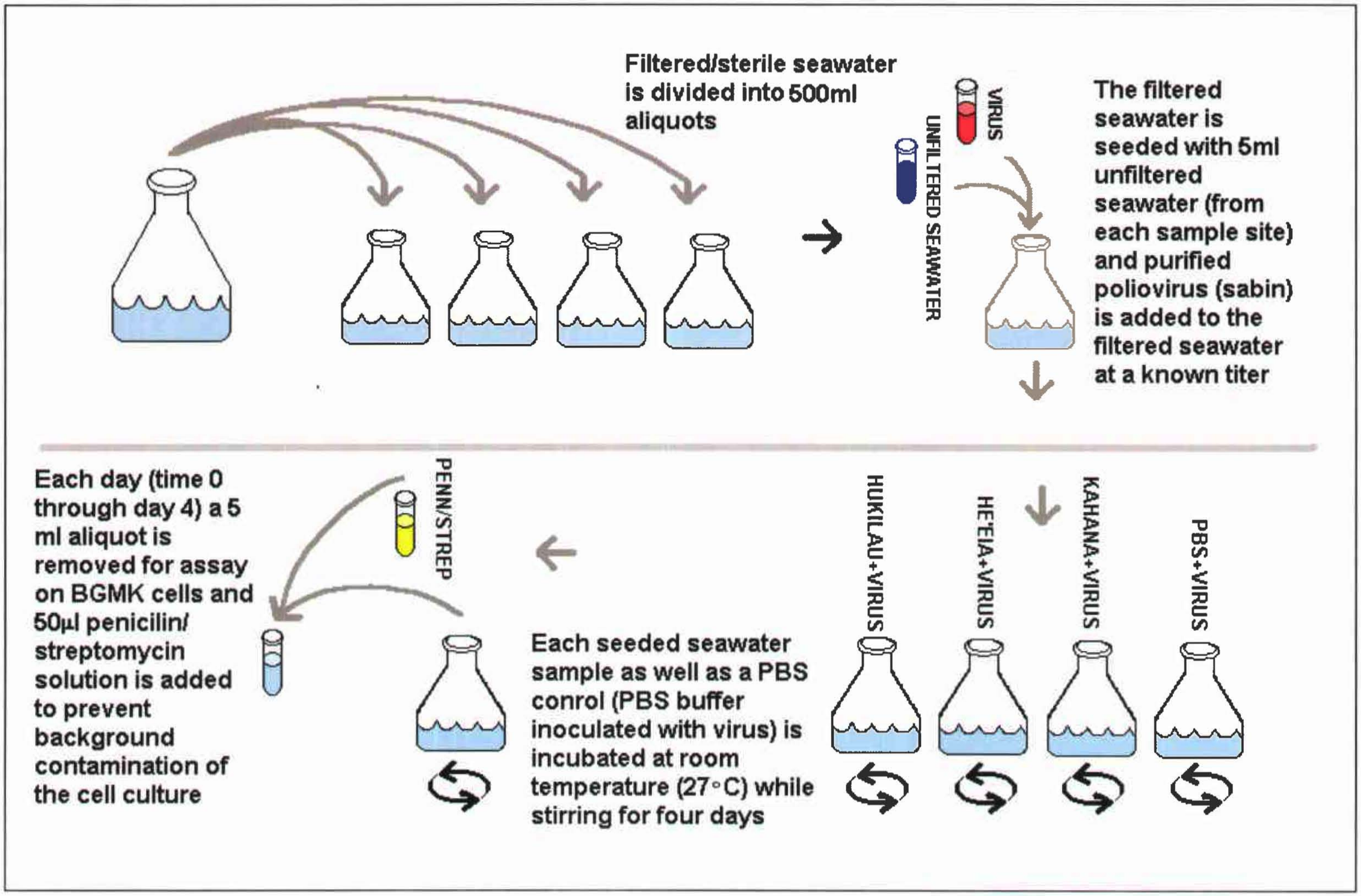


Figure 4

Cell Culture Assay for MAVA

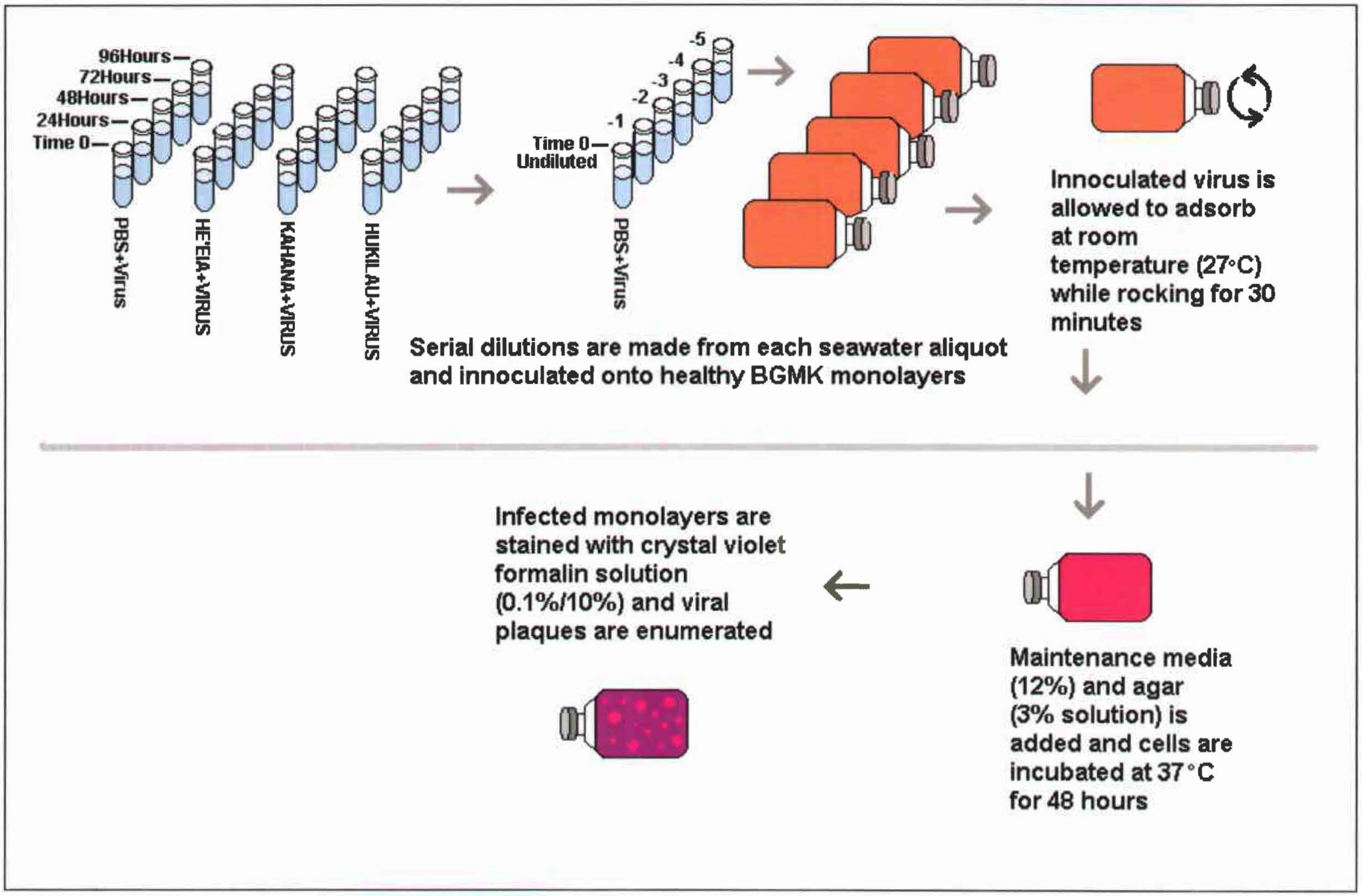


Figure 5

Bacterial Screening for MAVA

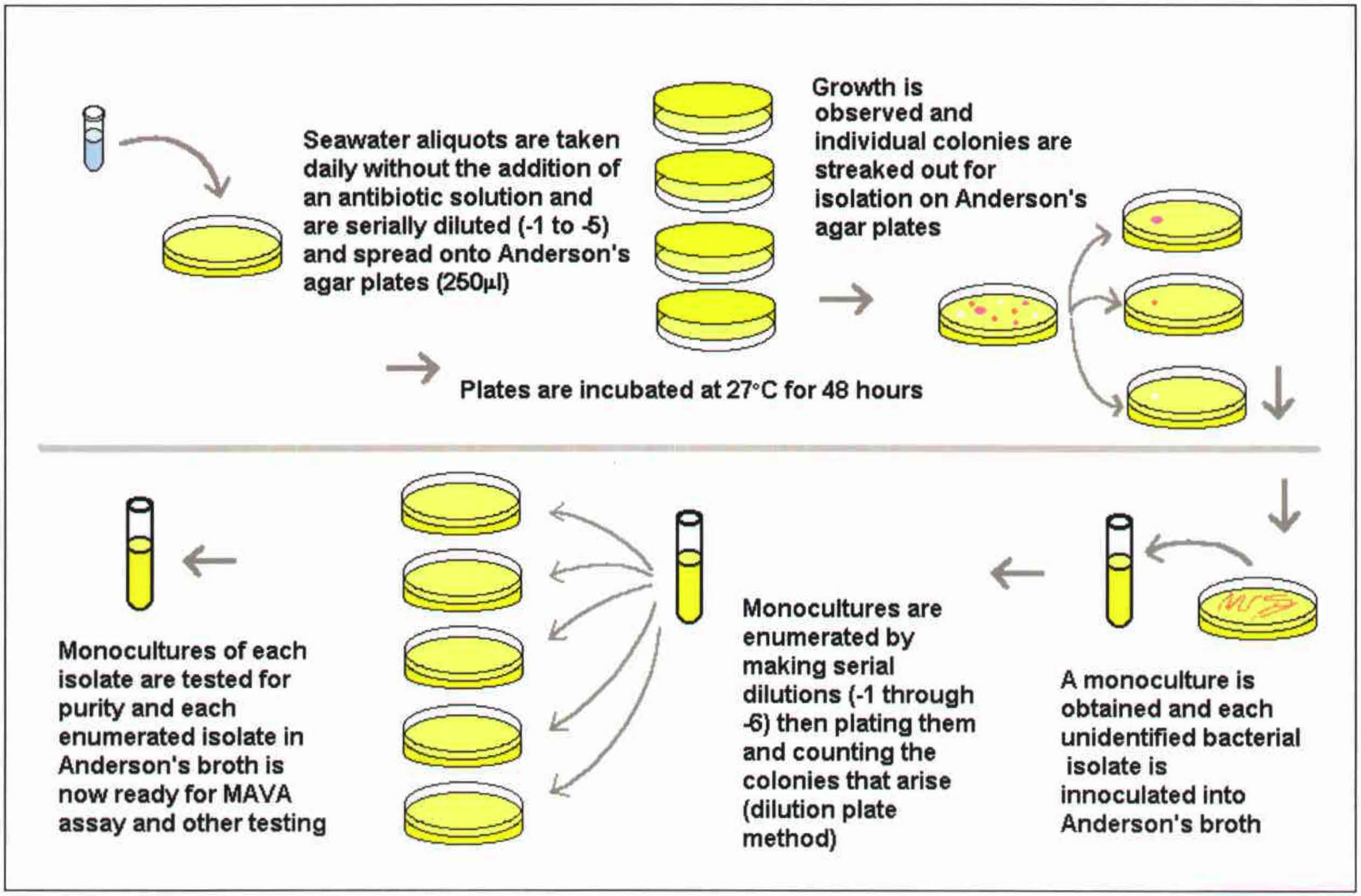


Figure 6

Assay for MAVA Using Purified Isolates

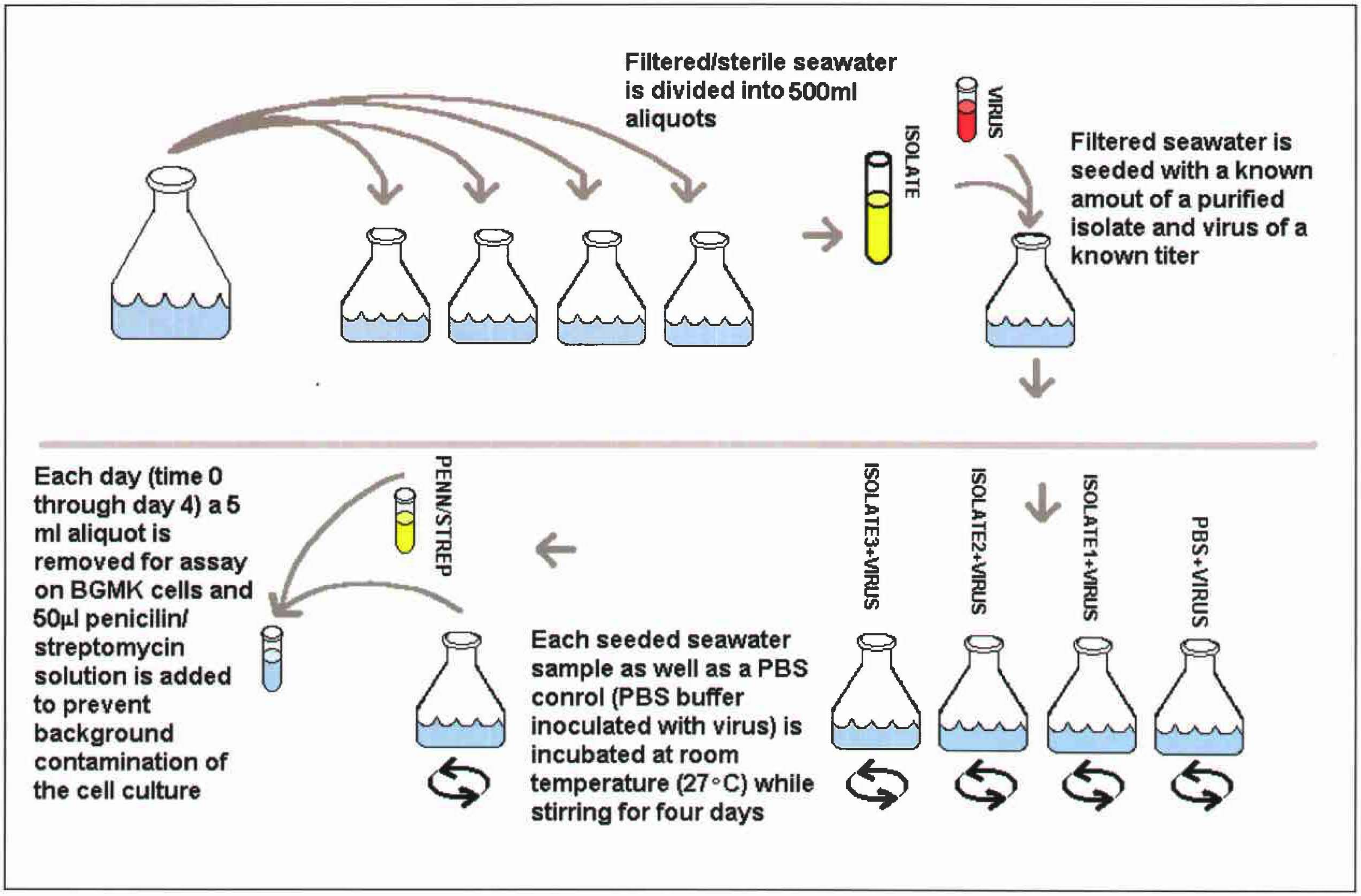
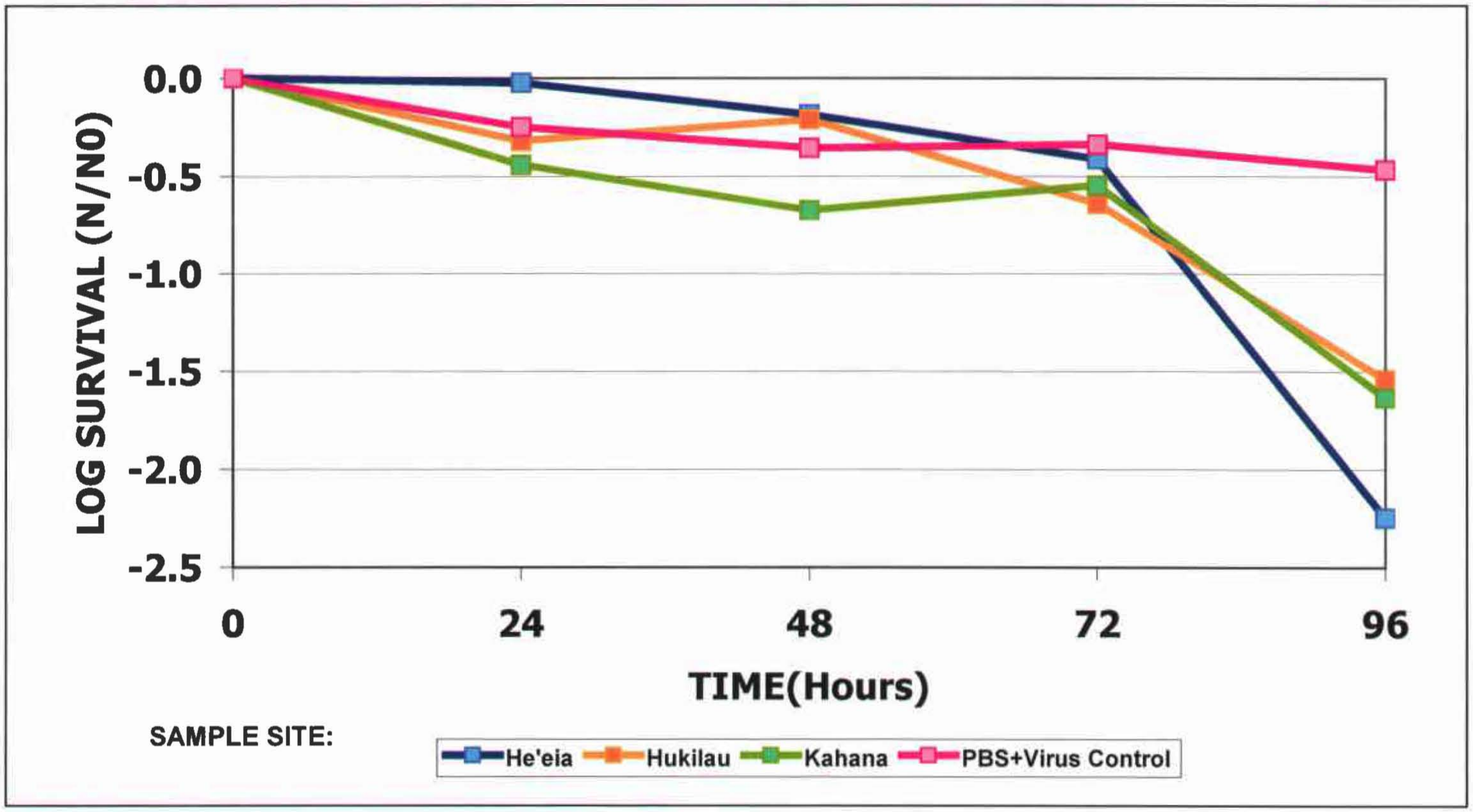


Figure 7

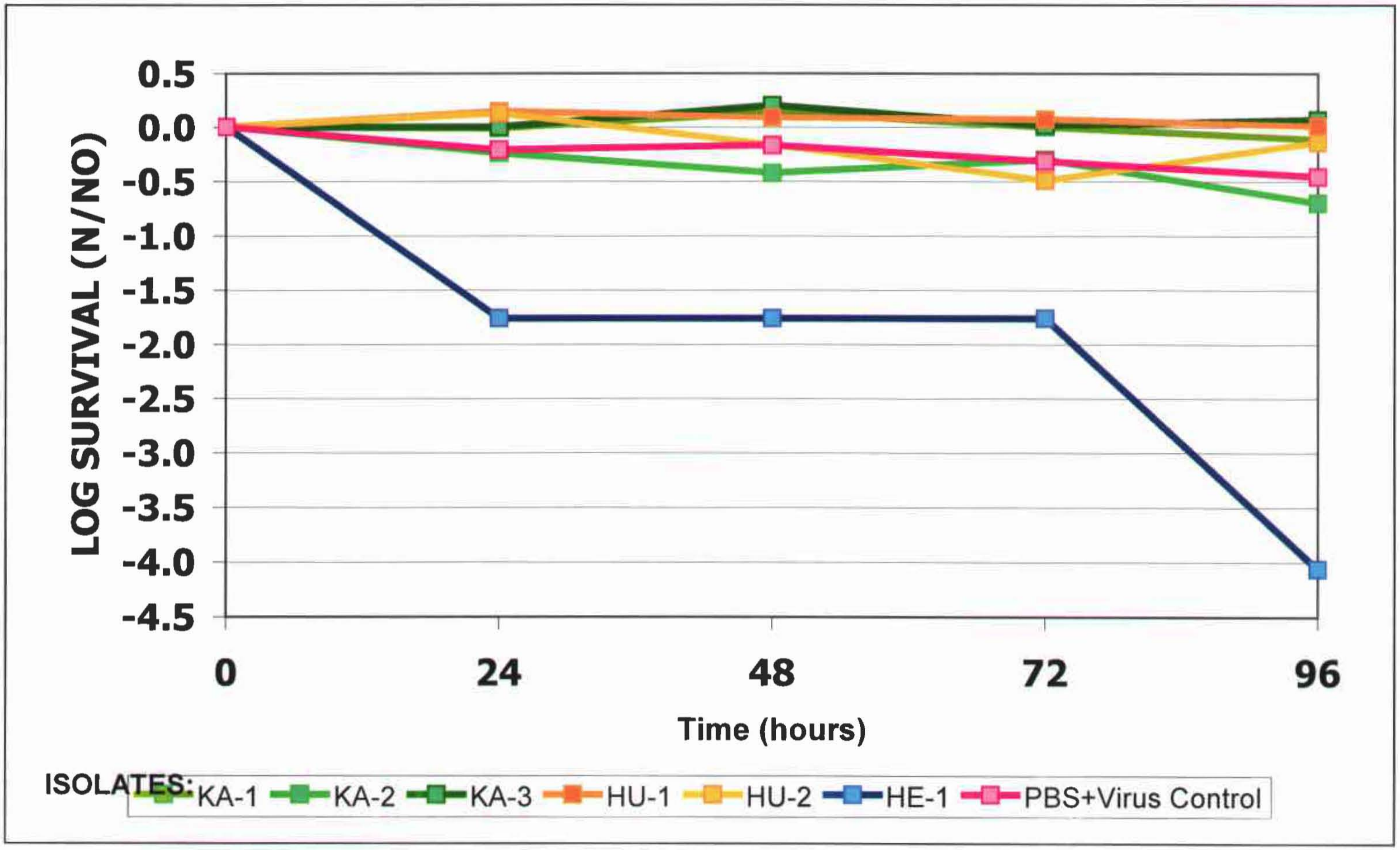
Effect of Oahu Coastal Seawater on Poliovirus Type 1 Survival



N=8

Figure 8

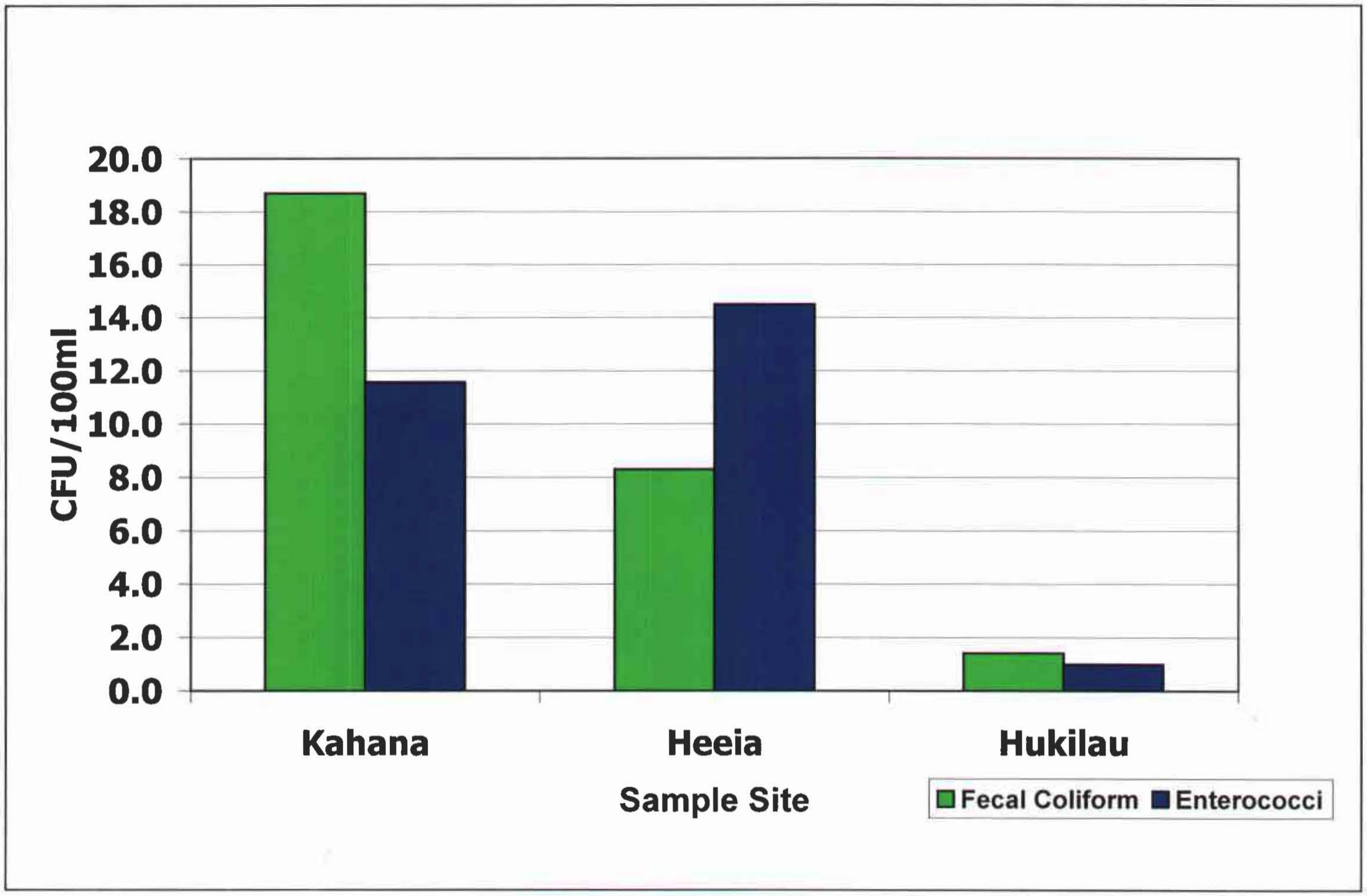
Effect of Purified Microbial Isolates from Oahu Seawater on Poliovirus Type 1 Survival



N=2

Figure 9

Presence of Fecal Indicators in Seawater from Three Sample Sites on Windward Oahu



N=8

Figure 10

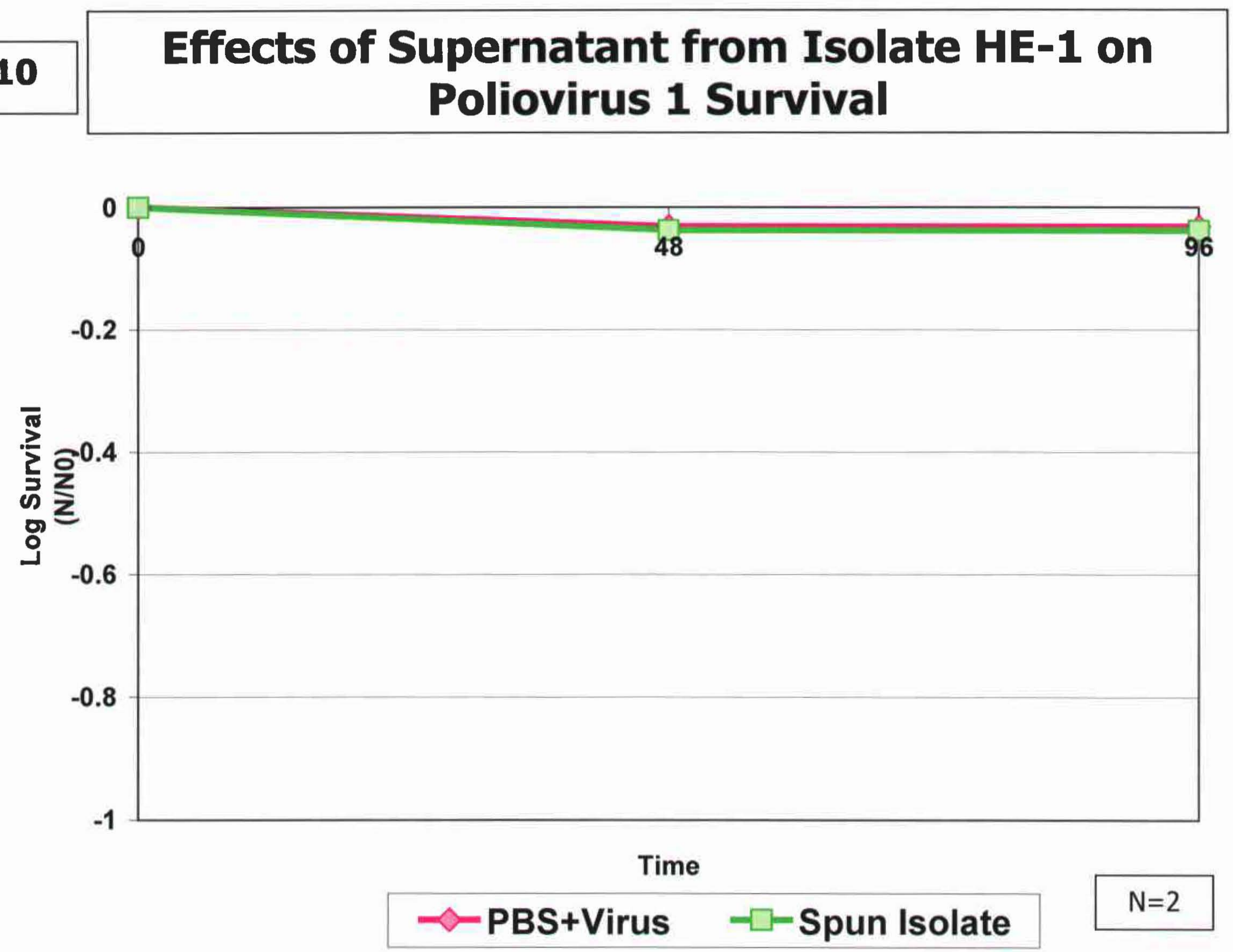
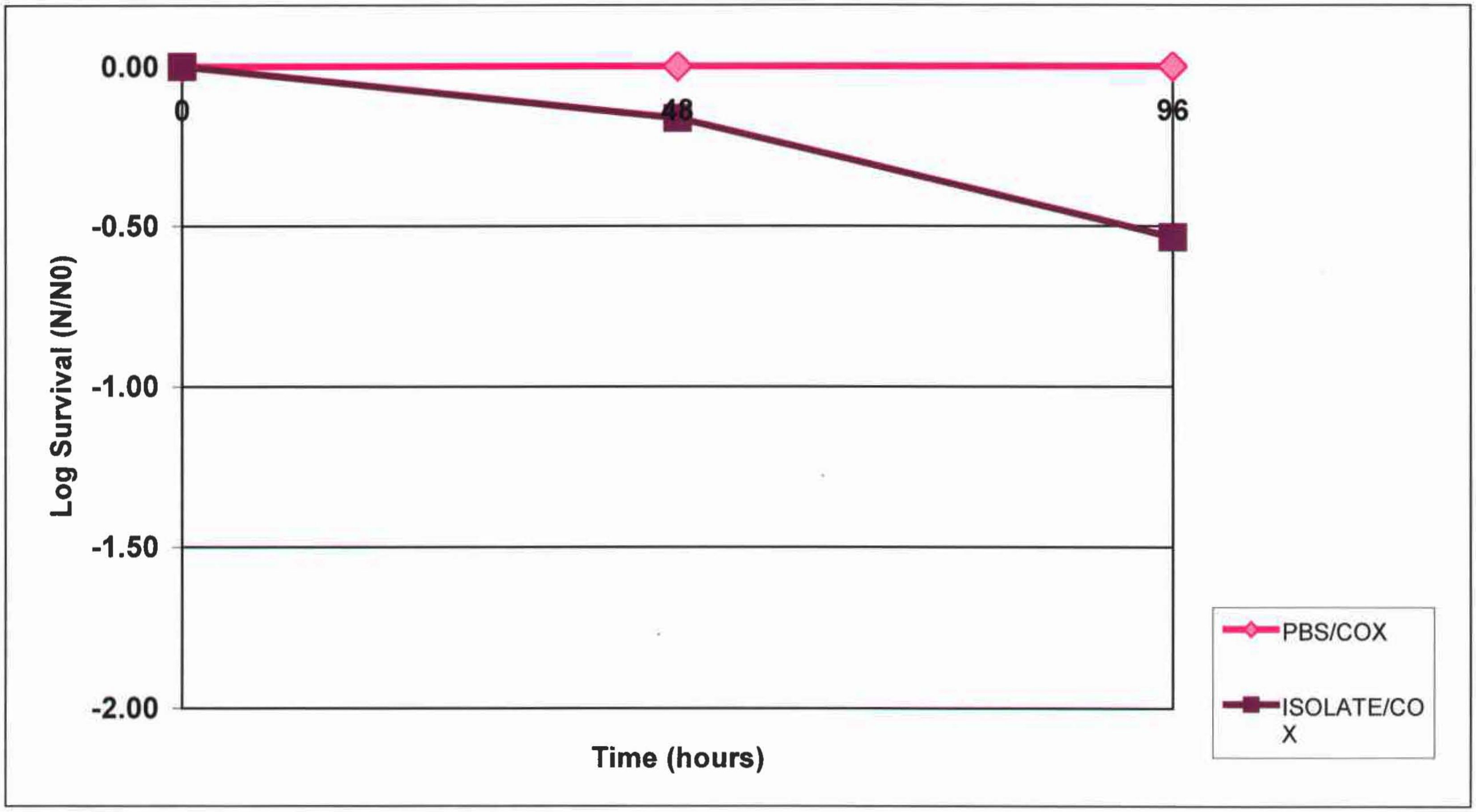

Effects of Supernatant from Isolate HE-1 on Poliovirus 1 Survival
0
-0.2
-0.4
-0.6
-0.8
-1
Log Survival (N/N0)
0
48
96
Time
PBS+Virus
Spun Isolate
N=2

Figure 11

Effect of Microbial Isolate HE-1 on Coxsackie B2 Virus Survival



N=2