Development of an Autonomous Sample Filtration and Archival device for Aquatic Microbiology: Assessment of Potential Preservation Buffers

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
Marine Science
Biological Oceanography

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2011

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ABSTRACT

Ongoing advances in molecular techniques have certainly increased our understanding of marine microbial ecology. However, much of this knowledge could be enhanced by increased sampling frequency. This work presents the first development phase of a small autonomous *in situ* water sampling device. The system will enable the autonomous collection of water samples to be preserved for subsequent downstream laboratory based molecular techniques. The Sample Filtration and Archiving (SaFA) system is being designed to collect and filter 24 user defined time-stamped water samples of between ~200-500 ml. During this phase, simulated SaFA tests using several commercial and noncommercial buffers were conducted to evaluate their capacity to preserve and stabilize DNA from natural seawater samples. The best performers of the commercially available buffers were PrepProtect, RNALater, Ethanol, and Isopropanol. These buffers were effective for 3 to 8 days without appreciable DNA degradation and significant microbial community changes. Only one non-commercial buffer (HRNAL pH 5.2) was efficient up to 5 days however this formulation represents a homemade version of the commercially available RNALater. These results indicate that future deployment of SaFA could be limited for periods up to a week under the condition of our tests. To facilitate adoption by end users, the SaFA is being constructed using predominately commercially available parts, and all information relating to its construction and operation will be downloadable under an open source license. The open source nature of the SaFA will enable end users to evolve the instrument by providing feedback on new designs or applications.

RESUMEN

Los continuos avances en técnicas moleculares han aumentado indudablemente nuestra comprensión de la ecología microbiana marina. Sin embargo, gran parte de este conocimiento podría ser mejorado aumentando la frecuencia de los muestreos. Este trabajo presenta la primera fase de desarrollo de un pequeño dispositivo autónomo de muestreo de aguas *in situ*. El sistema permitirá la recogida autónoma de muestras de agua y su conservación para posteriores análisis moleculares en el laboratorio. El sistema de Filtrado y Archivo de muestras, SaFA (siglas en inglés) está siendo diseñado para recoger y filtrar 24 muestras de entre ~ 200 y 500 ml con una frecuencia de muestreo delimitada por el usuario. Durante esta fase, se realizaron pruebas simulando el sistema SaFA con varios tampones comerciales y no comerciales cuya capacidad de conservar y estabilizar el ADN de muestras de agua de mar fue evaluada. Los mejores tampones comerciales fueron PrepProtect, RNAlater, etanol e isopropanol. Estos tampones fueron efectivos preservando el ADN de 3 a 8 días sin degradación significativa, y sin cambios en la comunidad microbiana. Sólo un amortiguador no comercial (HRNAL pH 5.2) ha sido eficaz hasta cinco días. Sin embargo, esta fórmula representa una versión casera de tampón comercial RNAlater. Estos resultados indican que el futuro uso de SaFA, podría limitarse a un período de hasta una semana, en las condiciones de estas pruebas. Para facilitar el uso del sistema, SaFA se está construyendo utilizando principalmente piezas comerciales, y toda la información relativa a su construcción y operación se podrá descargar bajo una licencia de código abierto. La naturaleza de licencia de código abierto del SaFA permitirá a los usuarios realizar cambios en el instrumento, proporcionando información sobre los nuevos diseños o aplicaciones.

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Para empezar un gran proyecto, hace falta valentía. Para terminar un gran proyecto, hace falta perseverancia…

Desconocido

ACKNOWLEDGEMENTS

During the development of my graduate studies at the University of Puerto Rico- Mayagüez several persons and institutions collaborated directly and indirectly with my research. Without their support it would be impossible for me to finish my work. That is why I wish to dedicate this section to recognize their support.

I want to start expressing a sincere acknowledgement to my advisor, Dr. Matthew Smith because he gave me the opportunity to research under his guidance and supervision. I received motivation; encouragement and support from him during all my studies. With him, I had the opportunity to present at international and national conferences to share my ideas and research to the scientific community. I also want to thank the support and knowledge I received from Dr. Schizas and Dr. Otero through the completion of my work. To the Mechanical Engineering Department, UPRM, Dr. Pedro Quintero, Esteban Suárez, Tania S. Cordero, Andrés Nolasco, Angel Gomez and Mike Hall from Q-Proto in Florida for sharing knowledge and help during the development of the instrument. To the Marine Science Department and Isla Magueyes's staff, especially to Nebo, Zulma and Taty thank you. Also a special thanks to Tania Lado Insua, Dania M. Rodríguez, Karen González, and Dr. Mark Brown for the help provided in the preparation of this manuscript. Special thanks to McLane Laboratories and Biotium Inc. to grant permissions to use their illustrations.

At last, but the most important I would like to thank God for being my strength. Also to my family and beloved husband, for their unconditional support, inspiration and love. Without them I couldn't have done it.

This research was supported from NSF Award # 0852787 providing the funding and the resources for the development of this research.

Table of Contents

Table List

Tables **Page**

Figure List

Figures **Page**

Chapter 1. Introduction to marine instrumentation and molecular microbiology

1.1 Motivation

The evolution of the field of microbial ecology has undoubtedly been accelerated by advances in technology. From the development of the microscope and culturing techniques to the application of molecular based methods and the most recent advances in high throughput sequencing, the microbial ecologist has experienced exponential growth in the number and type of tools that can be used to study of microbes and the role they play in the environment. Similarly, advances in automated sensing technologies (AST's) that can make observations in real-time or near real-time are enabling aquatic microbial ecologists to couple microbial structure, function with associated environmental observations on temporal scales that are more relevant to microbial processes (Shade et al., 2009). While AST's that make rapid physical and chemical measurements are robust and commercially available. The next frontier and challenge will be to develop autonomous sensors capable of performing molecular analysis that can approach the resolution of their sensors that measure abiotic parameters. A small number of researchers are approaching this challenge, however the molecular biological sensors they have developed are inherently complex, have high production and running costs, and are limited by the types of assays that they can perform. Additionally, the complex operational procedures for system set up, deployment, and retrieval, restricts their broad scale use. The aim of the research described here is to develop a small "open source", autonomous *in situ* sampling and archival device that will enable autonomous high resolution sampling of aquatic environments to be performed *in situ*. The device will therefore fill a much needed niche, until fully autonomous molecular biosensors become commercial reality. The proposed instrument, the Sample Filtration and Archiving (SaFA) system is designed to collect and filter 24 user defined time-stamped water samples of between ~200-500 ml over a period of 3 days. It is proposed that the captured biological material will be stabilized with a preservative buffer solution and following retrieval will be available for downstream laboratory processing.

1.2. Approaches to water sampling and analysis

One of the major limitations to the scope and scale of investigations into the microbial ecology of aquatic systems is the sampling regime. A major bottle neck in obtaining high resolution water samples is limited due to inadequate personnel requirements. Traditional sampling approaches require high personnel requirements and therefore potentially limit the scale of microbial ecology studies particularly during dangerous or inconvenient sampling periods. Conversely, automated technological approaches to sample acquisition have typically focused on the collection of zoo- and phyto- plankton.

1.2.1 Traditional manual sampling strategies

In most studies that investigate pelagic microbiology, the sampling of water is typically performed by a person who captures discrete water samples, with the retrieved samples typically being filtered on site or stored for subsequent filtration in the laboratory. For surface sample collection, water can simply be collected using a bucket or bottle. Alternatively, there have been many methods used to sample water from different depths in the water column include the use of pumps to feed water from different depths (Rusch et al., 2007) and the construction of segmented pipe samplers (Sutherland et al., 1992). However, for samples taken at different depths in the water column, the most commonly used device is the Niskin bottle (Figure 1.1). The basic design

of the Niskin bottle is an open plastic tube, at each end of the tube there is an endcap that are connected by a stretched rubber or elastic cord. When the Niskin bottle is being deployed the endcaps are held open by plastic cords that are linked to a release mechanism. The Niskin bottle is attached to a line and lowered into the water, when the Niskin bottle reaches the desired depth a small weight that encircles the line called a "messenger" is released. When the messenger reaches the release mechanism, the endcaps are released and seal the openings of the tube. In some applications, Niskin bottles can be strung together to obtain samples at different depths. In this case a second messenger is positioned below the Niskin bottle, when the first messenger triggers the releases mechanism it also releases the second messenger, which then triggers the next Niskin bottle in the line.

Figure 1.1. A Niskin bottle being deployed from the side of a boat.
Reproduced under public domain release. Source: NOAA and
http://en.wikipedia.org/wiki/File:Niskin-bottle.jpg (7 March 2011).

1.2.2 Automated sampling technologies

While traditional sampling approaches such as observed in Niskin Bottle sampling require high personnel requirements which potentially limit the scale of microbial ecology studies. The concept of automated sample acquisition is not new, with the first designs and use of automated samplers for the collection of zoo- and phyto- plankton being reported in the 1930's (Hardy, 1935; Hardy, 1936). Since that time various designs for the acquisition of zoo- and phyto- plankton been developed and applied to marine and fresh water studies (O'Hara, 1984; Lewis and Heckl, 1991; Reid et al., 2003). More recently, instruments for the autonomous acquisition of water zoo- and phyto- plankton samples have become commercially available. Probably a leading manufacturer of such instruments is McLane Research Laboratories which produce 4 different automated systems (Figure 1.2) capable of collecting and storing discrete water samples. The different product lines are configured to collect anywhere from single to 24 independent samples. Of these three systems (the phytoplankton sampler, zooplankton sampler

and large volume water transfer system (Morrison et al., 2000) are designed to undertake water filtration, and are predominantly aimed at zoo- or phyto- plankton fractions of the water column. The Remote Access sampler (Honda and Watanabe, 2007) (Figure 1.2B) is acquire grab samples which are held in Teldar Bags. Of the systems manufactured by McLane Research Laboratories, the Phytoplankton sampler (PPS) (Figure 1.2 A) is the most similar to the instrument tested here. The PPS is configured to acquire and filter 24 samples (up to 10 L) through GFF 47 mm filters. The system allows for the integration of optional fixative reservoirs. While the instrument is designed to filter and potentially fix phytoplankton no data are available on the system's ability to sample smaller fractions of the marine microbial community (e.g., Bacteria and Archaea) and subsequently preserve the samples for molecular analysis.

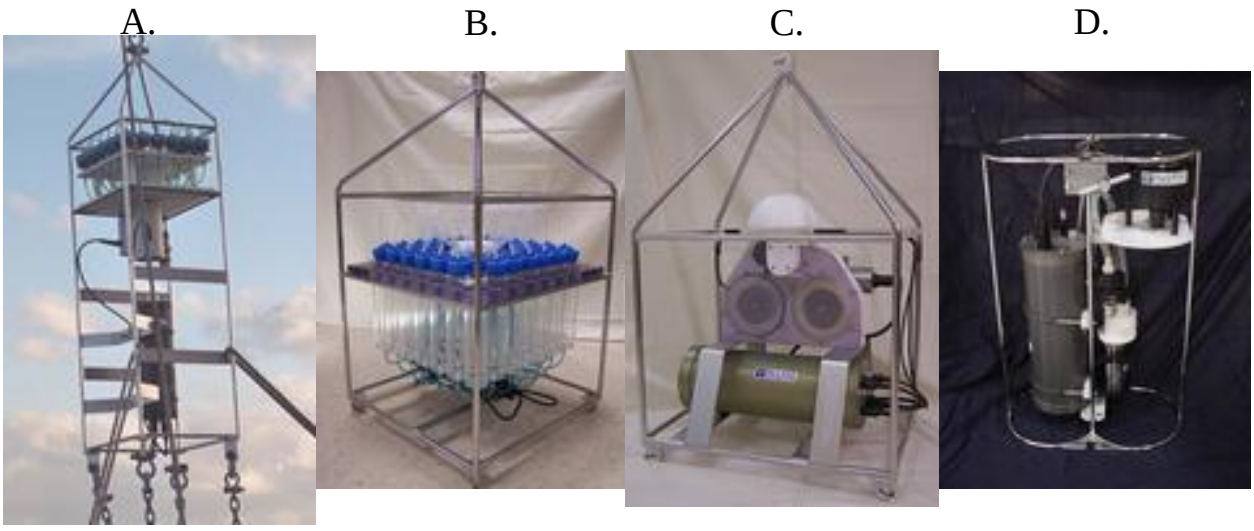


Figure 1.2. Different autonomous in situ sample acquisition systems manufactured by McLane Research Laboratories. A. The Phytoplankton Sampler (PPS). B. Remote Access Sampler (RAS). C. ZooPlankton Sampler (ZPS). D. Large volume Water Transfer System (WTS-LV). Reproduced with permission from McLane Research Laboratories, source www.mclanelabs.com/product-type/samplers (7 March 2011).

1.2.3 *In situ* sample analysis

Currently, optical detection is the most common approach that in situ instruments use for biological detection in the environment. For example, in situ optical sensors that measure pigment (e.g., chlorophyll, phycocyanin, phycoerythrin) concentrations via fluorescence spectra are now commercially available (e.g., YSI, WET labs, etc.) and commonplace in the aquatic sciences (Paul et al., 2007). A number of emerging sensor technologies that combine flow cytometry, imagine analysis or spectrometry to analyse the optical characteristics of bulk water samples or to identify or enumerate particles that pass across a particular detector. For example, commercially available FlowCam from Fluid Imaging Technologies (http://www.fluidimaging.com/) is capable of imaging phyto- or zoo-plankton using flow cytometry principals combined with detection using microscopy and digital photography. A similar approach is being undertaken by researchers at the University of South Florida who are developing a system called the Shadowed Image Particle Profiling Evaluation Recorder

(SIPPER) (Remsen et al., 2004) which also uses flow cytometry principals with detection using a line scan camera. Other optical based devices include an underwater flow cytometer called the FlowCytobot. The FlowCytobt was developed at the Woods Hole Oceanography Institution and has been used to monitor picoplankton populations in situ (Olson et al., 2003). Researchers at Mote Marine Laboratories have developed another class of optical instrument to detect the presence of *Karenia brevis* (red tide forming dinoflagellate in Florida). The instrument called the BrevBuster analyzes the absorbance of a water sample along a waveguide capilary and calculates a similarity index, characteristic of *K. brevis* (Robbins et al., 2006). Although these optical techniques are relatively mature and are routinely used in aquatic sciences, they lack the ability to distinguish microbial groups at resolutions relevant to most microbial ecologists (e.g., species level or higher), and except from the FlowCam, they are not readily accessible to the general scientific community.

The incorporation of molecular biological techniques into *in situ* marine sensors is a current goal because they offer sensitive detection, enumeration and identification of microbes. To our knowledge, only two *in situ* systems that employ genetic analysis have been applied to the aquatic sciences. Both systems share common attributes such as: robotic-like mechanical operations and use of syringe pumps to sample water. However the two systems differ in their operation, capabilities and genetic techniques employed.

The Environmental Sample Processor (ESP) developed by Dr. Chris Scholin and co-workers at MBARI (www.mbari.org/microbial/esp) is the most developed and robust molecular based sensor, and has recently undergone commercialisation through Spyglass Biosecurity, Inc. (http://www.spyglassbio.com/products/autonomous_platforms.shtml (accessed 8 March 2011) and McLane Research Laboratories (www.mclanelabs.com/product-type/samplers (accessed 8 March 2011)). The ESP performs a number of molecular analyses in situ, based on custom reaction "pucks" with molecular analysis performed using sandwich hybridization techniques on specialized probe arrays printed on reinforced nitrocellulose filters. The ESP has been successfully applied in the marine environment to detect *Pseudonitzschia australis* (Greenfield et al., 2006), and invertebrate larvae including *Carcinus maenas, Mytilus* sp., *Balanus* sp. and the polychaetes species *Osedax* sp. and *Ophelia* sp. (Jones et al., 2008). The ESP is capable of sample archival and downstream laboratory interrogation through storage of the filtration pucks. While the ESP has been deployed for prolonged periods with success, its size and weight (Figure, 1.3) potentially require specialized deployment equipment for use by researchers.

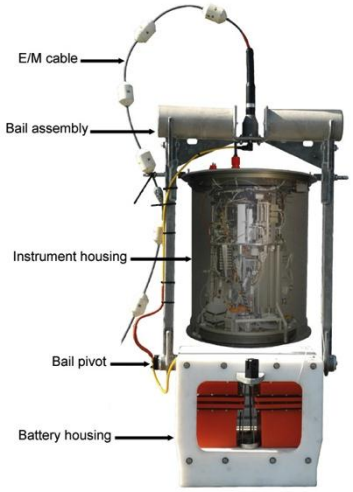


Figure 1.3. Image of the ESP highlighting the components of the system used in deployment of the instrument. The assembly weighs >370 kg (>800 lbs), but air trapped in the housing makes it sufficiently buoyant, when anchored. Reproduced with permission from McLane Research Laboratories, source www.mclanelabs.com/product-type/samplers (7 March 2011)..

` The second biological sensor platform, the Autonomous Microbial Genosensor (AMG) (Fig. 1.4) is under development by the University of South Florida's College of Marine Science (http://www.marine.usf.edu/systems/?q=amg). The AMG is the first microbiological detection buoy to be designed based upon nucleic acid amplification (Nucleic Acid Sequence-Based Amplification - NASBA). The core operation of the AMG resides in processing individual (30 ml) water samples being sequentially passed through 2 interacting offset rotating wheels. The first wheel contains custom filter/RNA purification columns and is responsible for sample filtration, cell lysis, RNA purification/concentration and sub-sampling of the eluted RNA. The second wheel contains 200 µl PCR tubes that hold the eluted nucleic acid and stores freeze-dried amplification chemistry. Additionally this wheel interacts with a heated fluorometer that performs and detects RNA amplification. Currently, capacity of the AMG limits the number of samples that can be processed to 12, including 4 negative controls that check for system contamination. In principle the AMG can be tailored to many different microbial targets by changing the primer/probe and cell lysis protocols. However, as the filter/purification columns used in the AMG are constructed from dismantled RNeasy columns of unknown pore size, it is unknown if the AMG is sited to effectively capturing bacterial or archaeal targets. In its current configuration, the AMG is detecting *Karenia brevis*. The AMG is approximately 1.5 m high and 20.5 cm in diameter and weighs approximately 40 kg. Therefore, compared to the ESP, it is more amenable to routine deployment from small craft using limited numbers of personnel. However, its overall design and current configuration has a number of inherent limitations that are limiting its widespread application including: it is difficult to calibrate and set up for deployment, it contains a number of custom parts that are not readily available (e.g., NASBA chemistry) or that require the user to manufacture them (e.g., sample filtration and extraction columns); sealing and stability of the lyophilized NASBA chemistry; and mechanical alignment issues between robotic parts (e.g., injector and wheel alignment). Finally, since all liquid processed by the AMG is currently held inside the unit, it is limited to taking small sample volumes.

While the AMG is still at the prototype stage, the basic system design has been licensed and is currently being marketed by Bioplex Technologies Inc. Florida, USA as an automated sampler preservation and archival system for chemical, biological (microbes and larvae) or particulate matter. However there does not appear to be any peer reviewed literature or manufacturer supplied data on sample preservation performance for subsequent molecular or microbiological analysis.

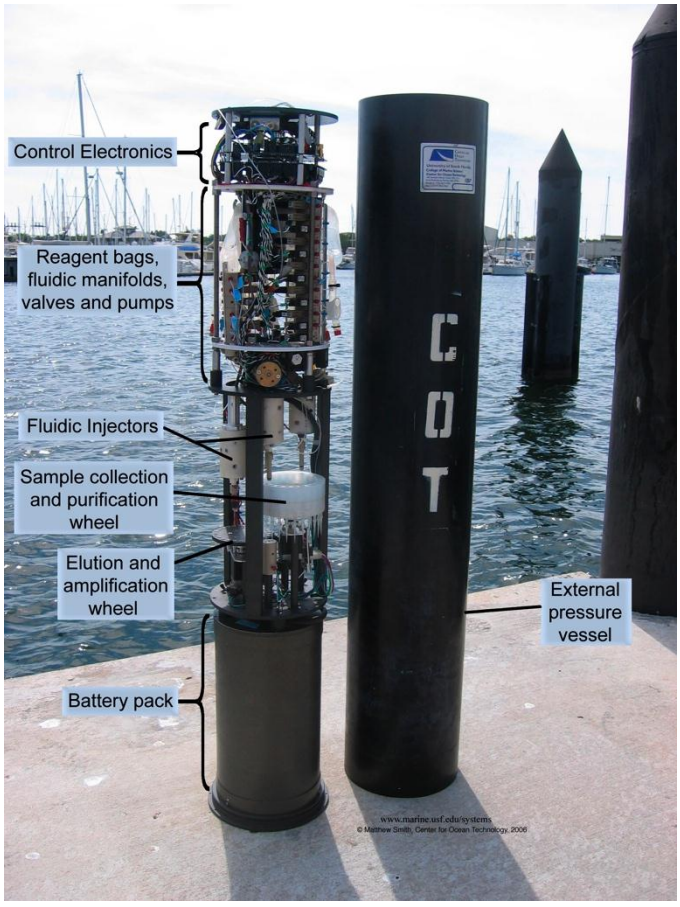


Figure 1.4. The Autonomous Microbial Genosensor. Image courtesy of Matthew Smith

1.3 Molecular Analysis of Marine Microbial Communities

Over 30 years ago, marine microbial ecologists were quick to use and apply the emerging field of culture independent molecular techniques to investigate the structure, composition and evolutionary relationships of marine microbial communities (DeLong, 2005). The development and application of molecular based approaches such as DNA sequence analysis, quickly expanded our ability to investigate the "great plate count anomaly" (Staley and Konopka, 1985). The pioneering work using rRNA sequencing to unravel evolutionary relationships (Woese, 1987) and the composition of microbial community assemblages (Pace 1997; Olson et al., 2003), was the seed to modern molecular approaches genomic, transcriptomic and high throughput sequencing methods (DeLong, 2005; Sogin et al., 2006; Vila-Costa et al., 2010). While a comprehensive review of current molecular methods is outside the scope of this thesis a number

of excellent reviews exist that focus on modern approaches that use molecular techniques to study marine microbial ecology (Doney et al., 2004; DeLong, 2005; Poretsky et al., 2009). Undoubtedly, the phrase "A revolution is occurring in biology" at the opening on Dr. Carl Woese's seminal paper on bacterial evolution (Woese, 1987), is just as fitting now as it was then. A brief overview on the methods used in this study is outlined.

1.3.1 The Polymerase Chain Reaction (PCR)

Perhaps the largest advance to the field of molecular microbiology was the development of the Polymerase Chain Reaction (PCR) by Kary Mullis (Saiki et al., 1985), which provided the ability to amplify small amounts of DNA *in vitro*. In the initial embodiment of PCR, the Klenlow fragment of *Escherichia coli* DNA polymerase I was used in conjunction with gene specific primers, and deoxynucleotide triphosphates (dNTP's) to copy and amplify a segment from the human β-globin gene (Saiki et al., 1985). Under this strategy, the enzymatic exponential amplification of DNA occurred under repeated temperature cycles of denaturation, primer annealing and extension. One major problem with the method was the degradation of the Klenlow fragment under the high denaturation temperatures, which required the continued addition of fresh enzyme at each extension cycle. However, the method was improved to overcome this in 1988, by using a thermostable DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* (Saiki et al., 1988). While there have been subsequent variations in PCR methodology developed, this much simplified method has remained the basic mechanism for all PCR protocols ever since, and today remains the basis of the many molecular applications. The general PCR principal (Figure 1.5) identifies and targets a specific DNA sequence. The reactions typically contain a buffer, dNTP's, thermostable DNA polymerase and target primers held in a final volume. Subsequent predetermined thermal cycling phases enable the extension and amplification of the target sequence. It must be noted that many more types of PCR have been developed. This includes techniques for the analysis of RNA by adding a Reverse Transcription (RT) step prior to the PCR protocol (RT-PCR) (Chelly et al., 1988). Additionally since the development of the PCR reaction a number of alternative amplification methodologies have been developed, that include strand displacement amplification (SDA), ligase chain reaction (LCR), and nucleic acid sequence-based amplification (NASBA) and rolling circle amplification (RCA). While these techniques are out of the scope of this study, these new techniques offer alternative approaches to molecular detection to the microbial ecologist, here two derivatives of PCR used in this study are discussed in further detail. However the reader is pointed to an excellent review on the advantages and disadvantages of these various amplification methodologies (Schweitzer and Kingsmore, 2001).

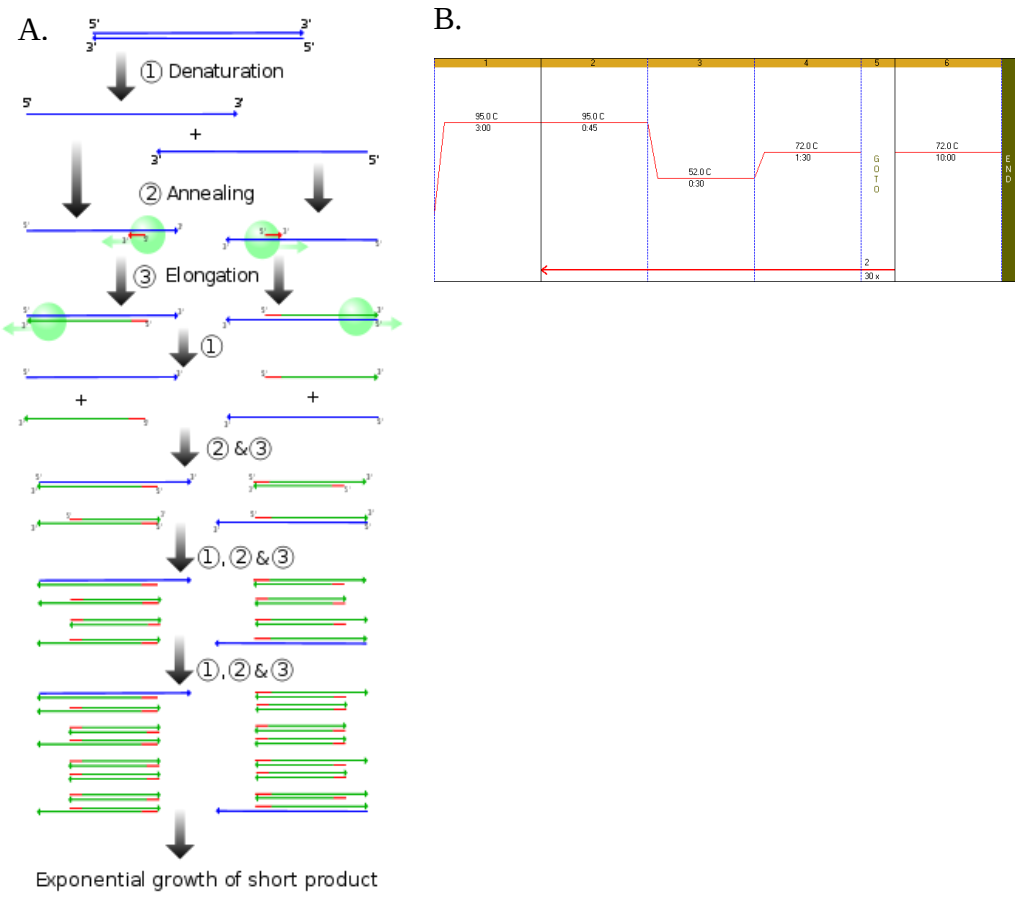


Figure 1.5. Schematic overview of the PCR reaction. A. The reaction proceeds through sequential cycles of denaturation, annealing and extension. The denaturation phase melts the double stranded DNA template (blue lines) forming singe stands. The annealing phase enables short oligonucleotide primers (red arrows) to bind to complementary regions on the DNA strands. The extension phase enables the thermostable DNA polymerase (green circles) to synthesize a new DNA strand (green line) in the 5' to 3' direction that is complementary to the DNA template. Upon completion of the extension phase. Subsequent cycles of denaturation, annealing and extension are performed where the newly synthesized DNA can form the template for primer binding and DNA synthesis. B. An example thermocycling profile for PCR. Figure 1 A Reproduced under global usage agreement, source: http://en.wikipedia.org/wiki/File:PCR.svg#globalusage (14 March 2011).

1.3.2 Quantitative Real Time PCR

Traditionally, the detection and analysis of PCR amplification was performed, at the completion of the PCR protocol by visualization of the DNA products by electrophoretic separation and staining with a DNA intercalating dye (e.g., ethidium bromide) or by labeled probes (e.g., Southern blotting). More recently, methods for quantitative real time PCR (qPCR) has become common place to amplify and quantify gene abundance and expression from environmental and clinical samples. These new qPCR methods have the advantage over the traditional end-point techniques in that detection is performed at the completion of each PCR

cycle in real time. The most common form of qPCR relies on the measurement of fluorescence emitted from a reporter molecule contained in the reaction; however other methods using electrochemical detection have also been used (Fang et al., 2009). Fluorescent reporter molecule types fall into 2 broad categories, namely probes and intercalating dyes. The most common probe based detection methodology is based around hydrolysis probes which are also known as TaqMan probes (Figure 1.6) (Holland et al., 1991). Quantitative PCR using hydrolysis probes involves the use of a 5' fluorescently labeled oligonucleotide probe and a non extendable quencher at the 3' end, the probe is designed to hybridize to one strand of the denatured target gene. During the PCR reaction the probe anneals to form a double stranded molecule. At the extension stage of the PCR reaction the 5' to 3' exonuclease activity of the DNA dependent polymerase (e.g., Taq Polymerase) degrades the probe and releases the fluorophore into the reaction solution. As the fluorophore is no longer in close proximity to the quencher, it emits a higher fluorescence than fluorophore bound to the probe.

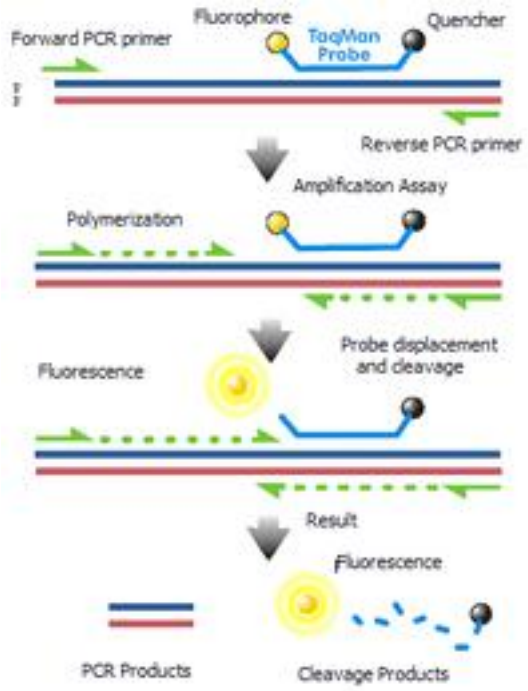


Figure 1.6. Operation of a qPCR reaction using hydrolysis probes.
Reproduced from wikipedia.org under public domain release
http://en.wikipedia.org/wiki/File:Taqman.png (22 March 2011).

Another common method to monitor the PCR amplification through the measure of fluorescence is the use of molecular beacons (Tyagi and Kramer 1996). A molecular beacon consists of a short oligonucleotide sequence (20-30 bases) that is complementary to the target sequence of the gene of interest. At both the 3' and 5' ends short self complementary sequences (6-8 bases) are placed so that the molecule forms a hairpin loop structure (Figure 1.7). In addition to the short self complementary regions a fluorophore and a quencher are placed at each end of the molecule. When the probe is free in solution the molecular beacon is in the hairpin-loop structure and the fluorophore is held close to the quencher. In this case little fluorescence is

emitted. As the amplification reaction proceeds and the amount of target gene sequences increases, the molecular beacon can hybridize to its target sequence. When this occurs, the fluorophore is separated from the quencher and the fluorescence signal increases. Unlike TaqMan PCR the molecular beacon is not degraded in the reaction as detection occurs at the end of the annealing step rather than at the end of the extension step.

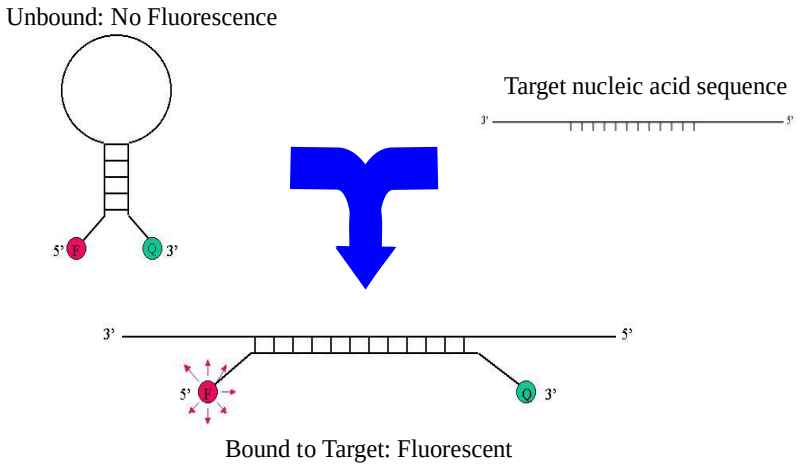


Figure 1.7. Operation of molecular beacon based detection. In the unbound conformation, the fluorophore (F) is positioned close to the quencher (Q) and little fluorescence is emitted. Upon hybridization, the molecular beacon changes conformation and separates the fluorophore from the quencher enabling the emission of fluorescence. Figure adapted from (Tyagi and Kramer, 1996).

Methods that rely on the use of intercalating dyes use a reporter molecule that is able to bind to double stranded DNA. In their unbound state, the dye displays little fluorescence, however, when the molecules form a dye-DNA complex they become fluorescent. A number of intercalating dyes have been applied to qPCR, the most commonly used are SYBR green and more recently EvaGreen (Khan et al., 2011). The main advantage of using DNA binding dyes in qPCR over probes based approaches is the reduction in cost. However they are limited by their ability to quantify the amplification of multiple gene targets in a single reaction. Similarly probe based approaches can be more specific to particular gene targets (Khan et al., 2011). However, the dye based approach enables melt curve characteristics of the amplified product to be obtained.

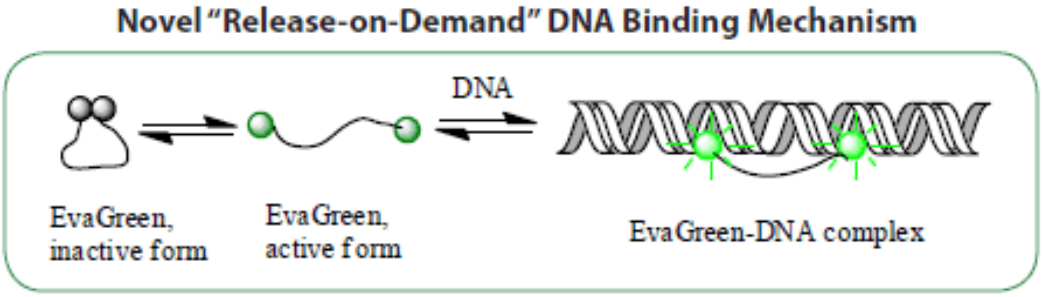


Figure 1.8. Example of the operation of the intercalating DNA dye EvaGreen. Reproduced with permission from Biotium Inc., source: http://www.biotium.com/product/product_info/Newproduct/EvaGreen.asp (accessed 22 March 2011).

Melt curve analysis is performed by examining the fluorescence properties of the amplified product over a temperature gradient. As the thermal denaturation characteristics of the PCR amplicons are dependent on both DNA sequence composition and amplicon length the melt curve characteristics can be informative. Changes in melt characteristics can highlight nonspecific binding of primers, the accumulation of primer dimers, or small differences in amplicon sequence composition or length. More recently advances in instrumentation and intercalating dyes has enabled the development of High Resolution Melt (HRM) Curve analysis. HRM operates on the same principle as melt curve analysis however it is much more sensitive and has been applied to mutation and genotyping studies (Witter et al., 2003).

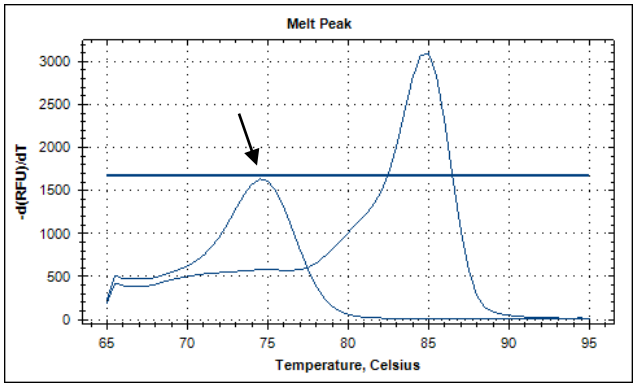


Figure 1.9. Example of a melt curve analysis performed on a Bio Rad CFX 96 real time thermocycler. The plot shows various scenarios for characterizing primer dimers or DNA with differing sequence/length. The arrow points to the primer dimer, the second peak is the target amplicon.

Despite the type of fluorescent reporter used in the reaction (i.e., probes or intercalating dyes), the output from the qPCR reactions has the same characteristics. The fluorescence signal emitted from the qPCR reaction is directly proportional to the amount of fluorescent reporter that has interacted with the accumulating PCR product. In the case of the hybridization probes, the fluorescence signal is a result of the release of the reporter molecule into solution, in other probe

based chemistries (e.g., molecular beacons) or intercalating dyes it is as a result of the reporter molecule directly bound to the DNA. Therefore the plotting of the fluorescent signal (y-axis) in respect to the cycle number (x-axis) will yield a characteristic amplification plot (Figure 1.10A). The amplification plots in Figure 1.10 highlight the different phases that occur in the qPCR reaction. The region defined as the baseline occurs in the initial cycles of the reaction where DNA concentrations are low and there is little fluorescence emitted, this background signal is due in part to the small amounts of fluorescence emitted from the reporter molecules, reaction reagents and plastic tubes as well as instrumentation noise (Bustin et al.,2009). The baseline for the reactions enables the calculation of the threshold value which will be used in quantification. As the PCR reaction proceeds and amplification product is synthesized, an increase in fluoresce is observed at this stage the exponential growth of product is best observed as a semi log plot (Figure 1.10B). As the fluorescence increases and crosses the threshold value, the fractional point that the reaction crosses the threshold value is defined as the Cycle threshold (C_t) value. The C_t value enables the quantification of the input DNA or relative changes in target abundances to be calculated. As there is inconsistency nomenclature to describe the C_t value with crossing point (Cp), and take-off point (TOP) also currently used in the literature, Bustin et al., (2009) have proposed that the term quantification cycle (Cq) be used which is in agreement with the RDML (Real-Time PCR Data Markup Language) data standard (http:// www.rdml.org). In this thesis, the nomenclature of Cq will be used. The linear and plateau stages occur as the reaction reaches product inhibition or limiting reagents.

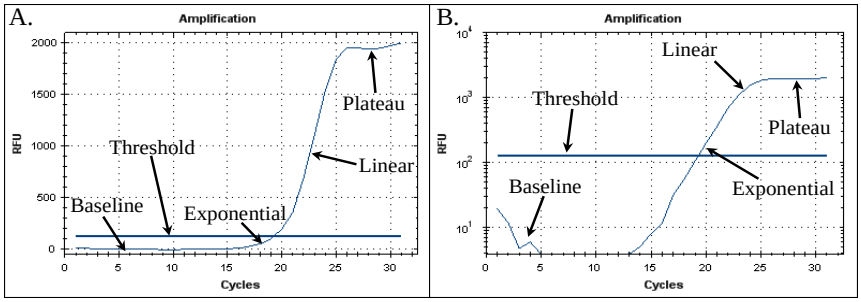


Figure 1.10. Example of real time qPCR amplification plots observed on a Bio Rad CFX 96 real time thermocycler. The various stages of the reaction are highlighted including the reaction baseline, exponential, linear and plateau. The C(q) value is determined at the intersection of the amplification curve with the threshold line. A. Standard plot of relative fluorescence intensity (RFU) against cycle number. B. Semi-log plot of RFU against cycle time.

1.3.3 DNA Finger printing techniques

For the microbial ecologist, the use of DNA finger printing methods has enabled the efficient and cost effective analysis of microbial community diversity, particularly from large numbers of samples (Brown et al., 2005). Numerous DNA fingerprinting methods have been developed, that rely on numerous intrinsic properties of an organisms DNA, and these methods can be divided into exploiting the broad properties of:

- The presence of restriction endonuclease sites (e.g., Restriction Fragment Length Polymorphism (RFLP) (Botstein et al., 1980), Terminal RFLP (T-RFLP) (Avaniss-Aghajani et al., 1994).

- The denaturation properties of the DNA (e.g., Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993), Temperature Gradient Gel Electrophoresis (TGGE) (Rosenbaum and Riesner, 1987)).
- The PCR amplification characteristics of the DNA (e.g., Random Amplified Polymorphic DNA (RAPD) (Williams et al., 1990), Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999).

Unlike clone library construction and subsequent DNA sequencing that can provide a specific taxonomic assignment of particular organisms in a microbial assemblage, community DNA finger printing techniques typically only enable the overall complexity of the microbial community or changes in community composition to be examined. However some methods such as T-RFLP and ARISA can provide tentative taxonomic assignments (Brown et al., 2005). Similarly, while DNA fingerprinting techniques offer a considerable time and cost advantage over clone library analysis, the different approaches have various advantages and disadvantages. For example, methods such as DGGE and TGGE use the electrophoretic migration of DNA molecules through a denaturing polyacrylamide gel. The denaturant can be a chemical or temperature gradient and it forces a mobility shift in the DNA as it transitions from double stranded to single stranded at its melting point in the denaturing gradient. As the melting point is sequence specific, the technique can resolve sequences with as little as a single base pair difference (Fischer and Lerman, 1983). However typically the techniques are limited to small DNA fragment sizes, additionally especially in the case of chemical denaturing gradients can be hard to reproduce.

More recent approaches have taken to adapt the automated sequencing capillary electrophoresis technology to perform molecular fingerprinting enabling higher throughput for to be undertaken. Two popular methods that use this approach are ARISA and T-RFLP. Both of these methods rely on PCR amplification of a specific gene target with a primer that is fluorescently labeled. Unlike ARISA, the T-RFLP method requires post amplification digestion with restriction enzymes prior to being loaded onto a capillary sequencer. DNA size fractionation occurs in the sequencer and the lengths of the DNA size fractions are measured from the fluorescent signal emitted from the labeled primer incorporated into the DNA during PCR amplification. As the ARISA technique was used in this study the following section will outline its operation.

1.3.3.4 Automated Ribosomal Intergenic Spacer Analysis (ARISA)

The method of Automated Ribosomal Intergenic Spacer Analysis (ARISA) was developed by Fisher and Triplett (1999) as a higher throughput method of Ribosomal Intergenic Spacer Analysis (RISA) (Borneman and Triplett, 1997), to enable the assessment of community diversity more rapidly and more efficiently. The ARISA technique uses conserved primers found on the 16S and 23S ribosomal RNA (rRNA) genes to amplify the internal transcribed spacer (ITS) region between the two subunits. The ITS region is hyper variable in length and sequence composition among organisms. The ARISA technique exploits this length heterogeneity to produce amplified products with DNA fragment lengths that are representative of the organisms present. The total number of distinct fluorescent peaks in the ARISA data within a given sample is taken as an estimate of species diversity, and the sizes of the fragments can be compared to those in the GenBank database to provide a tentative taxonomic assignment (Doringo et al., 2005). The automated fingerprinting technique ARISA has also been suggested to be more sensitive than other fingerprinting methods, such as denaturing gradient gel electrophoresis or

single-strand conformational polymorphism analysis, especially for the ability to detect less abundant taxa within bacterial assemblages (Danovaro et al., 2006).

Chapter 2. Assessment of Chemical Stabilization Methods for DNA Preservation

2.1 Summary

A number of commercially available and home-made chemical preservative solutions were tested for their ability to stabilize DNA contained in filtered water samples. A total of 16 formulations were tested over different time periods ranging from 1 to 8 days. All buffers tested were divided in two main categories: commercial and non-commercial buffers. Quantitative PCR of the 16-23S ITS region was used to assess DNA degradation or changes in community composition of the samples. Untreated samples or samples treated with buffers such like 10x TE, 10x TEG, IDTE, SSC, SSC-CU, SSC-ZN, SSD, Lugol's, HRALater pH 8.0 and MO showed poor preservation of the bacterial DNA. Other buffers like Ethanol, Isopropanol, and HRNALater pH 5.2 might be promising depending on the time spam and sensitivity needed by the user. **The best performing buffers were PrepProtect and RNALater which preserved DNA for up to 3 days with extended storage possible up to 8 days with PrepProtect.**

2.2 Introduction

The aim of this project is to produce an *in situ* instrument that will enable the sampling regimes in molecular microbial ecology studies to be increased. The instrument is being designed to autonomously filter water and store the filters for subsequent molecular analysis. Therefore, it is important that methods for sample preservation be characterized. The successful chemical stabilization of the sample is an important factor in the development of this instrument as it will influence the time that the instrument can be deployed as well as the downstream molecular applications that can be performed. High quality DNA or RNA are a prerequisite for virtually every molecular technique. One of the major problems is that DNA and RNA are very sensitive to chemical and enzymatic degradation. Additionally, if improperly stored changes in the microbial community structure may occur due to microbial growth or cell lysis. In aquatic molecular microbial ecology studies where researchers filter water samples, degradation is commonly overcome by immediate freezing of the filters at -80˚C (DeLong, 1992; Fuhrman et al., 2006) or immediate storage in a lysis solution containing high concentrations of guanidine based chaotropic salts and freezing (Paul et al., 2000). While these techniques remain the gold standard practice in aquatic microbial ecology, it is energetically impractical to maintain freezing temperatures in a small autonomous sampling instrument.

One mechanism to overcome the need for freezing samples to prevent degradation of nucleic acids is to chemically stabilize the samples. A number of buffers have become commercially available to achieve this. Probably the best known example of these buffers is RNAlater (Ambion Inc., TX, USA), however a number of other formulations are also available such as PrepProtect (Miltenyi Biotec GmbH, Germany) and RNASafer (SA Biotech). For our project we will address this problem by the application of different commercial and "home-made" buffers to samples. For successful application in the prototype instrument, the storage buffers need to meet a number of criteria including:

1. Be stable at room temperatures
2. Preserve DNA with little or no loss of quality for 3 days
3. Inhibit microbial growth, or community changes in the sample
4. Be relatively non-toxic and non-corrosive/reactive to instrument components
5. Does not leave residues that inhibit downstream molecular protocols

Here the ability of various chemical formulations to preserve filtered water samples is examined, both quantitatively and qualitatively. Each formulation is tested in a manner that mimics the operation of the prototype instrument. The efficiency of a formulation to preserve the sample was then tested using quantitative PCR (qPCR) of the 16S-23S rDNA ITS region, and by Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999). This approach will identify was to stabilize DNA and large scale changes in microbial community structure to be addressed.

2.3 Materials and Methods

2.3.1 Water Collection

Water obtained for most trials was collected from an open water site off the coast of South West Puerto Rico at approximate coordinates 17°53'2.22"N, 67° 3'41.23"W (Figure 2.1). When weather conditions were not favorable for collection at this site, water was obtained from the small boats dock at the Department of Marine Sciences Isla Magueyes, La Parguera Puerto Rico at approximate coordinates 17˚58'14.16"N, 67˚02'48.30"W (Figure 2.1). Water was collected in a 4 liter (L) rinsed amber glass bottles and returned to the laboratory in the dark at ambient temperature.

Figure 2.1. Locations for water sampling near La Parguera, Puerto Rico.

2.3.2 Preservation Buffers

Table 2.1 shows the composition of the preservation buffers used in this study. Where proprietary or commercially available buffers were used the manufacture information is provided. With the exception of Ethanol and Isopropanol, all in-house buffers were sterilized by either autoclaving at 121 ˚C for 15 minutes or by filter sterilization through a 0.22 µm nylon filter (Fisher Scientific Inc, MA, USA).

Table 2.1 Composition of preservative buffers used in the study

Preservative Buffer	Composition or Manufacturer	Amount
10x TE Buffer (Sambrook et al., 1989)	1 M Tris 0.5 M EDTA H_2O pH 8.0, Autoclaved	10 ml 2 ml 88 ml
10x TE-Guanidine Hydrocloride (This study)	10x TE Buffer Guanidine Hydrochloride H_2O	100 ml 15.3 g 88 ml
20x SSC (Saline Sodium Citrate) (Sambrook et al., 1989)	Sodium Chloride Sodium Citrate H_2O pH 7.0, Autoclaved	173.3 g 88.2 g 800 ml
SSC-Zn (This Study)	20x SSC Zinc Sulfate Filter Sterilized	100 ml 20 g
SSC-Cu (This Study)	20x SSC Copper Sulfate Filter Sterilized	100 ml 20 g
HMRNAL (Lader, 2001)	Ammonium Sulfate 1 M Sodium Citrate 0.5 M EDTA H_2O pH 5.2 or pH 8.0, Autoclaved	700 g 25 ml 40 ml 953 ml
SSD (Salt Saturated DMSO) (Seutin et al., 1991)	20% DMSO 0.5 M EDTA pH 8.0 Saturated NaCl H_2O pH 7.5, Filter Sterilized	20 ml 50 ml ~42.3 g 53.1 ml
Lugol's Solution (Stock Solution) (Willén 1962)	Glacial acetic acid Iodine Potassium Iodine H_2O Filter Sterilised	1 ml 0.5 g 1 g 9 ml
3% Lugol's Solution (Willén 1962)	Lugol's Solution (Stock Solution) H_2O (autoclaved)	3 ml 97 ml
RNAlater	Ambion Inc., TX, USA	
Mineral oil	Fisher Scientific, PA, USA (Cat #BP2629-1)	
Ethanol	Spectrum Chemical MFG Corp., CA, USA (Cat#: ET107)	
Isopropanol	Acros Organics, NJ, USA (Cat# 327270010)	
IDTE pH 8.0 (Sambrook et al., 1989)	Integrated DNA Technologies Inc, IA, USA. (Commercial formulation of 1x TE Buffer)	
PrepProtect	Miltenyi Biotec GmbH, Germany	

2.3.3 Water filtration and Preservation Buffer Addition

Water was filtered through either 25 mm or 47 mm diameter 0.22 µm Durapore filters (Millipore, MA, USA). Filters were housed in plastic 25 mm Swinnex (Millipore Corporation, MA, USA) or 47 mm (Advantec, ToyoRoshi Kaisha Ltd, Japan) filter holders. Prior to use the filters and filter housings were assembled and autoclaved at 121˚C for 15 min. Filtration and preservative buffer addition was performed in a manner that mimics the operation of the proposed instrument. For each water collection, an equal volume of seawater (ranging from 150 - 400 ml) was filtered through each filter using a 60 ml syringe (Becton Dickinson, NJ, USA) attached to a leur connector on the filter housing. To reduce variability between filters and the representative water, the 4 L of water collected was homogenized by gently mixing and pouring in a 1000 ml beaker on a stirring plate.

Following filtration of the water, the preservation buffer under examination was added to a randomly chosen filter. Each potential preservation buffer was tested using either a duplicate or triplicate randomly chosen filter. A total of 4 ml and 10 ml (for 47 mm) of preserving buffer was added to the filter using a 10 ml syringe (Becton Dickinson, NJ, USA). The filter was first flushed with 2 ml and 5 ml (for 47 mm) of preserving buffer; the remaining 2 ml and 5 ml (for 47 mm) of buffer was left to immerse the filter. With the exception of the Day 1 sample, the filters were stored in the dark at room temperature. Typically samples were stored for 1-5 days, with samples tested on days 1, 3 and 5, although other testing regimes were also performed. Day 1 samples served as the baseline water sample and underwent DNA extraction (Section 2.2.4) on the day of filtration. To maintain randomness among the remaining stored filters, no identification was made other than the preservative buffer used. On the corresponding day of storage, replicate filters for each preservative treatment were chosen and the DNA was extracted. These steps were done for the continuous days until all filters had undergone DNA extraction. Following DNA extraction all DNA was stored at -20 ˚C until analysis. Filters not treated with any preservation buffer were processed in the same way however the buffer addition was omitted.

2.3.4 DNA Extraction

A modified protocol using the Ultra Clean 15 DNA purification kit (MO BIO Laboratories Inc, CA, USA) was used to extract and purify DNA from the filters. DNA extractions were performed by aseptically removing the filters from the filter housings and transferring them to sterile 2.0 ml screw cap tube (Fisher Scientific Inc, MA, USA) containing 0.2 g of 0.1 mm glass beads (Research Products International Corp, IL, USA). Cell lysis was performed by mechanical disruption. First, 200 µl of IDTE pH 8.0 buffer (Integrated DNA Technologies Inc, IA, USA) and 600 µl of Ultra Salt Buffer (MO BIO Laboratories Inc, CA, USA) was added to each tube. Each tube was then bead beaten 3 times at full speed (10, 000 rpm) with 1 minute on ice between beatings in a mini beadbeater-1 (BioSpec Products, OK, USA). Following bead beating the tubes were briefly vortexed and then centrifuged at full speed for 2 min. The supernatant carefully removed to avoid collecting filter material and glass beads and transferred into a new sterile 1.5 ml micro centrifuge tube. The ultra bind matrix was then resuspended by vortexing and a 5µl aliquot of the homogenate added to each tube. The Ultra Clean-15 DNA purification protocol for DNA from a solution was then followed as described by the manufacturer. DNA was eluted in 100 µl of molecular biology grade water (MO BIO Laboratories Inc, CA, USA) and stored at -20^{o} C until analysis.

2.3.5 PCR

PCR amplification of all samples was performed using bacteria-specific primers to amplify the ITS region of the 16S-23S rRNA operon (Table 2.2) (Fisher and Triplett, 1999). All primers used were synthesized by Integrated DNA Technologies, Inc (IA, USA), and resuspended at a concentration of 100 µM in IDTE pH 8.0 (Integrated DNA Technologies, Inc, IA, USA). To prevent damage to the primer stocks from extraneous DNA contamination or from repeated freeze thaw cycles, aliquots of the primers were prepared prior to freezing at -20 ˚C. Preparation of all PCR reactions was performed in a PCR hood (Labconco Corporation, MO, USA) that was fitted with an ultra violet light to reduce the possibility of extraneous DNA contamination. Reactions were performed in white low profile PCR tubes and sealed by flat optical caps or in clear 96 well plates sealed with optically clear microseal 'B' film all of these PCR consumables were obtained from Bio-Rad Laboratories, Inc. (CA, USA).

Table 2.2. Primer sequences used in this study.

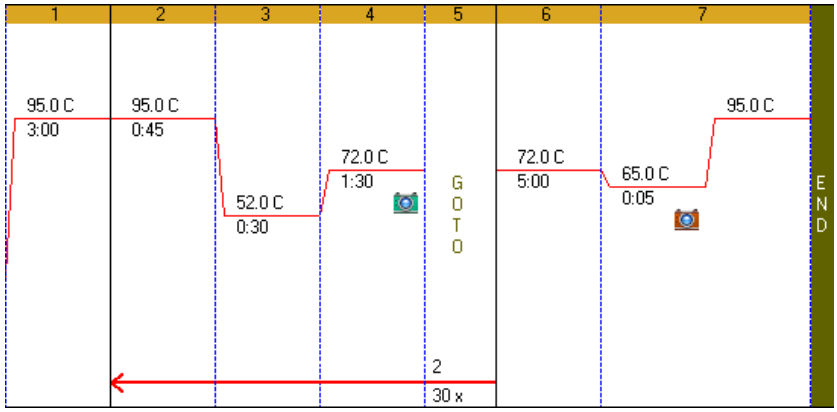
Primer	Sequence (5' to 3')
1406F	TGY ACA CAC CGC CCG T
ARISA-1406F	[FAM]-TGY ACA CAC CGC CCG T
125R	GGG TTB CCC CAT TCR G

The qPCR reactions were performed in 10 µl volumes and contained 5 µl of 2x EvaGreen Supermix (Bio-Rad Laboratories Inc, CA, USA), 0.05 µl of each primer 1406F (100 µM) and 125R (100 µM), 1 µl of extracted DNA and 3.8 µl of molecular biology grade water (MO BIO Laboratories Inc, CA, USA). No template control (NTC) reactions were also prepared with each qPCR experiment, in this case the 1 µl of input DNA was replaced with 1 µl of molecular biology grade water (MO BIO Laboratories Inc, CA, USA). Quantitative PCR analysis was performed in a real time CFX 96 thermocycler (Bio-Rad Laboratories Inc, CA, USA). The thermocycling parameters (Figure 2.2A) consisted of an initial incubation at 95.0 °C for 3 min, followed by 30 cycles of 95.0 °C for 45 s, 52.0 °C for 30 s, and 72.0 °C. for 90 s. Optical detection in the SYBR channel was performed immediately following the 72 ˚C extension incubation. At the completion of the 30 cycles, the reaction was incubated at 72.0 °C for 5 min to ensure complete extension of the amplified products. Immediately following the amplification stage of the qPCR protocol, melt curve analysis was performed on the amplified product which consisted of detection in the SYBR channel every 0.1 ˚C for 0:05 s over the temperature range of 72.0 °C to 95.0 °C.

PCR amplification for ARISA followed a similar thermocycling protocol to qPCR (Figure 2.2 B) with the exception that the ARISA protocol did not include optical detection or melt curve analysis. PCR reactions for ARISA were performed in 20 µl reaction volumes and contained 10 µl of 2x Biomix (Bioline, MA, USA), 0.1µl of each primer ARISA-1406F (100 µM) and 125R (100 µM), 1 µl of extracted DNA and 8.8 µl of molecular biology grade water (MO BIO Laboratories Inc, CA, USA). NTC reactions were also prepared with each ARISA PCR experiment, in this case the 1 µl of input DNA was substituted with 1 µl of molecular biology grade water (MO BIO Laboratories Inc, CA, USA). To ensure the PCR had worked and that no amplification products were obtained in the NTC sample, a 3 µl aliquots of the PCR products were quality checked by gel electrophoresis using a 1% TAE agarose (Fisher Scientific, PA, USA) gel containing 10 µl of GelGreen (Biotinum, CA, USA). Visualization of the PCR

products following gel electrophoresis was performed on a blue light Dark Reader transilluminator (Clare Chemical Research, Inc. CO, USA). ARISA analysis was performed at the Sequencing and Genotyping Facility at the University of Puerto Rico, Rio Piedras using an ABI 3130 capillary sequencer (Applied Biosystems, CA, USA). Fragment length analysis was performed using GeneMapper v4.0 (Applied Biosystems, CA, USA).

A.



B.

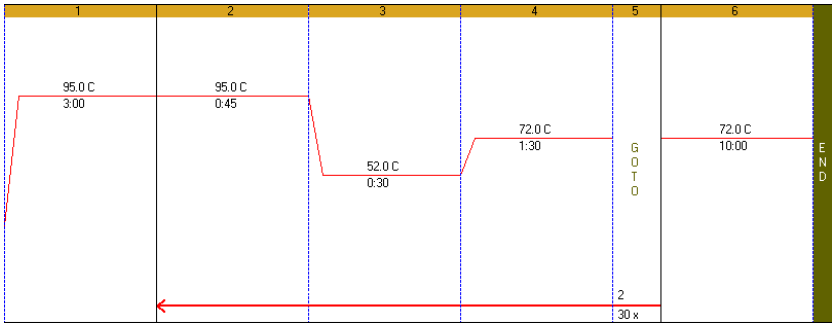


Figure 2.2. Thermocycling parameters for qPCR and ARISA. The numbers highlighted in orange represent the various stages of the protocols. Common to both protocols are steps 1 to 6. Step 1 is the initial denaturation phase, steps 2 to 4 represent the multiple cycles of denaturation, annealing and extension and step 6 enables complete extension or fill in of amplified products. A. Characteristics of the qPCR protocol, optical detection for amplification is performed in step 4, and melt curve analysis is performed in step 7. B. Characteristics of the ARISA protocol.

Reaction efficiencies for the qPCR reaction were calculated, the template for these reactions was previously amplified ITS PCR product obtained from environmental samples. Prior to use the template was purified to remove unincorporated primer from the previous reactions using the Ultra Clean 15 DNA purification kit (MO BIO Laboratories Inc, CA, USA), purification was performed according to the manufacturers instructions. The template DNA was quantified on a NanoPhotometer (Implen, CA, USA). Serial dilutions of the DNA were performed in molecular grade water prior to preparing the reactions described previously for qPCR The reaction efficiency is automatically calculated by the CFX 96 thermocycler as percentage efficiency (E%), using the method described by Pfaffl (2001). To convert E% to the E value described by Pfaffl, (2001) the equation E = (E% x 0.01) + 1 is used (Bio-Rad, 2008).

2.3.6 Statistical Analysis

Statistical Analysis was performed using the statistical software package R (Ihaka and Gentleman, 1996). Due to the small sample sizes used in this study, the data could not be assumed to have a normal distribution; therefore statistical testing of Cq values was performed using the non-parametric Wilcoxon method (Yuan et al., 2006). Bray Curtis similarities were performed for ARISA as is commonly performed with ARISA data (e.g., Kent et al., 2007).

2.4 Results

Quantitative PCR amplification of the 16-23S rDNA internal transcribed spacer (ITS) regions was used to examine the ability of 16 preservation buffers to stabilize DNA in filtered seawater samples. Both the Cq and melt curve parameters from the qPCR, was used to relate to the quality of the preservation. Comparison of the changes in Cq from samples stored over time and their initial control, can provide insight into the ability of the buffer to preserve the sample. The preservation of DNA can be marked if the Cq remains the same through time within the same sample. Higher Cq values in stored samples compared to its control are indicators that the DNA is being degraded by chemical or enzymatic processes, or there is a net loss of DNA due to cell lysis. Conversely, lower Cq values indicates that there is a greater amount of target DNA present in the sample, suggesting that a sample is improperly fixed and microbial growth is occurring on the filter. A further method that can be used to evaluate this latter aspect of community changes due to microbial growth on the filter is through melt curve analysis. As the ITS region is of variable length in different microorganisms, large changes in community composition due to microbial growth can potentially be visualized by changes in melt curve characteristics within the sample over time. It is expected that a properly preserved filter will display the same community same melt curve characteristics, while improperly stored filters will display different communities melt curve characteristics of function of time.

2.4.1 Reaction efficiency

The reaction efficiency of the PCR was tested to ensure that the qPCR assay was reproducible. The reaction was shown to be linear over 6 orders of magnitude and reproducibly gave efficiencies of approximately 93% (E=1.93). An example of qPCR amplification plots and standard curves used to perform the qPCR efficiency is shown in Figure 2.3.

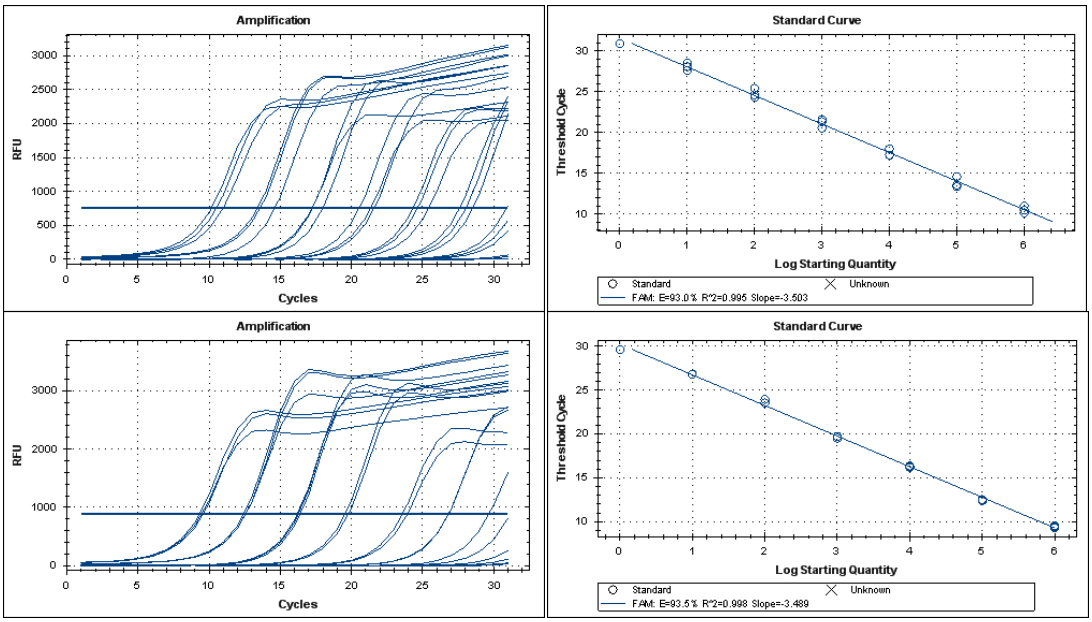


Figure 2.3. Quantitative PCR plots used to calculate PCR efficiency. Triplicate reactions were constructed using input wild type DNA concentrations ranging from 1 ng to 1 fg.

2.4.2 No Buffer

Samples that were stored without the presence of a preservation buffer showed a significant increase in the Cq values (W=0, p=0.03125) after being stored for 1 day (Figure 2.4A). These samples continued to show increasing Cq times over a period of 4 days storage. Similarly, the samples showed a major shift in the characteristics of the melt curves occurs following 2 days storage (Figure 2.3B) where strong peaks on day 2 at 86.60 and 82.20 ˚C transition to peaks at 82.20 and 82.10 ˚C on days 3 and 4.

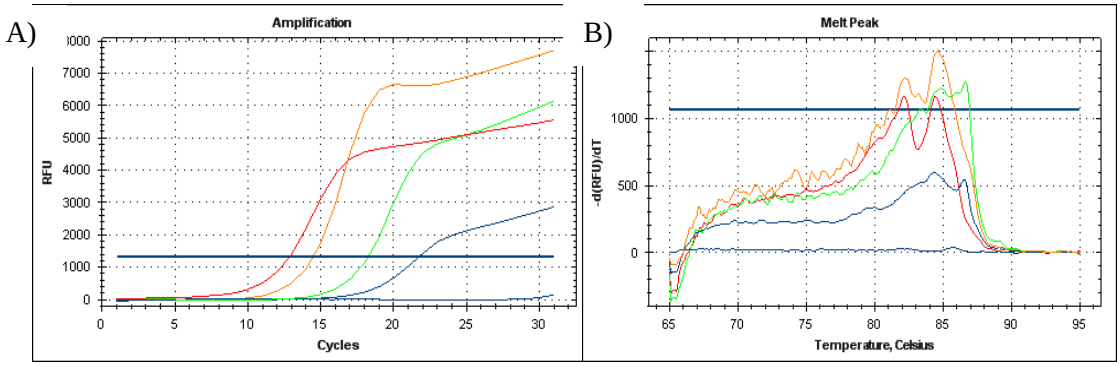


Figure 2.4. Example qPCR amplification plots and melt curve analysis for samples stored without buffer. (A) Amplification plot (B) Melt curve. Storage day is represented as follows: Day1 (blue), Day2 (green), Day3 (orange), Day4 (red).

2.4.3 IDTE Buffer and TE Buffer Variations

Three different variations of TE buffer were tested, they included a commercial formulation of 1x TE buffer (IDTE), 10x TE and 10x TE containing 1.6 M Guanidine

hydrochloride. For samples treated with IDTE, pH 8.0 produced similar results to samples not treated with any preserving agent. A large decrease in Cq values was observed following 1 day of incubation which was accompanied by shifts in melt curve characteristics (Figure 2.5). Also, a large decrease in Cq values after day 3 indicating that microbial growth is occurring on the filter.

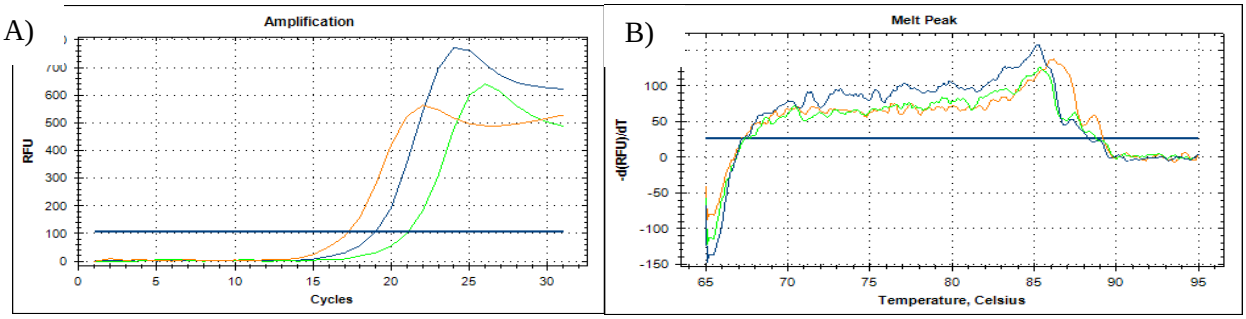


Figure 2.5. Example qPCR amplification plots and melt curve analysis for samples stored with IDTE pH 8.0 added to the filter. (A) Amplification plot (B) Melt curve. Storage days presented: Day1 (blue), Day3 (green), and Day 5 (orange).

The buffer 10x TE showed similar results to 1x TE buffers, showing a significant decrease in Cq values (W= 45, p=0.0039) after Day1. The formulation of 10x TE containing 1.6M Guanidine hydrochloride also showed significantly faster Cq times than the no buffer control after 1 day (V = 42, p-value = 0.01953). Additionally, this buffer did not homogenize completely and precipitated from solution after several days.

2.4.4 PrepProtect

Samples treated with the commercially available buffer PrepProtect showed an initial increase in Cq values, however this increase was not significant (W = 20, p-value = 0.0625). This could be indicating possible opening of cells leading to DNA loss. There was no statistically significant change in the Cq values ($p>0.05$) when samples were stored over a period of 8 days (Figure 2.6A), indicating a more stable preservation of DNA. The observed Cq values were for Day1- 26.63, Day3- 26.98, Day6- 27.18 and Day 8- 26.47. Additionally, the melt curve did not show any evidence for bacterial shifts or degradation of DNA (Figure 2.5B).

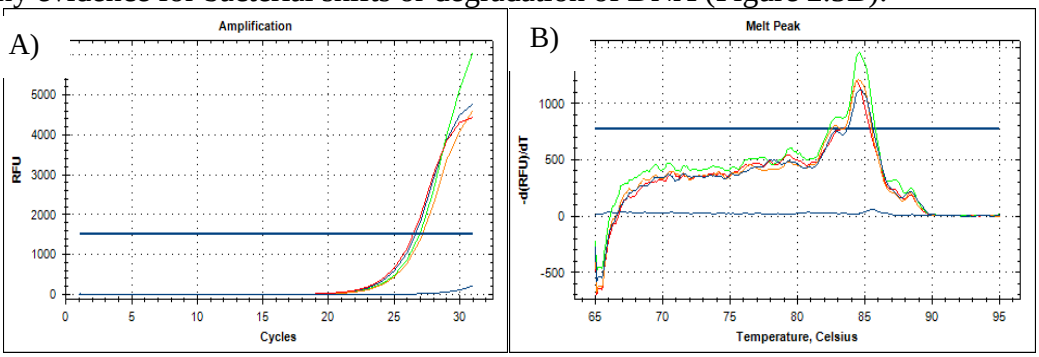


Figure 2.6. Example qPCR amplification plots and melt curve analysis for samples stored with PrepProtect added to the filter. (A) Amplification plot (B) Melt curve. Storage days presented: Day1 (blue), Day3 (green), Day6 (orange), and Day 8 (red).

2.4.5 RNALater

Following 3 days incubation, RNALater did not exhibit initial DNA loss nor bacterial shifts in the community present. However on day 5 a significant decrease in Cq is detected (W = 0, p-value = 0.03125) indicating degradation of DNA in the sample (Figure 2.7).

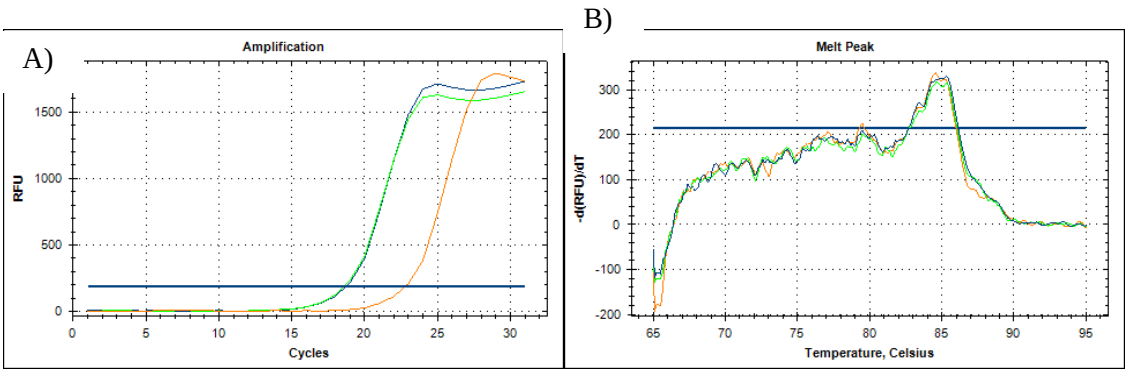


Figure 2.7. Example qPCR amplification plots and melt curve analysis for samples stored with RNALater added to the filter. (A) Amplification plot (B) Melt curve. Storage days presented: Day1 (blue), Day3 (green) and Day 5 (orange).

2.4.6 HMRNAL pH 5.2 and HMRNL pH 8.0

A comparison of different versions of HRNAL at a different pH was preformed. HMRNAL at pH 5.2 did not exhibit any initial DNA loss and it seems promising for up to 5 days without any community changes (Figure.2.8). HRNAL pH 8.0 showed a significant loss of DNA on day one compared to the non-treated control (W = 0, p-value = 0.003906). Similarly after day 3 the Cq values significantly decreased (W = 0, p-value = 0.00909)(Figure 2.9).

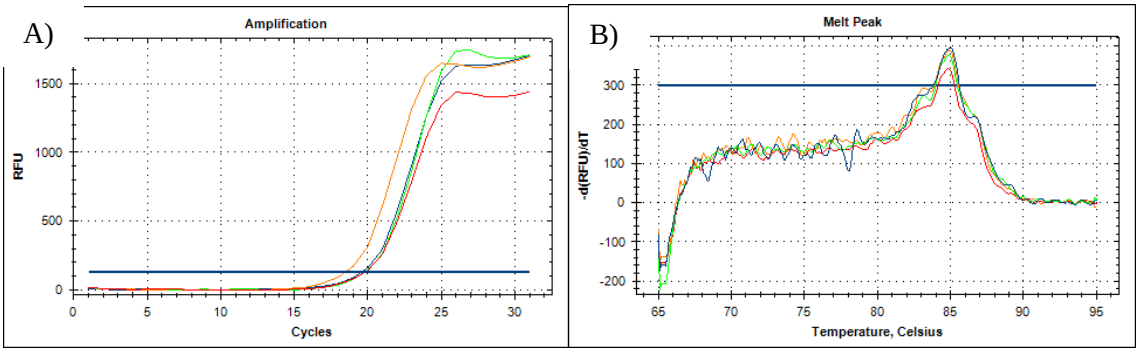


Figure 2.8. Example qPCR amplification plots and melt curve analysis for samples stored with HRNALater pH 5.2 added to the filter. (A) Amplification plot (B) Melt curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red).

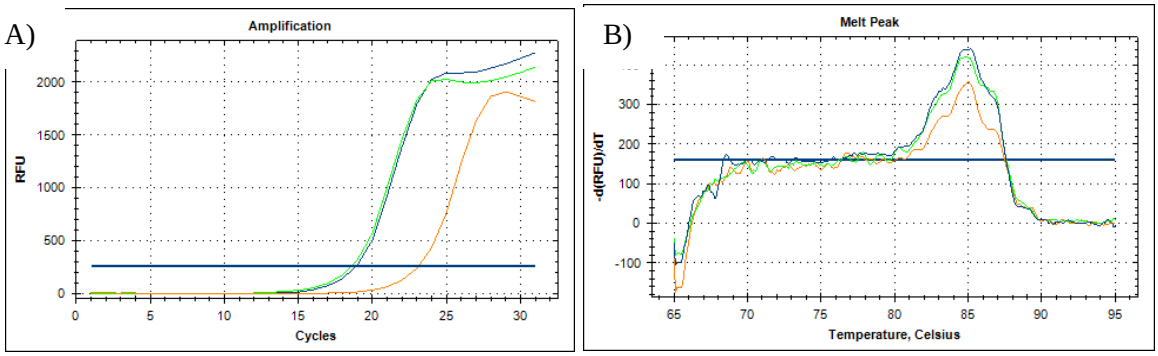


Figure 2.9. Example qPCR amplification plots and melt curve analysis for samples stored with HRNALater pH 8.0 added to the filter. (A) Amplification plot (B) Melt curve. Storage days presented: No Buffer (blue), Day1 (green) and Day3 (orange).

2.4.7 20x SSC and 20x SSC variations

Initial testing of the buffer 20x SSC showed no statistical difference on the first day of treatment when compared to an untreated control (W = 10, p-value = 0.125) this was also observed after 4 days storage. Upon re-testing of the buffer a statistical difference was observed after storage for 3 days. Further testing of the buffer showed a large decrease in Cq on day Day 1 when compared to the no buffer Day1 control. This indicates an increase in DNA in the sample. Additionally the melt curve indicated a difference between the treated and untreated samples two samples (Figure 2.10B). Because of this the buffer was checked for contamination. A volume of 50 ml was filtered and analyzed by qPCR, the results indicated contamination of the buffer (data not shown).

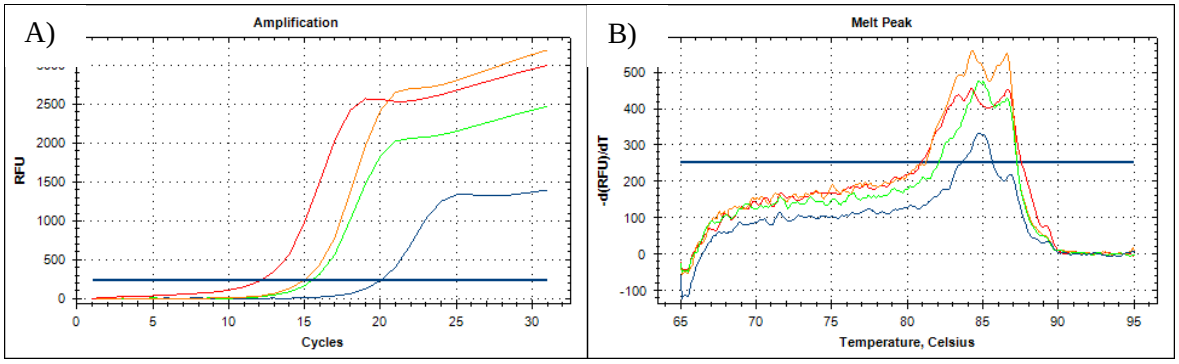


Figure 2.10. Example qPCR amplification plots and melt curve analysis for samples stored with 20x SSC added to the filter. (A) Amplification plot. (B) Melt curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red)

The SSC buffers containing zinc and copper precipitated out of solution and left a residue in the filter holders. Samples treated with the copper containing buffer did amplify following the 3 and 5 day incubation. The SSC-zinc formulation showed a large decrease in Cq times over the 5 days storage.

2.4.8 Lugol's Solution

This buffer was tested at two different concentrations. Both concentrations (3% and 100%) had suboptimal results in preserving DNA. On the first day of treatment, the 100% Lugol treatment was significantly different (W = 0, p-value = 0.003906) to the no buffer control. Following the day 1 treatment, no amplification was observed in the day 3 and 5 samples. The treatment with 3% Lugols solution did not show inhibitory results but it did show large decreases in DNA over 5 days storage with an associated shift in melt curve characteristics (Figure 2.11).

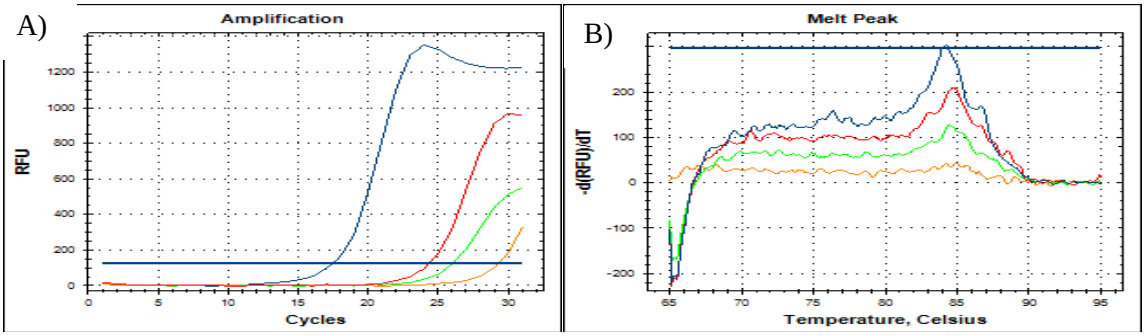


Figure 2.11. Example qPCR amplification plots and melt curve analysis for samples stored with 3% Lugol's added to the filter. (A) Amplification plot. (B) Melt curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red).

2.4.9 Alcohols

Two types of alcohols, ethanol and isopropanol were tested for their ability to preserve the filtered water samples. The qPCR graphs indicate that ethanol and isopropanol have the same pattern of preservation. Both alcohols show no initial DNA loss after Day 1 (Figure 2.12). But when compared with a no buffer Day1 a shift in community structure was observed. This is noticeable in the melt curve, comparison of both buffers with a no buffer Day1 control which has peaks at 85.10 and 82.60 ˚C, whereas ethanol has peaks at 84.70 and 86.70 ˚C, and isopropanol has peaks at, 86.00 and 84.90 ˚C. This shift can be due to community change or possible precipitation of DNA what can't be recuperated during DNA extraction. Based on our observations no major differences were detected between these two buffers, although the decreasing Cq times using Ethanol seems indicates that it is less effective than isopropanol.

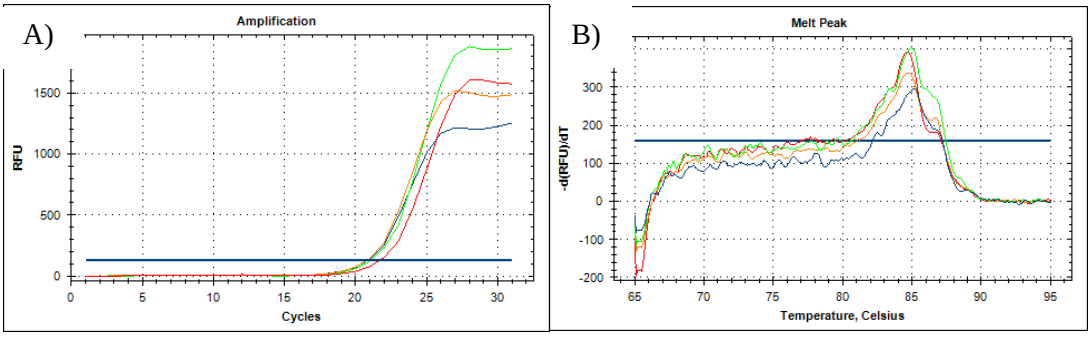


Figure 2.12. Example qPCR amplification plots and melt curve analysis for samples stored with Ethanol added to the filter. (A) Amplification plots.

(B)Melt curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red).

2.4.10 Mineral Oil

The possibility of using Mineral oil as a buffer was tested. The amplification plots indicate a considerable loss of DNA. The Cq for the mineral oil treatment values with-in itself did not vary over 5 days, but the melt curve indicates a large change in community composition (Figure 2.13). Indicating hypothetically the same amount of DNA but from different communities. In addition to these results, the mineral oil was shown to degrade the rubber components on the syringe plunger.

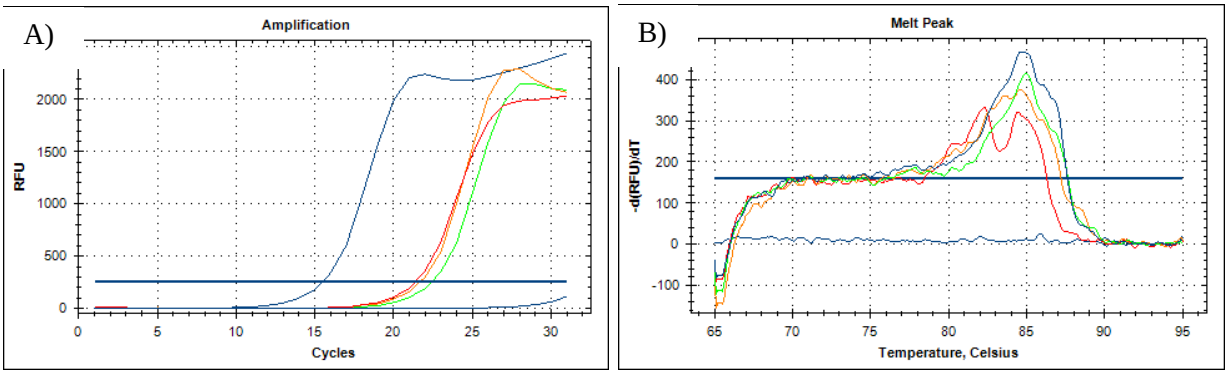


Figure 2.13. Example qPCR amplification plots and melt curve analysis for samples stored with Mineral oil added to the filter. (A) Amplification plot. (B) Melt curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red).

2.4.11 Salt Saturated DMSO (SS-DMSO)

The formulation of SS-DMSO was tested. This buffer presented increased Cq values when compared with no treatment plots. This is a potential indication that the buffer was contaminated, however this is not supported in the melt curve plots as there did not appear to be a change in melt characteristics compared to the no buffer control. Although with-in the buffer treatment it doesn't report significant changes until day 5 (Figure 2.14) where there is a large increase in Cq values indication the loss of preservation effectiveness of the buffer.

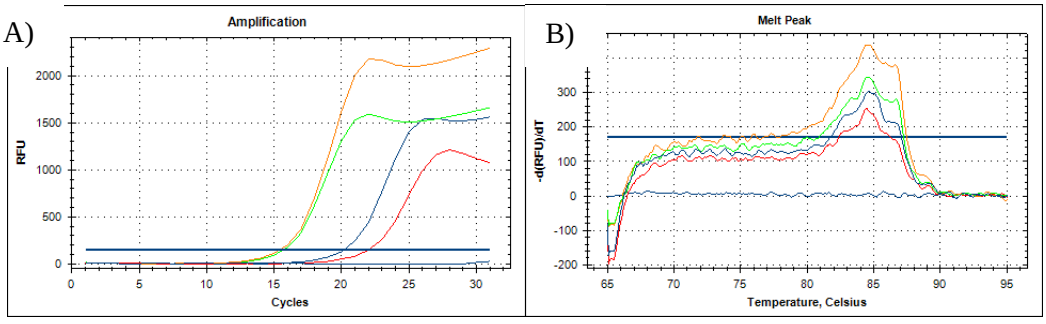


Figure 2.14. Example qPCR amplification plots and melt curve analysis for samples stored with SS-DMSO added to the filter. (A) Amplification. (B)Melt

curve. Storage days presented: No Buffer (blue), Day1 (green), Day3 (orange) and Day5 (red)

2.4.12 Comparison of Buffer Performance

Having in mind the parameters that had been established for a buffer to be successful, the most promising buffers can be further characterized and compared. The evaluations of buffers were compared using the ARISA technique. However this technique showed large variability between not just buffers but also within replicates. For example, comparing samples stored with RNALater and Preprotect, using Bray Curtis similarity results from ARISA showed that there was only 40% similarity between replicate samples from PrepProtect on day 1 (Table 2.3). In a similar situation using RNALater, the Day 1 replicates showed no similarity between each other. Visualization of the ARISA using a heat map showing the peak height as a color highlights the difference between fragment lengths for each of the fragment lengths (Figure 2.15). Upon analysis of the electrophoretograms, many of the samples had low quality scores. Attempts to trouble shoot the cause of these problems have not yet been successful. It is unknown if the cause is something inherent with the PCR chemistries being used. However, purifying the reactions has not appeared to produce better quality scores in the electrophoretograms.

Table 2.3 Bray Curtis similarities of ARISA analysis for samples treated with PrepProtect and RNALater.

Bray Curtis similarity	D1 PP F1	D1 PP F2	D1 RNL F1	D1 RNL F2	D3 PP F1	D3 PP F2	D3 RNL F1	D3 RNL F2	D5 PP F1	D5 PP F2	D5 RNL F1	D5 RNL F2
D1_PP_F1												
D1_PP_F2	40											
D1_RNL_F1	30	40										
D1_RNL_F2	0	0	0									
D3_PP_F1	40	40	80	0								
D3_PP_F2	30	40	80	0	70							
D3_RNL_F1	40	50	80	0	80	80						
D3_RNL_F2	40	40	80	0	80	70	80					
D5_PP_F1	40	40	80	0	80	70	80	90				
D5_PP_F2	40	40	80	0	70	80	90	80	80			
D5_RNL_F1	40	50	80	0	80	80	90	80	80	80		
D5_RNL_F2	0	0	0	0	0	0	0	0	0	0	0	

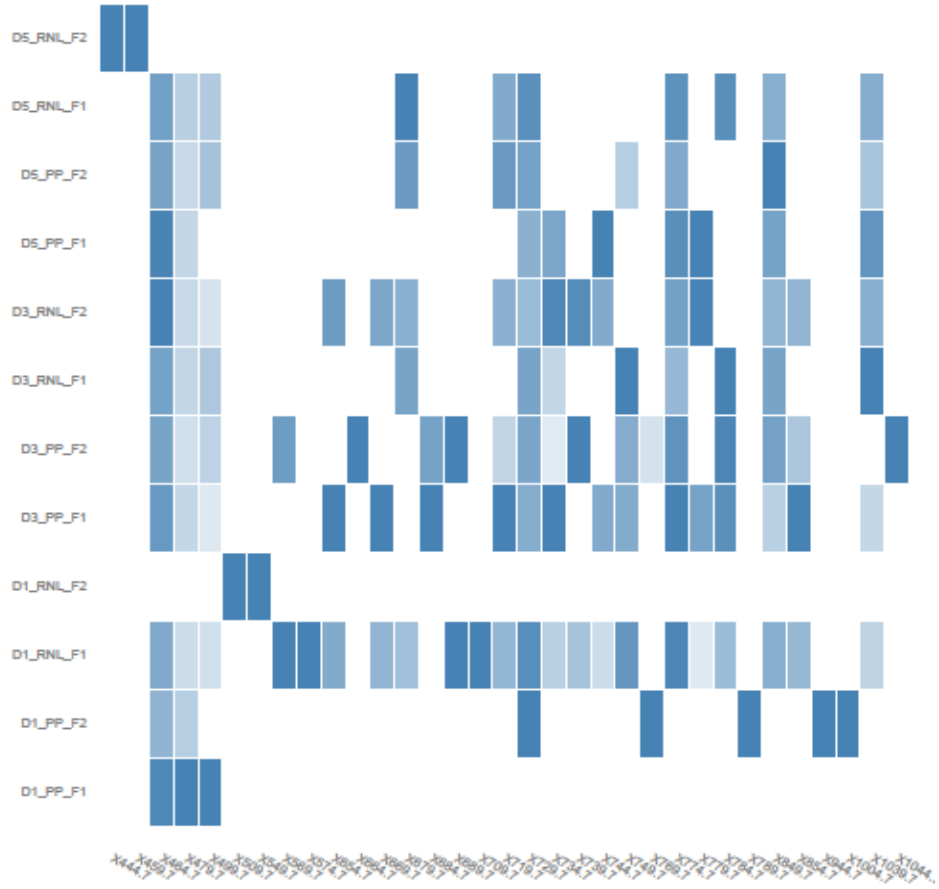


Figure 2.15. Heat map of ARISA peak heights and fragment lengths. Peak height is normalized to values between 1 and 0, where 0 is represented by white and 1 is dark blue.

Within the commercial buffer group the buffers can be classified in two sub-categories: alcohol based and non-alcohol based. In the non-alcohol based buffer, we had three buffers PrepProtect, RNALater and IDTE pH 8.0. For PrepProtect, a maximum of eight days was tested without any significant change in Cq nor melt curve (see above). But an initial loss of DNA can be detected in some trails in Cq values (Figure 2.5). On the other hand, RNALater buffer seems to lose efficiency after Day 3 (Figure 2.15).

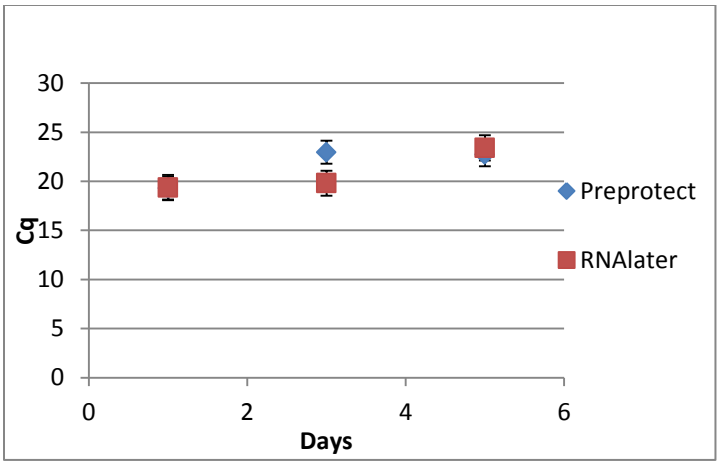


Figure 2.16. Comparison of Preprotect and RNALater

Other commercial buffers such as Mineral oil and IDTE have been used to output microbial growth. In the direct comparison of this two buffers it seem to be more efficient the use of Mineral Oil (Figure 2.16). But observations have indicated that it interacts with the rubber in the syringe.

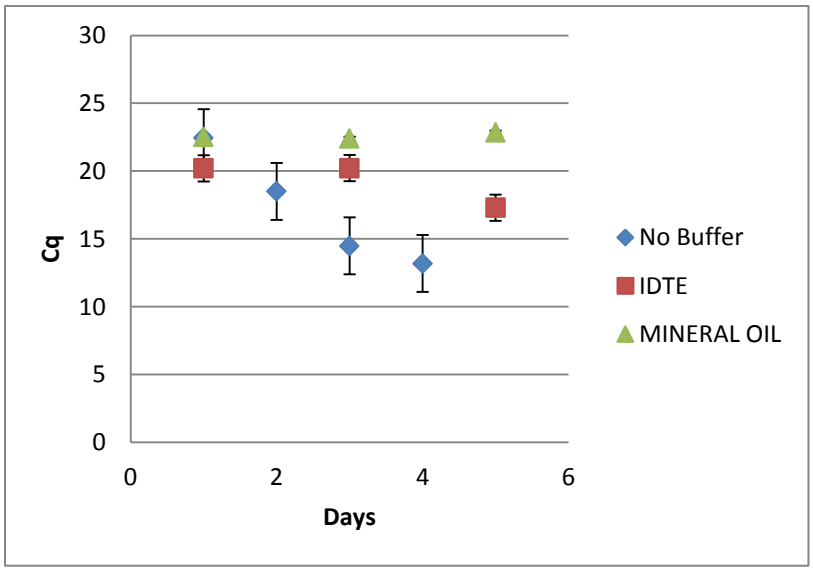


Figure 2.17. Comparison of IDTE and Mineral Oil

The second division of the commercial buffers is the alcohol based. These two alcohols (Ethanol and Isopropanol) have been projecting similar results indicating not much difference between them (Figure 2.17).

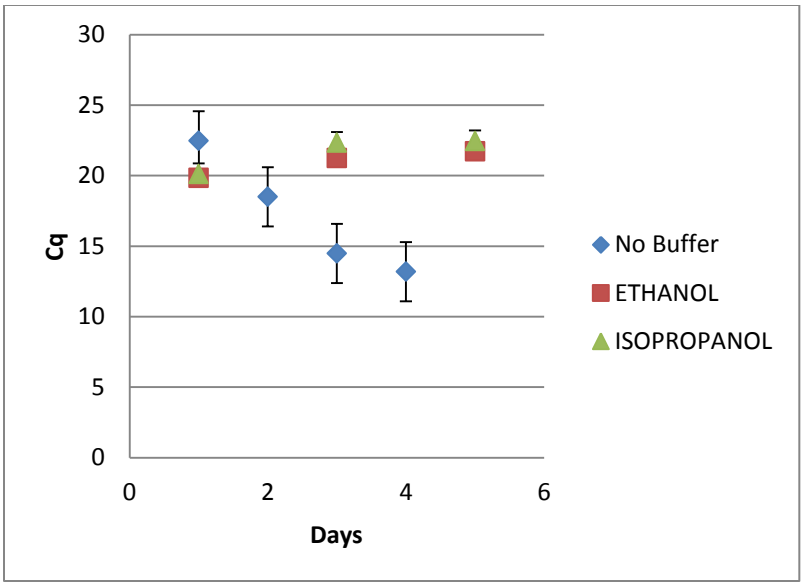


Figure 2.18. Comparison of commercial alcohols based buffers.

On the other hand, some home-made buffers were produced and evaluated. Two HRNALater were produced with different pH concentrations. Only HRNALater pH 5.2 buffer did meet the criteria for potential buffers (Figure 2.6). In the comparison a tendency of degradation can be seen for HRNALater pH8 (Figure 2.18).

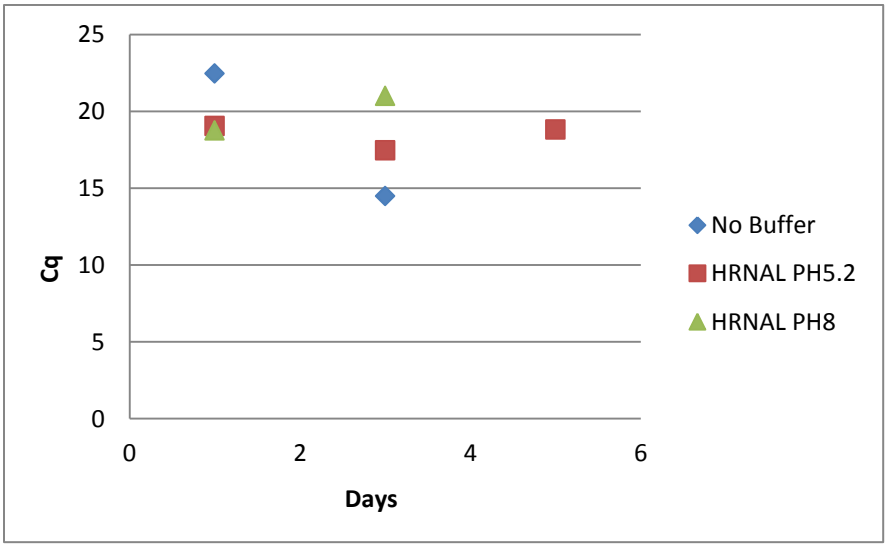


Figure 2.19. Comparison of different pH concentrations of HRNALater buffers.

Another home-made buffer was the SSC and its variations SSC-CU and SSC-ZN. Despite being contaminated, the SSC buffer appears to produce stable Cq values over the 5 days. In other trials with this buffer, no initial reduction in Cq values were observed. .

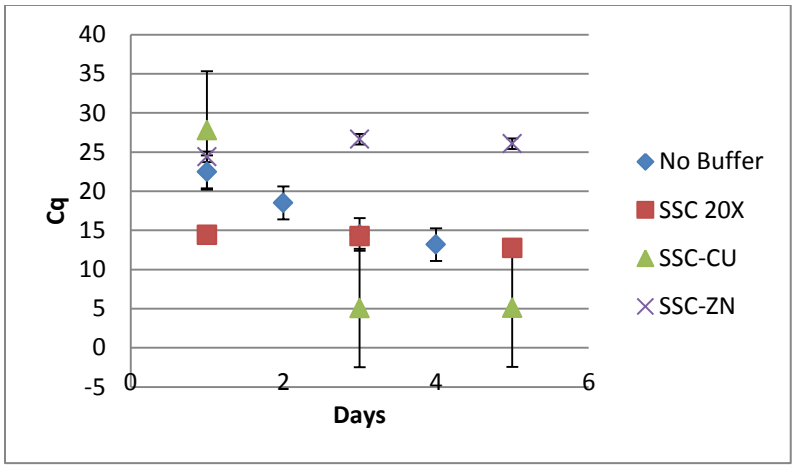


Figure 2.20. Comparison of commercial alcohols based buffers.

The rest of the home-made buffers were SS-DMSO and TE with its variations. Neither TE nor its variations preformed as expected. While SS-DMSO buffer seems to preserve at least for 3 days but had an initial decrease in Cq values compared to the no buffer control.

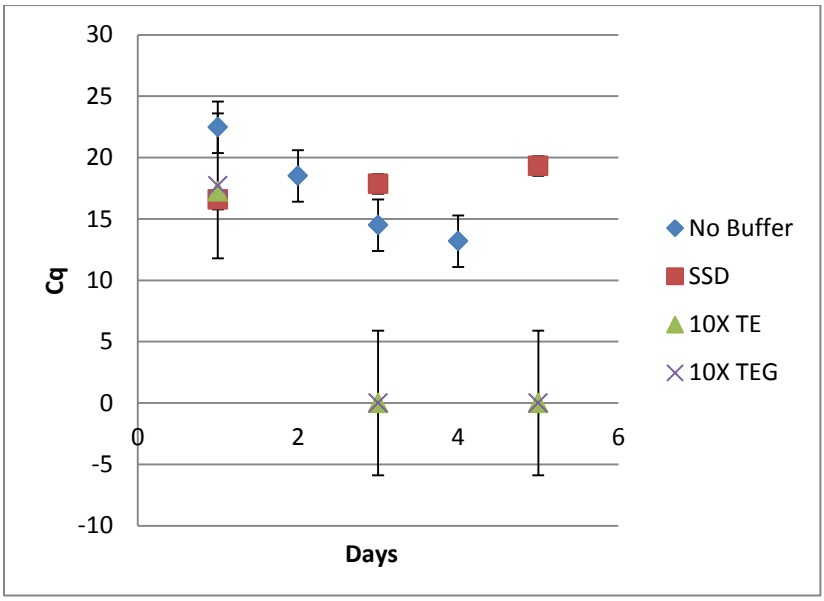


Figure 2.21. Comparison of SSD and TE variations buffers.

2.5 Discussion

It is evident that without the presence of a preservative buffer there are three potential outcomes: 1. The sample on the filter will not change; 2. There will be a microbial community change; or 3.The DNA will start to degrade. The results of this study showed that in the case here there was a change in the microbial community (with increasing biomass) was obtained without any form of preservation. Therefore there is a requirement to stabilize the sample. A total of 16

buffers were tested to stabilize the samples held on the filters. As the ARISA technique was providing problematic results, all buffers tested were compared and analyzed based on qPCR amplification and melt curve analysis. For the buffer to be deemed appropriate using this technique, the Cq values and melt curve characteristics should not change over time. A reduction in Cq values over time implies an increase in the concentration of DNA. While an increase in Cq values implicates DNA reduction or loss in the sample. The potentiality of a good buffer was based on 5 criteria discussed above (Section 2.2). If a potential buffer appeared to stabilize the microbial community after a period of 5 days, a longer extension time was performed to confirm the buffers performance, PrepProtect was the only buffer that met this criteria.

A potential range of buffers was analyzed based on chemicals/agents related to stabilize DNA. From the available buffers tested IDTE (a commercial formulation of TE buffer), was classified as a poor preserving agent. The use of IDTE is common in preserving agent for extracted DNA in molecular biology laboratories. However, it is not efficient to use as a sample preservation buffer, as it appears to support bacterial growth particularly after 3 days. Increasing the concentration of the buffer 10-fold (10x TE) did not have any influence on the storage ability. The effectiveness of TE buffer to stabilize DNA in the molecular laboratory is due to its ability to scavenge metal ions that are required for the activity of DNase. This is achieved by the EDTA which helps inactivate these enzymes by acting as a chelating agent of divalent cations like Ca^{+2} and Mg^{+2}. These cations are required for the regularity and functionality for enzymes.

Another buffer commonly used in molecular biology SSC showed initial promise as a preservation buffer. However this buffer was inconsistent in its performance, with some samples having no significant changes in their Cq values, while others showed a significant decrease in the Cq values indicating microbial growth is occurring. Additionally the SSC appeared to become easily contaminated which indicates that the buffer may not be a good preservative. To address this, 2 modifications of the SSC buffer were made that contained high concentrations of zinc and copper which was aimed at inhibiting microbial growth. While the inhibition of microbial growth may have occurred using these treatments, the copper treatment either inhibited the PCR reaction or the levels of DNA was reduced to undetectable limits. Similarly Zinc showed an increase in the Cq values overtime suggesting the buffers inability to archive the DNA in the sample.

Lugols solution was tested at 2 differing concentrations, 100% and at 3%. This buffer is commonly applied to preserve phytoplankton samples for later analysis. While these applications are typically using microscopy identification, mixed results have been observed in the literature regarding its use for subsequent molecular analysis (Godhe, 2002). This includes work that has said that DNA is not suitable for use as it inhibits PCR. Alternatively, some work suggests that the buffer will not inhibit PCR. This work shows that samples stored with 100% Lugols solution could not be detected following 3 days incubation. A 3% solution had better reults, but a steady increase of Cq times was observed. This may be adding evidence to the literature that while the Lugols solution may preserve the phytoplankton sample it may still enable DNA to degrade. Another treatment with Mineral oil showed similar results, paraffin embedded samples are a common preservative method and mineral oil is a liquid form of paraffin. The added detriments of using the mineral oil treatment was that it degraded the rubber components in the syringe and also that it was extremely hard to move through the filter.

The two most successful buffers were PrepProtect and RNALaater. PrepProtect seems to be effective for eight days. The buffer had a relatively low viscosity compared to other buffers which may have aided keeping the filter submerged and also avoiding early evaporation. Based

on the Cq values, a slight reduction of the DNA present in sample is evident. This buffer can be used for possible users if the interested target is highly abundant in the sample. Although, it might not be accessible to all possible users due to the high cost of the product. Another formulation is RNALater while this buffer also has a high cost it presents no initial DNA loss in the sample but its effectiveness hold up to three days. The effectiveness and duration makes it a potential buffer for users that are mainly interested in a low abundance target.

While literature for RNALater focuses on its application to preserving RNA, it was noted from information in the products patent (Lader, 2001) that describes the formulation that the potential pH of the formulation was low. It is known that DNA is susceptible to depurination at low pH (Lindahl and Nyberg, 1972), 2 formulations based on information the composition of RNALater in the patent were developed. The first HRNAL pH 5.2 was the same as described in the patent and was used as a control formulation. The second was the same formulation but the pH was raised to 8.0. While the theoretical evidence that DNA is degraded faster at low pH, the evidence in this study suggests that the DNA was degraded faster over time.

The SS-DMSO buffer potentially warrants further investigation, this buffer showed a significant decrease in Cq on day one compared the no buffer control but retained the same melt curve characteristics. This decrease in Cq values is interesting as it may represent contamination of the buffer, however if the buffer was contaminated it could be expected that a change in melt curve characteristics could be observed, this was seen in the contamination of the SSC buffer. One explanation for this could be that the buffer is helping making the cells fragile and upon DNA extraction is helping cell lysis. In this study we are under the assumption that that we have reproducible DNA extraction efficiencies for all samples, however this may not be the case particularly for some cells that may be hard to lyse.

It must be noted that there are a number of other potential experimental factors that may influence the results of this study. One major factor is due to sampling error, each filter was manually filtered by hand this entailed manually refilling the syringe before passing it through the filter, this usually occurred 6 times per filter. Undoubtedly this could introduce an error in the final volume filtered. Also when sampling, it is assumed that the bacterial community is homogenous and a representative portion of it is present in the sample taken. Similarly the influence on filtration pressure breaking sensitive cells is also assumed to be the same within collections. For these reasons inter collection comparisons between buffers were not made. Similarly we have provided data on the reproducibility of the PCR reaction. The reaction efficiency of the qPCR reaction was found to be approximately 93%, or E = 1.93 which indicates that the reaction has just under a perfect doubling every cycle, a perfect doubling would have a E value of 2 (Pfaffl, 2001). This reaction efficiency is good considering that the PCR reaction is not designed for real-time applications. In particular the heterogeneous lengths of the PCR amplicons are potentially suboptimal for the assay especially considering that some of the amplicon lengths can be up to 1400 base pairs. This may lead to the case that Cq values may be obtained at different rates if 2 samples contain different ITS lengths as longer amplicons will bind more of the intercalating dye than shorter strands. However, for our purposes this would only help to differentiate changes in samples. Evidence that the PCR assay did not bias the results was that the efficiency reactions were performed with a number of different samples containing heterogeneous mixtures of ITS lengths, with little change in the reaction efficiencies.

Chapter 3. Design and operation of the Sample Filtration and Archiving (SaFA) instrument.

3.0 Preface

This chapter is the result of interdisciplinary research, if information from this chapter is used it should be viewed as a joint publication and cited as follows:

Colon Padilla, B.L., Suarez Velez, E., Gomez Vallejo, A., Castilla, A¸Cordero Figueroa, T.S, Velasco, A., Quintero, P.O., Hall, M., and Smith, M.C. (2011). Design and operation of the Sample Filtration and Archiving (SaFA) instrument. *In:* Colon Padilla (2011). *Development of an Autonomous Sample Filtration and Archival Device for Aquatic Microbiology*. Masters of Science Thesis in Biological Oceanography, Department of Marine Science, University of Puerto Rico, Mayagüez.

3.1 Summary

The initial design parameters for the sample filtration and archiving (SaFA) instrument that have been influenced by this study are described. The SaFA (SaFA) system is being designed to collect and filter 24 user defined time-stamped water samples of between ~200-500 ml over a period of 3 days. Filters will be stabilized by the addition of a preservative buffer that will inhibit DNA degradation and changes in community composition on the filters. Seawater will be filtered through standard 47 mm filter holders that contain 0.22 µm filters. Two options for fluidic movements in the system have been developed and are being investigated. The system is in the initial stages of construction and is focusing on the use of commercially available parts.

3.2 Introduction

The aim of this study was to aid in the construction and design of the SaFA, by identifying potential preservation buffers for use in the instrument. Therefore the experimental design used to test the sample preservation buffers was based around the proposed operation of the instrument. The study was also of use to identify potential design and part configurations of the instrument including fluidic organization, filter holders and connectors.

Prior to starting this study, the initial design requirements of the instrument had been established which was used to for the experimental design outlined in Chapter 2. These design requirements were as follows:

- Collect and filter 24 water samples of between 200-500 ml using a 0.2 µm filter
- Filter the water samples in approximately 10 minutes
- Stabilize samples for a minimum of 3 days without appreciable DNA degradation or microbial community change.
- Use primarily commercially available parts.
- Be relatively low cost, small and light weight.

This chapter describes the initial work on constructing the SaFA system. The development of the SaFA instrument relies on interdisciplinary research. For the SaFA to operate properly the outcomes from the biological oriented studies has to be merged with engineering principles. This chapter aims to highlight how this is occurring and provides a summary of the progress toward building the SaFA. Much of this chapter contains work by Dr. Matthew Smith at the Department of Marine Science, University of Puerto Rico, Mayagüez (UPRM) and

colleagues at the Department of Mechanical Engineering UPRM, who are working toward building the SaFA system that is based in part on the work performed in this study. Therefore the engineering team of Dr. Pedro Quintero, Esteban Suarez, Andres Velasco, Ángel Gómez Vallejo, and Tania S. Cordero Figueroa are specifically acknowledged for their input of material in this chapter.

3.3 Materials and Methods

3.3.1 Fluidic Design

Many components for the SaFA system have been chosen based on prior work by Dr. Matthew Smith. The proposed system will sample water and pass it through individual filters using an NE-511 high pressure syringe pump (New Era Pump Systems, Inc., NY, USA). The water will be sent to individual filters using a 26 position rotary valve (C35Z-41826EUTA, VICI Valco Instruments Co. Ltd, TX, USA). To reduce contamination with previous water samples one fluidic port will not be connected to a filter as it will be used to flush the system with a small amount of the ambient water prior to filtration. A number of small solenoid valves (Part # 458302422ES, ASCO Valve Inc., NJ, USA) will also be used to direct water flow in and out of the system. Two types of filter housings were proposed, 25 mm Swinnex (Millipore Corporation, MA, USA) or 47 mm (Millipore Corporation, MA, USA; and Advantec, ToyoRoshi Kaisha, Ltd, Japan) were tested as potential filter housings. Two potential fluidic organizations based on pushing or pulling water through the system are proposed (Figure 3.1). A system organized to push water through the system (Figure 3.1A) the pump is placed up stream of the filter housings, the pump samples water directly from the environment and then forces the water through the filters. In the alternative method, where water is pulled through the system (Figure 3.1B), the pump is positioned after the filter; water from the environment is first drawn through the filter and into the pump.

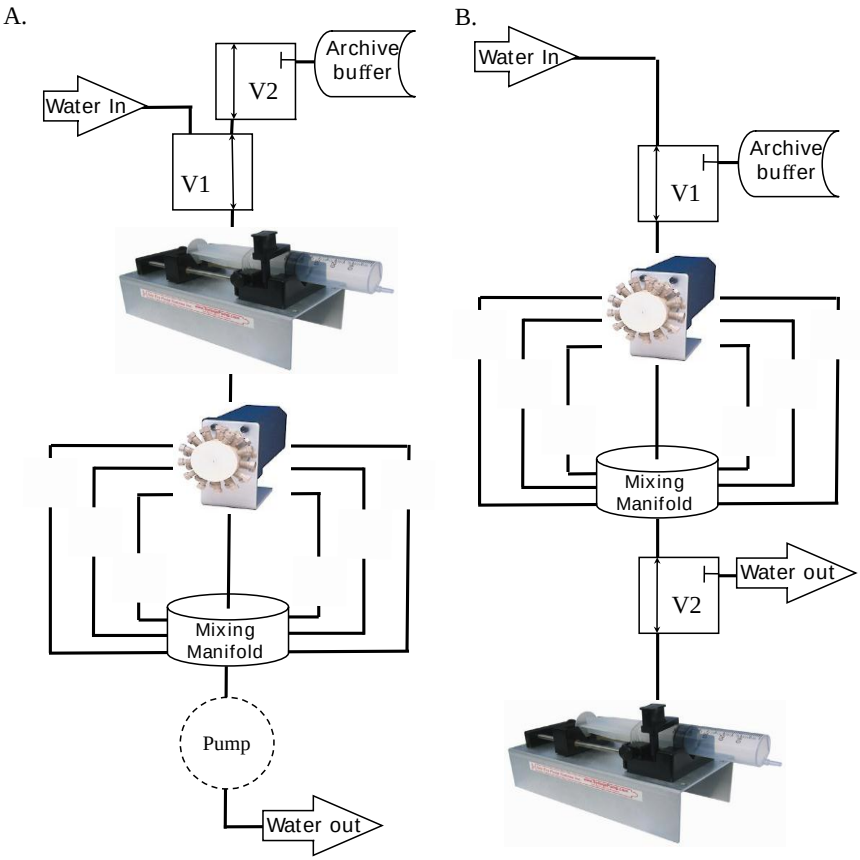


Figure 3.1. Potential fluidic designs of the SaFA system. Key fluidic components of the system are shown including the syringe Pump and rotary valve used in this study. For clarity, only 6 of the 24 filtration streams are shown. A. Fluidic design for pushing water through the filters. B. Fluidic design for pulling water through the filters.

3.3.2 Mechanical and Electronic Design and Power Requirements

The mechanical design and configuration for the internal components of the SaFA positioning of components was performed using the 3D computer Aided Drafting (CAD) software SolidWorks (Dassault Systèmes SolidWorks Corp. France). While specific descriptions of the operating software and electronic design are out of the scope of this chapter it is proposed that the SaFA is will be controlled using a Programmable Logic Controller (PLC) with power being supplied by UBI-2590 sealed lithium ion rechargeable batteries (Ultralife Batteries, NY, USA).

3.4 Results

3.4.1 Fluidic design

Filtration using 25 mm filter 0.2 µm Durapore filters contained in 25 mm Swinnex housings produced too much back pressure for the pump to adequately push water through. Increasing the filtration surface area by using 47 mm filters overcame this problem and for the water used in this study (Section 2.2.1) up to 500 ml of water can be easily filtered using this pumping system. Having decided on using 47 mm filters due to the decrease in back pressure, a suitable filter housing design needed to be chosen. A number of manufacturers make various styles of 47 mm housings. A number of different designs from different manufacturers where characterized, and we identified two different manufacturers (Advantec, ToyoRoshi Kaisha Ltd, Japan; and GE Osmonics, MN, USA) that had a suitable design for the SaFA application. The design of these tow filter housings is the same and they could be used interchangeably in the prototype SaFA. The design of both filters was chosen primarily because the inlet and outlet both had a 1/4" Male NPT thread on it which would enable fittings to be securely fastened to the housings, thereby reducing the possibility of leaks.

Having formalized a filter holder type, the fluidic connections and tubing for use in the plumbing of the instrument could then be addressed. Tubing will be connected to the individual components using barbed fittings. This will form a closed fluidic system which will also serve to reduce the possibility of leaks inside the pressure vessel. As each individual component uses a different type fitting, the challenge was to find a common barb size that would enable the same size tubing to be used throughout the system. Table 3.1 gives the details of the fittings used in the prototype SaFA that use the common barb size of 1/8 inch, this enables the use of 1/8 inch internal diameter tubing throughout the instrument (Table 3.1).

Table 3.1. Fluidic fittings and components used for the prototype SaFA

Part#	Description	Vendor	Use
SS-300-1-OR	3/16 OD bulkhead O-seal	Swadgelok, OH, USA	Connects to Outside water
A1032-2NK	10/32 Male UNF/1/8" barb	Eldon James, CO, USA	Connects to all valves
LF-2NN	Female leur/1/8" Barb	Eldon James, CO, USA	Connects to syringe
XO-2NK	4 way manifold 1/8" barb	Eldon James, CO, USA	Connects tubing post filtration
FLXC 2-3	Tubing 1/8" ID x 3/16" OD	Eldon James, CO, USA	Tubing
EW-31500-41	Female 1/4" NPT x 1/8" barb	Cole Palmer, IL, USA	Connects to Filter holder
EW-06623-22	Polypropylene filter holder	Cole Palmer, IL, USA	Contains the 47 mm filter

Based on the potential fluidic designs (Figure 3.1) the ability of the pump to pull or push water through the system was assessed. There did not appear to be any difference in the performance of the pump to filter water through a 0.2 µm filter contained in a 47 mm housing using either the push or pull mode. Based on these results, the decision was made to develop the prototype instrument based on the pulling of water through the system as highlighted in Figure 3.1B.

The proposed plumbing for the entire SaFA system is highlighted in Figure 3.2. To aid in the development of the control software, this fluidic configuration was used to develop process control flow charts for the instruments operation. The fluidic logic is in part based on the laboratory process used to test the preservation buffers and is split into 3 distinct logic events termed system flush, water filtration, and buffer addition (Figure 3.3).

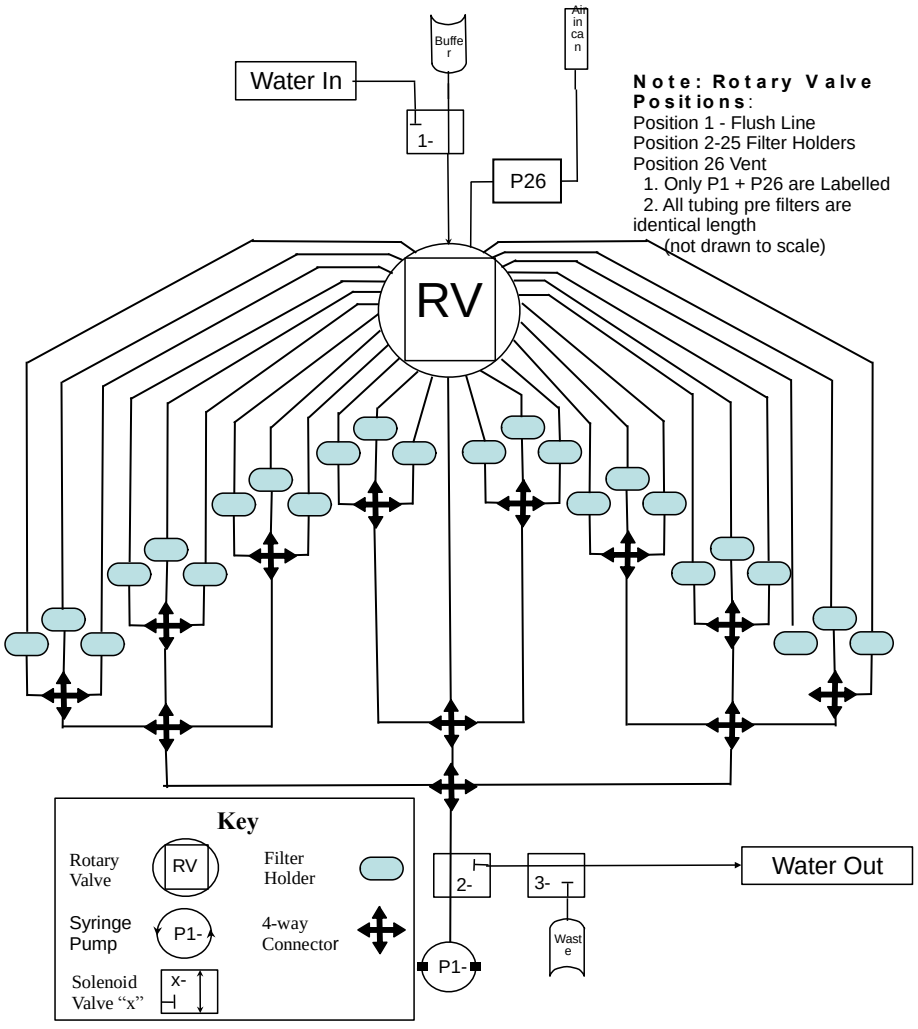


Figure 3.2. Fluidic organization of the prototype SaFA in the Pulling water configuration. All 24 filters are shown. A waste bag has been added to the design considerations to store flow through storage buffer. At this stage position 26 of the rotary valve is unused in the system. Adapted from Colon Padilla et al., (2011).

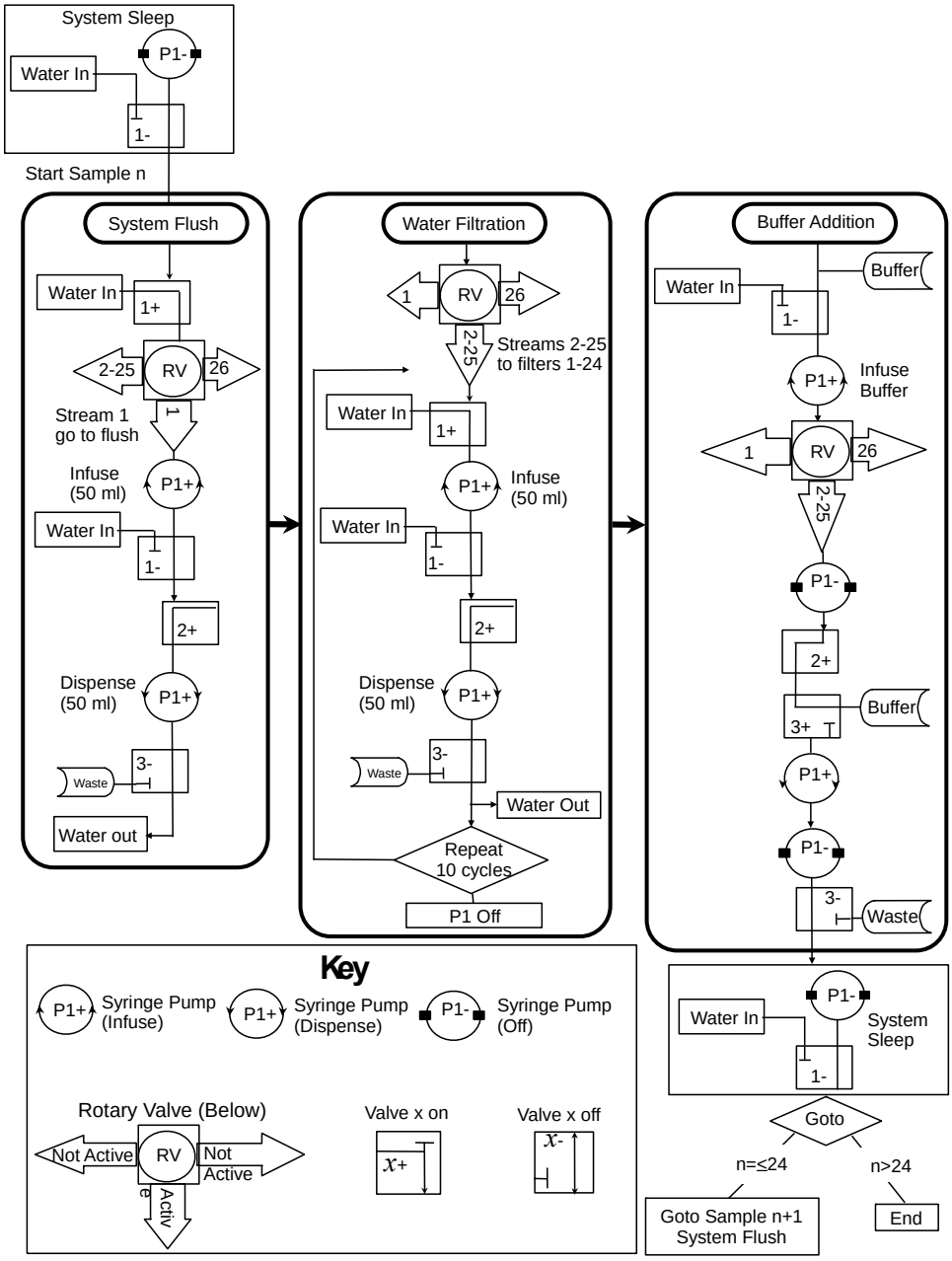


Figure 3.3. Prototype fluidic logic for the SaFa System. Adapted from Colon Padilla et al., (2011).

3.4.2 Mechanical design

With the major components of the SaFA system identified, a CAD model of the system could be constructed. The aim of the design was to use commonly available parts where possible. If custom manufactured parts were required, the designs were to be kept as simple as possible so that they could be manufactured cheaply and easily. From these design parameters, the SaFA chassis was designed using a series of simple plates that house the filter holders pumps valves and electronics (Figure 3.4). The plates are connected using common stainless steel 7/16" threaded rods. The distance between plates can be adjusted by moving locator nuts up and down the threaded rods. The diameter of the SaFA chassis was set to 9.4 inches. The external housing that the SaFA will fit into is the most complicated part that is required to be manufactured, it is anticipated to be manufactured from 10" schedule 80 PVC pipe which has an internal diameter of 9.5 inches.

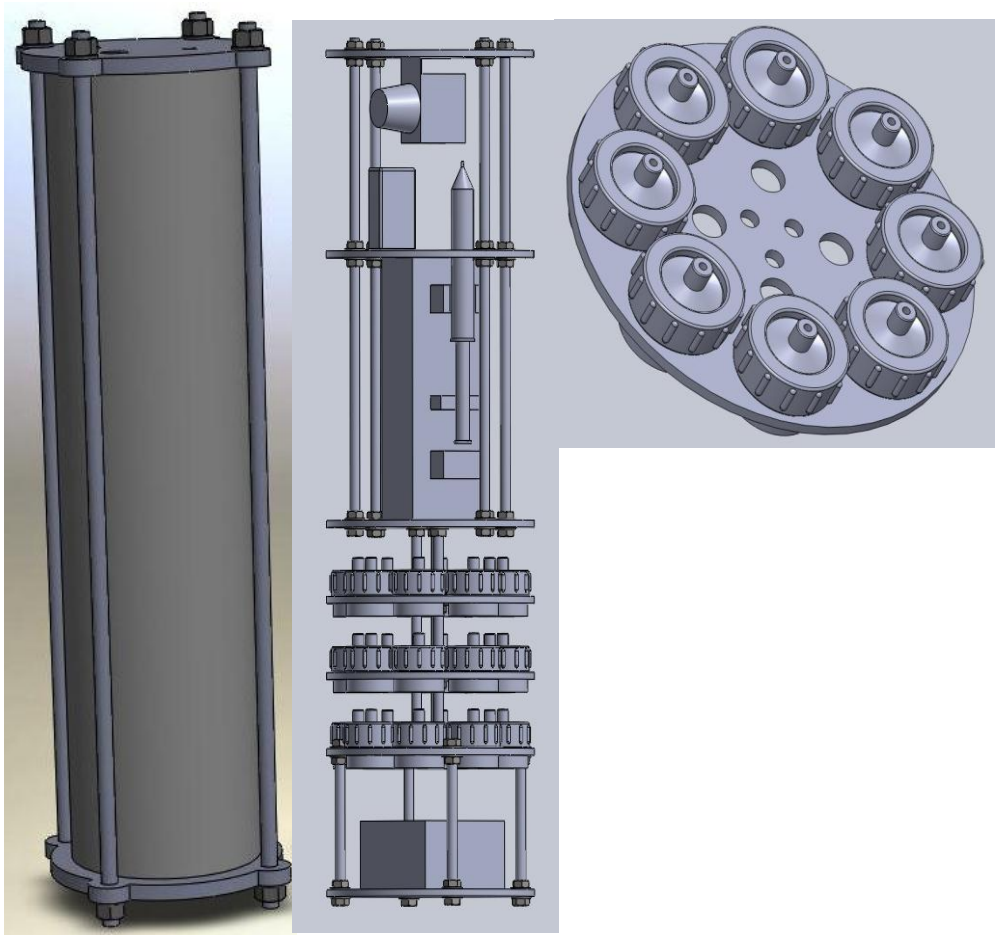


Figure 3.4 .CAD drawing of the prototype SaFA system. A. External pressure vessel and internal chassis design. B. View of the filter holding plate.

Based on these schematics outlined above the internal components are being constructed from 0.25 inch thick PVC sheets (Figure 3.5A & B). As the instrument is going to be designed to be open source all schematics to enable SaFA to be constructed will be available for download.

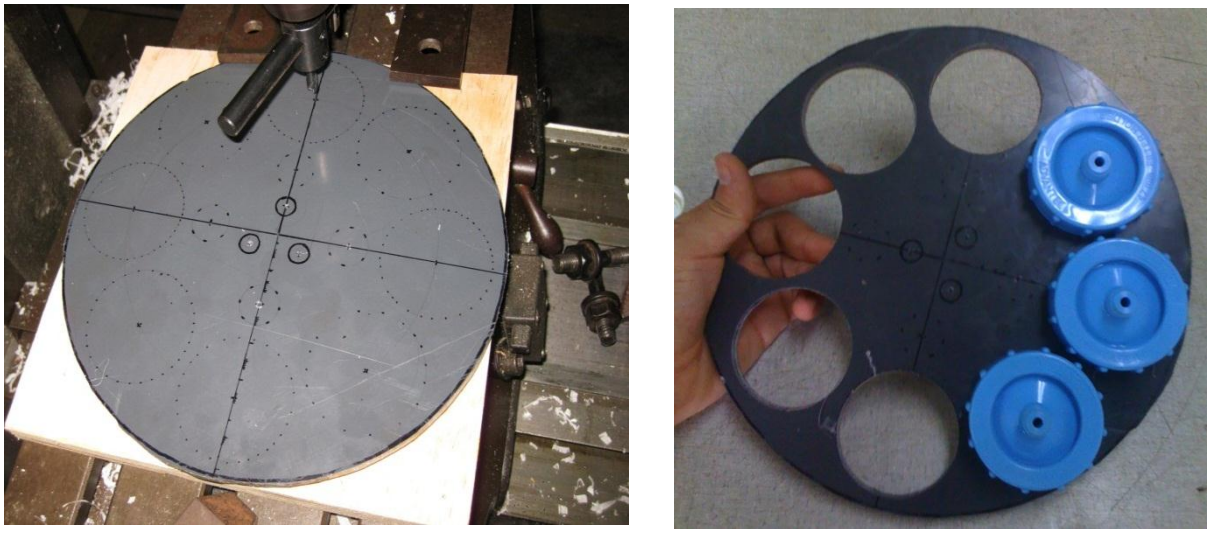


Figure 3.5 Construction of the prototype SaFA system. A. The plastic plate that holds the filter holders under construction B. Completed filter plate.

3.5 Discussion

The construction of the SaFA is currently underway; the approximate in water dimensions for the finalized SaFA prototype are 25.4 cm in diameter and 1 m in length. While the use of 25 mm filters may have reduced the overall size of the instrument, the increased back pressure problems that were observed with this sized filter would have required a larger pump capable of higher pressures to be implemented. This would potentially have negated the reduction in size as it potentially would have had increased power requirements, thereby requiring an increase in batteries.

The fluidic strategy of pulling water through the system offers a number of advantages. From a biological perspective, having the pump located downstream of the filters reduces the chance of the pump being a source of contamination from carry over water from the previous sample. Additionally, a reduced amount of water will be required to flush the system at the start of each sample, as there is only a small amount of tubing that is shared. This should serve to decrease power requirements of the system compared having to perform multiple washes on a pump that is situated before the filter. As the SaFA is to be deployed underwater, when the inlet valve is opened, there will be a positive pressure being exerted on the filter as water tries to enter the system, this pressure may potentially aid in the filtration process and reduce the amount of work that the pump has to perform. In the alternative scenario, the pump would be buffering this positive pressure. A final advantage of pulling the water through the system is that it potentially

will use less components such as a "jockey" pump that aids in removing water from the system, which also will potentially decrease the control software complexity and decrease the cost of manufacture.

Excluding labour or shipping costs and based on the pricing for the individual components currently being used to construct the prototype SaFA, is estimated to that it will cost approximately $9,000 in parts to build the system. Current approximate pricing for the major components of the system is highlighted in Table 3.2.

Table 3.2. Pricing of the prototype SaFA system

Category	Part #	Description	Unit ($)	No.	Total ($)
PLC	AFPORT32CT-FP0R	Panasonic PLC	368	1	368
	AFC8513-US 3M	Cable for PLC programming	69	1	69
Pump	NE-511	New Era Syringe Pump	590	1	590
Valves	C35Z-41826EUTA	Valco Rotary Valve (26 Position)	1515.00	1	1515
	458302422ES	ASCO 3-way universal	120.52	3	361.55
Filters	EW-06623-22	Advantec, Cole Palmer	61	24	1464
Batteries	UBI-2590 (UBBL02)	Ultralife Rechargeable Lithium Battery	353.13	2	706.26
	N/A	Ultralife Charger for UBBL02	275	1	275
	BAI-10G	UBBL02 Connectors Battery Axxessories, Inc.	13.50	2	27
Pressure vessel	Custom	Q-Proto, St Petersburg, FL	2900	1	2900
Misc.		Threaded rods, Fittings, Tubing etc.,	500	1	500
					Total cost
					8,775.81

As the SaFA system is will be available to individual researchers under an open source agreement it is hoped that it will enable researchers to increase the scope of their microbial ecology studies. Undoubtedly, as the system provides samples over time, a variety of researchers will be able to investigate their specific areas of interest with using a variety of methods. It is hoped that these researchers will continue to modify the system for new applications. The evolution of the system will hopefully further increase the user base of the SaFA system and help move ultimately advance our knowledge of the important role microbes play in the environment.

3.6 Summary and Recommendations

The SaFA has the potential to fill a gap in microbial ecology studies by increasing the temporal resolution that samples are obtained over. The ability of the instrument to obtain samples autonomously reduces the personnel requirements for people to be present in the field. This may reduce the cost of sampling, and also enable samples to be taken during conditions that may be unsafe to personnel. Similarly, the relatively small size and weight of the SaFA will enable it to be deployed relatively easily from small boats without the need for specialized equipment such as winches and A-frames.

The SaFA operates by filtering discrete water samples and then stabilizing them with a preservation buffer. The preservation buffer has the two-fold purpose of preventing changes in

the microbial community and, to stabilize the DNA in the sample. Upon retrieval of the SaFA, the filters are available for subsequent analysis in the laboratory using molecular techniques. The SaFA is therefore an assay independent device, enabling a wide variety of downstream molecular biological tests to be performed on the filters. However, end users will be encouraged to test and verify the performance of the storage buffer for their particular application. This includes the type of downstream molecular assay, and the type of environmental samples that the SaFA will be taking. However, it is obvious that the SaFA could have broad application in microbial ecology studies including microbial community structure and dynamics; anthropogenic impacts (e.g., fecal source tracking and public health); and investigating the microbial dynamics in biogeochemical cycling. While the SaFA design and construction has primarily focused on application in microbial ecology studies. The ability to obtain time stamped samples from aquatic environments may be applicable to other scientific disciplines such as chemical analysis of the water column. In this case the SaFA could be tuned to the end application by changing the type of filter and the preservation buffer.

The SaFA is being constructed as an open source instrument that uses commercially available components. The majority of the instrument is manufactured using commonly available tools, with only the pressure vessel requiring strict machining tolerances. As such it is hoped that the relatively low cost (~ $10,000) of the SaFA will enable broad integration of the device into the aquatic sciences. It is hoped that end users of this technology will provide feedback to other users of the SaFA providing information on new applications, preservation buffers or to improvements in the SaFA's design.

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