RNA Detection Technology for Applications in Marine Science: Microbes to Fish

Robert Michael Ulrich
University of South Florida, bobmiu@aol.com

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RNA Detection Technology for Applications in Marine Science: Microbes to Fish

by

Robert M. Ulrich

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
College of Marine Science
University of South Florida

Major Professor: John H. Paul, Ph.D.
Valerie J. Harwood, Ph.D.
Mya Breitbart, Ph.D.
Christopher D. Stallings, Ph.D.
David E. John, Ph.D.

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Keywords: NASBA, grouper, *Karenia mikimotoi*, Enterococcus

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DEDICATION

This dissertation is dedicated to my fiancée, Dr. Shannon McQuaig for inspiring my return to graduate school and her continued support over the last four years. On no other porch in our little town have there been more impactful scientific discussions, nor more words of encouragement.
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ABSTRACT

The accurate identification of taxa from mixed assemblages using genetic analysis remains an important field of molecular biology research. The common principle behind the development of numerous documented genetic detection technologies is to exploit specific nucleotide sequences inherent to each taxon. This body of work focuses on practical applications of real-time nucleic acid sequence-based amplification (RT-NASBA) in marine science, and is presented in four case studies. Each study represents novel work in the genetic identification of respective taxa of interest using RT-NASBA. Two case studies documented the development of an assay targeting mitochondrial 16S rRNA to discern legally salable grouper species in the U.S. from fraudulently mislabeled surrogate fish. This technology was first validated using lab-based, benchtop instrumentation, and was then adapted into a complete field detection system. The third study documented an internally controlled RT-NASBA (IC-NASBA) assay for the detection and quantification of the harmful algal bloom-causing dinoflagellate, *Karenia mikimotoi*, by targeting the ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO) large-subunit gene (*rbcL*). The final section of this dissertation details the preliminary development of an IC-NASBA assay targeting large subunit rRNA for the quantification of *Enterococcus*, which is a genus of bacteria commonly used as an indicator of fecal pollution in recreational marine water. My results show that RT-NASBA provides a suitable format for the accurate identification of target species from these taxa which include prokaryotes, as well as both unicellular and multicellular eukaryotes.
CHAPTER ONE:
INTRODUCTION

Nucleic acid sequence-based amplification (NASBA) is an isothermal method of RNA amplification first described by Compton (1991). It is similar to the polymerase chain reaction (PCR) in that both involve the amplification of nucleic acid targets via enzymatic reactions using two specific oligonucleotide primers. However, whereas conventional PCR employs a single DNA polymerase to amplify DNA templates, NASBA targets RNA templates and is catalyzed by three enzymes working in concert; T7 RNA polymerase, avian myoblastosis virus reverse transcriptase (AMV-RT), and RNaseH. Moreover, NASBA is carried out isothermally at 41°C, whereas PCR requires thermal cycling within large temperature ranges typically from 50°C to 95°C. In principle, the NASBA reaction begins when an oligonucleotide primer (P1) hybridizes to a complimentary region of the target RNA. This primer also contains a T7 RNA polymerase binding site on its 3’ end. The AMV-RT begins incorporating nucleotides on the 3’ end of the primer, creating a cDNA copy of the RNA template that forms a RNA/DNA hybrid. The RNaseH enzyme then recognizes this hybrid as substrate and degrades the RNA portion, leaving behind only single-stranded cDNA. A second primer (P2), also specific for a portion of the target sequence, then anneals to the cDNA template and is extended by AMV-RT rendering the T7 promoter region double stranded, and thus transcriptionally active. The T7 RNA polymerase now recognizes the functional promoter and produces multiple copies of anti-sense RNA, which can then act as templates themselves, and are again converted into transcriptionally active DNA
intermediates. Thus, an exponential increase in RNA copies occurs from each RNA target in a cyclical fashion. A schematic overview of the NASBA process can be seen in Figure 1.

![NASBA amplification pathway](image)

**Fig. 1.** NASBA amplification pathway. Wavy lines represent RNA and straight lines represent DNA.

An extension of conventional NASBA by the incorporation of fluorescently-labeled molecular beacons (Tyagi & Kramer, 1996) allows for real-time detection of NASBA amplicons (RT-NASBA) first described by Leone et al. (1998). Molecular beacons are fluorescently-labeled oligonucleotides that contain a homologous sequence to a specific target site, typically interior of the primer binding sites. Molecular beacons are constructed to form a unique hairpin-
loop structure which allows for internal quenching of the fluorophore when in their innate form. When in the presence of the complimentary target sequence, a hybridization event occurs that causes a conformational change of the hairpin structure, restoring fluorescence. This fluorescence is read by specialized fluorometers in real-time. Thus, increase in reaction fluorescence is proportional to the specific nucleic acid copy number being amplified via RT-NASBA. Using RT-NASBA over conventional NASBA, forgoes the need of any electrophoretic methods that typically extend the turnover times required for data acquisition. Moreover, RT-NASBA allows for the quantification of various microorganisms, either in pure culture or within heterogeneous communities. Quantification of RNA by RT-NASBA occurs by comparing time to positive (TTP) values of unknown fluorescence amplification plots to TTP values generated from a standard curve of known target RNA copy numbers or known concentrations of control organisms. A more detailed description of quantification by RT-NASBA will be discussed in following sections.

The number of published RT-NASBA assays has grown steadily over the past 20 years with assays developed for the specific detection and/or quantifications of a myriad of taxonomically diverse microorganisms and viruses. Most of these assays were developed (and are continuing to be developed) to target clinically relevant microorganisms, although applications of RT-NASBA targeting microorganisms with environmental relevancy are increasing. Some recent RT-NASBA applications targeting various important pathogenic bacterial species are assays for *Escherichia coli* (Kao & Durst, 2010), *Vibrio cholerae* (Fykse, Nilsen, Nielsen, Tryland, Delacroix, & Blatny, 2012), *Mycoplasma pneumonia* (Loens, Beck, Ursi, Overdijk, Sillekens, Goossens, et al., 2008), *Campylobacter jejuni* (Churuca, Girbau, Martinez, Mateo, Alonso, & Fernandez-Astorga, 2007), and *Salmonella enterica* (D'Souza &
Jaykus, 2003). Several clinically important RNA viruses have also recently been targeted using RT-NASBA including the avian influenza (HPAI) virus (Sakurai & Shibasaki, 2012), human rhinovirus (HRV) (Sidoti, Bergallo, Terlizzi, Alessio, Astegiano, Gasparini, et al., 2012), influenza A virus (H1N1) (Ge, Cui, Qi, Shan, Shan, Qi, et al., 2010), human immunodeficiency virus (HIV) (Forbi, Gabadi, Iperepolu, Esona, & Agwale, 2010), human noroviruses (Lamhoujeb, Fliss, Ngazoa, & Jean, 2008; Patterson, Smith, Casper, Huffman, Stark, Fries, et al., 2006), and enteroviruses (Casper, Patterson, Smith, & Paul, 2005). RT-NASBA assays have also been developed to detect pathogens from other taxonomic phyla including a fungal Aspergillus spp. (Kim, Park, Kwon, Shin, Kwon, Park, et al., 2012), the protozoan Trypanosoma brucei (Mugasa, Laurent, Schoone, Kager, Lubega, & Schallig, 2009), and a species of malaria-causing Plasmodium (Mens, Schoone, Kager, & Schallig, 2006).

Several microorganisms having more ecological relevance have also been targets for RT-NASBA assay development, including two harmful algal bloom-causing dinoflagellates; Karenia brevis (Casper, Paul, Smith, & Gray, 2004) and Karenia mikimotoi (Ulrich, Casper, Campbell, Richardson, Heil, & Paul, 2010), as well as the neurotoxin-producing diatom Pseudo-nitzschia multiseries (Delaney, Ulrich, & Paul, 2011). RT-NASBA has become a powerful tool, and its frequency of application is beginning to rival more conventional methods of specific RNA detection and quantification, such as reverse transcriptase quantitative PCR (RT-qPCR) (Fakruddin, Mazumdar, Chowdhury, & Bin Mannan, 2012). Moreover, there are attributes of IC-NASBA that make it more suitable for several molecular identification applications, some of which are detailed in subsequent sections of this dissertation.
Overview of Dissertation

The underlying theme of my dissertation research was to develop and validate RT-NASBA assays targeting several organisms that are important to both marine ecology and marine-related human resources. The work presented here is composed of three chapters that have been published in, or have been submitted to peer-reviewed journals. Each of these chapters is meant to stand alone as a complete research project including respective tables, figures, and references. The appendix section contains preliminary research towards the development of a fourth RT-NASBA assay, and is structured similar to the aforementioned chapters.

- Chapter Two details the development of a RT-NASBA assay utilizing commercial benchtop instrumentation to discern legally salable grouper species from impostor fish in regards to seafood mislabeling fraud. This work has been published in Food Control (Ulrich, John, Barton, Hendrick, Fries, & Paul, 2013).
- Chapter Three describes the integration of the RT-NASBA assay developed in Chapter Two, into a handheld sensor format for the identification of grouper in the field. This work has been submitted to Food Chemistry.
- Chapter Four details the development of an IC-NASBA assay for the detection and quantification of the harmful algal bloom-causing dinoflagellate, Karenia mikimotoi. This work has been published in Harmful Algae (Ulrich, Casper, Campbell, Richardson, Heil, & Paul, 2010).
- The Appendix describes preliminary research towards developing an IC-NASBA assay for the detection and quantification of Enterococcus, which is a genus of bacteria commonly used as an indicator of fecal pollution in coastal marine environments.
References


CHAPTER TWO:
ENSURING SEAFOOD IDENTITY: GROUPER IDENTIFICATION BY REAL-TIME NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION (RT-NASBA)

Note to Reader
This chapter has been published in full (Ulrich, John, Barton, Hendrick, Fries, & Paul, 2013), and is included with permission from Elsevier®.

Abstract
Grouper are one of the most economically important seafood products in the state of Florida and their popularity as a high-end restaurant dish is increasing across the U.S. There is an increased incidence rate of the purposeful, fraudulent mislabeling of less costly and more readily available fish species as grouper in the U.S., particularly in Florida. This is compounded by commercial quotas on grouper becoming increasingly more restrictive, which continues to drive both wholesale and restaurant prices higher each year. Currently, the U.S. Food and Drug Administration recognize 56 species of fish that can use “grouper” as an acceptable market name for interstate commerce. This group of fish includes species from ten different genera, making accurate taxonomic identification difficult especially if distinguishing features such as skin, head, and tail have been removed. This is leading regulatory agencies to employ genetic identification methods which tend to have much higher species-level resolution than phenotypic methods. Standard genetic identification methods are highly technical and require expensive
A continuing challenge facing the seafood industry is the accurate identification of fish sold by wholesalers, seafood markets, and restaurants. Cases of fraudulent mislabeling of lesser-valued species as higher-valued are becoming more common as commercial quotas become more restrictive. With finfish species, this type of fraud is often made possible due to similarities in texture, appearance, and taste when in fillet form (Buck, 2010). Accurate identification of fillets becomes even more difficult after the fish has been prepared and cooked to edible presentations served in restaurants. The U.S. Food and Drug Administration (FDA) is the principal federal agency responsible for ensuring the accurate labeling of food sold in interstate commerce, and is aided in international seafood fraud prevention by the Department of Homeland Security’s Customs and Border Protection (CBP), and the Department of Commerce’s National Marine Fisheries Service (NMFS).

One of the most important commercial seafood products in the state of Florida is grouper. According to the Florida Department of Agriculture and Consumer Services, this iconic seafood product was the most valuable finfish harvested in Florida in 2010, with a dockside value estimated at over $14 million (Putnam, 2010). The high demand and increased commercial
regulation posed on grouper has led to an increase in the purposeful mislabeling of cheaper
finfish as “grouper” in an effort to commit economic fraud by both wholesalers and restaurants.
In 2008, a $300,000 settlement was reached between the Florida Attorney General and a
prominent seafood wholesaler for the purposeful mislabeling of Asian catfish as “grouper”
(Nohlgren, 2008). In 2012, similar charges were brought against a California-based wholesaler
amounting to a fine of $1 million and a three year probation period (Staff, 2012). The fault of
seafood wholesalers in the consumer-level consumption of mislabeled grouper is not always
warranted, as several high-profile investigations uncovered purposeful substitutions by
restaurateurs (Copes, 2007; Nohlgren, 2007, p.A1; Staff, 2011). One investigation uncovered
that 17 of 24 Tampa Bay (FL) area restaurants surveyed were serving impostor fish instead of
grouper, as ordered from the menu. Substituted species were found to be to be Emperor
(generally a Lethrinus spp.), Hake (typically Urophycis or Merluccius spp.), Sutchi (Pangasius
hypophthalmus), Bream (numerous species are considered bream), Green Weakfish (Cynoscion
virescens) and Painted Sweetlips (Diagramma pictum) (Copes, 2007).

The proper identification of seafood relies on the correct use of standard market names as
many global species have differing indigenous vernacular names, and the use of scientific
binomial nomenclature is often impractical in the seafood industry. Putative naming of
commercially important seafood fish is aided by the standardization of common names
performed by professional societies (Nelson, 2004). Moreover, the NMFS and FDA have
cooperated in creating the “Seafood List” which is a compilation of acceptable market names
(including binomial nomenclature) for both domestic and imported seafood species (FDA, 2011).
Currently, there are 56 species (all in the family Serranidae) spanning ten distinct genera of fish
that can carry the acceptable market name “grouper” according to the FDA Seafood List (FDA,
Most of these species are included in three major genera: *Epinephelus* (32 species), *Cephalopholis* (8 species), and *Mycteroperca* (8 species). The rest are included in 7 minor genera: *Aethaloperca* (1 species), *Anyperodon* (1 species), *Capradon* (1 species), *Dermatolepis* (1 species), *Diplectrum* (1 species), *Plectropomus* (2 species), and *Variola* (1 species). This wide species diversity often makes accurate taxonomic identification difficult using only phenotypic characteristics, and can become even more difficult when in fillet form with the head and skin removed. Therefore, it is now common practice for seafood regulatory agencies to employ the use of molecular (protein, DNA, and RNA) identification techniques, which offer much higher taxonomic resolution than phenotypic identification.

Various protein-based techniques including immunologic, electrophoretic, and chromatographic methods have been employed to identify seafood species. Immunologic methods tend to be best suited for high-throughput sample analysis, whereas chromatographic and electrophoretic methods are technologically complex and require specialized facilities and instrumentation not routinely available to seafood regulatory agencies (Rasmussen & Morrissey, 2008). Until very recently (2011), protein isoelectric focusing (IEF) of soluble muscle proteins (electrophoretic technique) was the standard method for seafood species identification used by the FDA (AOAC, 1980). This method has been described as somewhat unreliable for species-level identification for heat-processed or dried fish products, and thus may not be suitable for use on cooked fish (Rasmussen & Morrissey, 2008). Recent research has been focused toward the use of nucleic acid-based technologies for more accurate identification of seafood species. Due to the degeneracy of some gene sequences and the presence of non-coding regions that are not translated to proteins, nucleic acid sequence analysis offers increased resolution in identifying fish species over protein-based techniques (Rasmussen & Morrissey, 2008). Typically, DNA-
based methods of fish identification employ the use of polymerase chain reaction (PCR) to amplify target genes for subsequent use in random amplified polymorphic DNA analyses (DNA-RAPD) (Asensio et al., 2002), PCR restriction fragment length polymorphism (PCR-RFLP) (Asensio et al., 2000), and PCR-single strand conformational polymorphism (PCR-SSCP) (Asensio et al., 2001). These techniques all produce electrophoretic DNA banding patterns which are referenced to a database generated from vouchered specimens also generated by their respective method. PCR-based methodologies require benchtop thermal cyclers which have cycling capacities ranging from approximately 50°C to 95°C. These techniques also require electrophoretic equipment, gel imaging apparatus (typically UV light generation), and expensive gel analysis software.

DNA barcoding is the latest evolution of DNA-based methods for seafood species identification. This technique involves PCR amplification of a specific gene locus, typically the mitochondrial cytochrome c oxidase subunit I gene (COI), and performing DNA sequencing reactions on the amplified gene target. This produces an exact DNA nucleotide sequence (~650-bp) of the COI gene for the tested sample. This unknown sequence is then referenced to an online database containing known reference sequences obtained from verified, or vouchered, specimens (Yancy et al., 2008). The FDA recently approved DNA barcoding as a regulatory protocol to detect seafood fraud, in consortium with the Fish Barcode of Life (FISH-BOL) Initiative (www.fishbol.org), which is an international effort to assemble a standardized reference DNA sequence library for all fish species, using only vouchered reference specimens (Hanner, 2011). DNA barcoding methodology has advantages, such as the use of a standardized gene locus, and standardized laboratory procedures for its amplification. However, this method still requires PCR amplification, and is even more daunting in required hardware, reagent, and
expertise. Thus, DNA sequence analysis is typically outsourced to only a few labs that have this capacity, increasing cost and turn-around time for results.

An alternate molecular technique termed nucleic acid sequence-based amplification (NASBA) is similar to PCR in that both involve the amplification of specific nucleic acid sequences via enzymatic reactions (Compton, 1991). However, NASBA targets ribonucleic acid (RNA) rather than DNA as in PCR, and NASBA is carried out isothermally (41°C) whereas PCR requires thermal cycling within large temperature ranges up to 95°C. An extension of conventional NASBA by the incorporation of fluorescently-labeled molecular beacons (Tyagi & Kramer, 1996) allows for real-time detection of NASBA amplicons (RT-NASBA) first described by Leone van Schijndel, van Gemen, Kramer, and Schoen (1998). Our lab has an extensive knowledge base in the development of RT-NASBA assays to detect several microorganisms (Casper, Patterson, Smith, & Paul, 2005; Casper, Paul, Smith, & Gray, 2004; Delaney, Ulrich, & Paul, 2011; Patterson, Casper, Garcia-Rubio, Smith, & Paul, 2005; Patterson et al., 2006; Ulrich et al., 2010), and we have recognized its potential applications in the accurate and timely identification of seafood species.

Both ribosomal (mitochondrial rDNA) and COI genes have previously been evaluated using both PCR and real-time PCR (RT-PCR) assays to accurately identify grouper species (Asensio, Gonzalez, Rojas, Garcia, & Martin, 2009; Chen et al., 2012; Trotta et al., 2005). There is evidence that the 16S rRNA gene is adequate for discriminating some *Epinephelus* and *Mycteroperca* species from non-target species (Trotta et al., 2005). However the COI target gene may be better suited for accurately identifying some of the Asian market groupers, such as *Plectropomus* and *Caprodon* species (Chen, et al., 2012). Taking this evidence into account, as well as the understanding that rRNA tends to be more resistant to degradation than messenger
RNA (mRNA), which is the form of COI RNA in tissue cells (Houseley & Tollervey, 2009; Mitchell & Tollervey, 2000), we chose to target 16S rRNA in the development of this assay. Moreover, there is an abundance of 16S rDNA sequence information from numerous fish species in public databases (i.e. GenBank) to aid in bioinformatic analysis used in gene target design. Here we report the development of a 90 min multiplex RT-NASBA assay targeting a portion of the mitochondrial 16S ribosomal RNA (rRNA) gene for the accurate identification of most grouper species on the FDA Seafood List with no cross-reactivity to non-target species. We also provide evidence that this assay can verify or negate the claim of grouper even when testing previously cooked fish.

**Materials and Methods**

**Sequencing 16S rDNA from grouper tissue**

Numerous fish samples were obtained from several sources including: The National Oceanic and Atmospheric Administration (NOAA), Southeast Fisheries Science Center (Charleston, SC); SCRIPPS Institution of Oceanography (SCRIPPS), Marine Vertebrates Collection (La Jolla, CA); University of Kansas (KU) Natural History Museum & Biodiversity Research Center, Division of Ichthyology (Lawrence, KS); University of South Florida (USF), College of Marine Science, David Mann Lab; and Bama (BAMA) Sea Products, Inc. (St. Petersburg, FL) (Table 1). Tissue samples were preserved in 99% ethanol (KU), 50% isopropanol (SCRIPPS), or frozen without preservatives (NOAA, BAMA, and USF). To verify species identity for each tissue sample, as well as provide additional sequence information for RT-NASBA primer and molecular beacon design, 16S rDNA sequence analysis was performed. DNA was extracted and purified from tissues using DNeasy® Blood & Tissue Kit (Qiagen,
Valencia, CA) per manufacturer’s instructions for animal tissues. A highly conserved portion of the 16S rDNA gene (~500-bp) was amplified using PCR utilizing oligonucleotide primers designed in our lab (forward primer, 5’-TATAAGAGGTCCCGCCTG-3’; reverse primer, 5’-ACAAACGAACCCCTTAATAGC-3’). The resulting amplicons were TOPO® TA cloned into a pCR®II cloning vector (Invitrogen, Carlsbad, CA) per manufacturer’s instructions and were sequenced using the Sanger method at the DNA Sequencing Core at the University of Florida.

**Table 1.** Fish species permitted by the FDA (2011) to be sold as grouper in the U.S. and tissue samples used for assay validation.

<table>
<thead>
<tr>
<th>Latin Binary Name</th>
<th>Common Name</th>
<th>GenBank Accession Number</th>
<th>Tissue Sample Designation from Source</th>
<th>Tissue Sample Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Epinephelus acanthistius</em></td>
<td>Gulf Coney</td>
<td>HQ010109</td>
<td>19-74-2^a</td>
<td>NOAA</td>
</tr>
<tr>
<td><em>Epinephelus areolatus</em></td>
<td>Persian Grouper</td>
<td>DQ088038</td>
<td>07-77</td>
<td>SCRIPPS</td>
</tr>
<tr>
<td><em>Epinephelus coioides</em></td>
<td>Orange-spotted Grouper</td>
<td>JF750751</td>
<td>64-235</td>
<td>SCRIPPS</td>
</tr>
<tr>
<td><em>Epinephelus diacanthus</em></td>
<td>Spinycheek Grouper</td>
<td>DQ088043</td>
<td>No designation</td>
<td>BAMA</td>
</tr>
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<td><em>Epinephelus fasciatus</em></td>
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<td>DQ088039</td>
<td>5716^a</td>
<td>KU</td>
</tr>
<tr>
<td><em>Epinephelus flavolimbatus</em></td>
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<td>AF297293</td>
<td>1142^a</td>
<td>KU</td>
</tr>
<tr>
<td><em>Epinephelus guttatus</em></td>
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<td>AF297299</td>
<td>ST159EG^a</td>
<td>NOAA</td>
</tr>
<tr>
<td><em>Epinephelus hexagonatus</em></td>
<td>Starspotted Grouper</td>
<td>DQ154106</td>
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<td>KU</td>
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</table>

GenBank accession numbers represent a portion of the 16S rDNA gene for each species used in Clustal alignments. Sequences obtained from tissue samples are not from the same specimens referenced in GenBank.

Minus sign designates species not available for tissue analysis.

a Tissue from vouchered specimens.

**Primer and molecular beacon design**

Target 16S rDNA sequences from grouper species obtained from GenBank and sequencing efforts in our lab as well as numerous non-target GenBank sequences from likely
 impostor fish (e.g. Tilapia, Emperor, Hake, Catfish, and Bream) were aligned using the Clustal W 1.6 algorithm (Thompson, Higgins, & Gibson, 1994) in the MEGA 5 software package (Tamura et al., 2011). Forward and reverse primers were designed to target a conserved, 136-bp region of the 16S rDNA gene. Primers were analyzed for self- and hetero-dimerization using OligoAnalyzer 3.1 primer design tool (http://www.idtdna.com/analyzer/applications/oligoanalyzer/) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 2).

<table>
<thead>
<tr>
<th>Table 2. RT-NASBA oligonucleotides</th>
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<tr>
<td>Oligonucleotide</td>
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<td>Grouper NASBA forward primer</td>
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<tr>
<td>Molecular beacon B</td>
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<tr>
<td>Molecular beacon C</td>
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</table>

Italicized text indicates T7 RNA polymerase promoter site.

Due to target sequence heterogeneity found with some grouper species, a total of three molecular beacon variants were designed to target as many FDA grouper species as possible, while still allowing for non-target discrimination. Molecular beacons were designed internal of the two primers and labeled with 6-carboxyfluorescein (FAM) on the 5’ end and DABCYL on the 3’ end (Table 2). The three beacons were synthesized by Eurofins MWG Operon (formerly Operon, Huntsville, AL).

**RNA extraction and RT-NASBA assay**

RNA was extracted and purified from both target and non-target tissues using a slightly modified RNeasy® Mini Kit protocol (Qiagen, Valencia, CA). Briefly, a small (5-10 mg) piece
of tissue was removed from samples using a sterile, 2 mm biopsy punch (Integra™ Milex®, York, PA) and placed into 500 µl RLT lysis buffer. After a 10 min incubation at room temperature with intermittent vortex agitation, 200 µl of 100% ethanol was added to the lysate. The entire volume of lysate (attempting to exclude any un-lysed tissue) was added to the RNA purification column, and the rest of the purification protocol was carried out per manufacturer’s instructions. Purified RNA was eluted from the column using 100 µl nuclease-free water, and stored on ice until its use as RT-NASBA template. All RT-NASBA reactions were carried out in triplicate for each tissue punch using NucliSENS EasyQ® Basic Kit (bio-Mérieux, Durham, NC). Primers were diluted to a final concentration of 400 nM, and the three molecular beacons were diluted to a final concentration of 100 nM each. The optimal KCl concentration was determined to be 80 mM (data not shown). To maximize the number of reactions per kit, 10 µl reaction volumes were used (half the volume recommended by bio-Mérieux) containing 5 µl reagent mix (containing primers and beacons), 2.5 µl RNA template, and 2.5 µl enzyme mix. RT-NASBA amplification and fluorescence detection were carried out at 41°C for 90 min using a NucliSENS EasyQ® analyzer (bio-Mérieux, Durham, NC). A defined fluorescence value was chosen as a positive signal level to be used in all subsequent assays. Typically, this value was 0.25 relative fluorescence units (rfu) above the final fluorescence generated from a negative control reaction (generally 1.35 rfu). The time at which target amplification curves reached the threshold level was recorded as the time to positivity (TTP) in minutes for each reaction.

**RT-NASBA on cooked samples**

We obtained three frozen fillets of *Epinephelus diacanthus* (Spineycheek Grouper) from Bama Sea Products, Inc., which is a major seafood wholesaler in Florida that regularly authenticates their imported fish samples using outsourced DNA lab testing. The species of each
fillet was confirmed by DNA sequence analysis performed in our lab as described above. Each fillet had approximately the same size dimensions (17 cm long × 7 cm wide × 2 cm thick) and also weighed approximately the same (110 g). Three separate tissue pieces (5 mg) were removed from each of the three fillets while still frozen using a sterile biopsy punch and RNA extraction and purification were carried out as described above. After allowing 3 hr to thaw, each of the three fillets was subjected to pan-frying, which is a common method used by many restaurants for grouper preparation. We performed a simplified version of a recipe for fried grouper recommended by the Florida Department of Agriculture and Consumer Services’ Division of Marketing and Development (http://www.florida-agriculture.com/consumers/fnr/recipes/Seafood-4332.html). Briefly, each fillet was coated in an all-purpose flour, corn meal, and egg wash mixture. The fillets were then pan-fried in canola oil (1-inch level) heated to 375°F (191°C) for 2 min on each side. Excess oil was removed from the fillets by resting on a bed of absorbent paper toweling for 5 min. Three separate pieces (5 mg) were removed from each cooked fillet using a biopsy punch, taking care not to retain a significant amount of the breading. RNA extraction and purification were again carried out on each cooked tissue sample as described above. RT-NASBA reactions were performed for each pseudo-replicate tissue piece from each fillet (raw and cooked) as described above. TTPs generated from each pseudo-replicate were averaged for each fillet and means were compared between raw and cooked forms of each fillet.

**Phylogenetic and statistical analysis**

Phylogenetic analysis was performed on portions of mitochondrial 16S rDNA gene sequences obtained from GenBank by comparing evolutionary relatedness between target grouper species, as well as non-target species commonly used as grouper surrogates using the Neighbor-Joining method (Saitou & Nei, 1987) within the MEGA 5 software package (Tamura,
Peterson, Peterson, Stecher, Nei, & Kumar, 2011). The tree is drawn to scale, using 1000 replicates in the bootstrap test and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) (Fig. 2).

Summary statistics including mean calculations, standard deviations, and significant relationships among means were computed for TTP using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA). Differences among average TTP between raw (preserved and non-preserved) and cooked grouper samples were determined using paired t-tests. All means were considered significantly different when $P < 0.05$.

**Results**

**RT-NASBA specificity**

To confirm the species identity of tissue samples used to test the specificity of the RT-NASBA, 16S rDNA sequences obtained from each sample were aligned with a member of their respective species entry in GenBank (Table 1). No tissue sample had less than 98% percent homology for the entire ~500-bp 16S rDNA amplicon to its respective GenBank entry, and no sample had less than 100% homology in the 136-bp NASBA target sequence to its respective GenBank entry (data not shown). Phylogenetic analysis comparing 16S rDNA sequences obtained from GenBank show cladal divergence between target and non-target fish, with minimal exceptions (Fig. 2). The RT-NASBA assays for all tissue samples were performed using a multiplex chemistry mix containing beacons A, B, and C using the same forward and reverse primers. The RT-NASBA assay was able to detect 29 of the 35 target grouper species tested. Target species not detected were; three Cephalopholis spp., one Plectropomus spp., one Variola spp., and one Diplectrum spp. All six of these non-detectable species have no less than
four mismatches to any beacon variant (Table 4). The average TTP (min) for species with no
more than one nucleotide mismatch for any beacon variant was 17.1 ± 2.3, 30.3 ± 1.8 for species
with no more than two mismatches, and 42.1 ± 2.4 for species with no more than three
mismatches (Table 4).

Typical RT-NASBA fluorescence signatures for the various levels of beacon
heterogeneity are shown in Figure 3. No target grouper species were detected having more than
three beacon mismatches for any beacon variant, and none of the 14 non-target species tested
were detected (Table 4). Seven of the 8 Florida grouper species listed by the Florida Department
of Agriculture and Consumer Services’ Division of Marketing and Development
(http://www.florida-agriculture.com/consumers/crops/seafoodproducts/species/grouper/types
.html) were positively detected using RT-NASBA including: Black Grouper (*Mycteroperca
bonaci*), Gag (*Mycteroperca microlepis*), Yellowfin Grouper (*Mycteroperca venenosa*),
Yellowmouth Grouper (*Mycteroperca interstitialis*), Yellowedge Grouper (*Epinephelus
flavolimbatus*), Snowy Grouper (*Epinephelus niveatus*), and Red Grouper (*Epinephelus morio*)
(Table 4). The one other Florida grouper we were unable to obtain tissue samples for testing was
Misty Grouper (*Epinephelus mystacinus*), which has zero nucleotide mismatches to at least one
of the three molecular beacons determined from GenBank alignments (data not shown).
Fig. 2. Phylogenetic dendrogram comparing mitochondrial 16S rDNA sequence relatedness from FDA Seafood List grouper species (highlighted in gray), as well as non-target species commonly used as impostors (not highlighted). Species representatives are accompanied by the GenBank accession number used in the comparison. Grouper species most commonly found in Florida waters are indicated with arrows.
RT-NASBA on cooked grouper samples

RT-NASBA was performed on three individual *E. diacanthus* fillets both before and after cooking. This species of fish was determined to have no more than one nucleotide mismatch to any molecular beacon included in the RT-NASBA multiplex chemistry (Table 4). Average TTPs were calculated from RT-NASBA assays performed on three pseudo-replicate biopsy punch samples obtained from each fillet (Table 3). All RT-NASBA reactions for all three cooked and non-cooked fillets gave positive detection results. The average TTP for all RT-NASBA reactions from frozen tissues was 15.7 ± 0.7; whereas the average TTP for all cooked tissues increased to 18.4 ± 1.5 (Table 3). There was a significant increase in average TTP for each of the three fillets after being cooked (all \(P\)-values < 0.05) however; rRNA from all cooked tissues was still able to be detected in less than 20 min.

### Table 3. RT-NASBA on cooked grouper samples and paired \(t\)-test analysis

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<th>Fillet Designation</th>
<th>Average TTP Pre-cooked (± STDEV)</th>
<th>Average TTP Post-cooked (± STDEV)</th>
<th>(P)-value</th>
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<td>Average from all fillets</td>
<td>15.7 ± 0.7</td>
<td>18.4 ± 1.5</td>
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Means are considered significantly different when \(P < 0.05\).
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<tr>
<th>Latin Binary Name</th>
<th>Common Name</th>
<th>Tissue Sample</th>
<th>Molecular Beacon Nucleotide Mismatches</th>
<th>RT-NASBA Detection</th>
<th>Average TTP (min) (± STDEV)</th>
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<td>16.4 ± 0.3</td>
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<td>Orange-spotted Grouper</td>
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<td>15.4 ± 0.7</td>
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<td>0 1 1 +</td>
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<td>0 1 1 +</td>
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<td>16.4 ± 0.8</td>
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<td>KU 5464(^a)</td>
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<td>21.3 ± 2.1</td>
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<td>Nassau Grouper</td>
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<td>22.8 ± 1.5</td>
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<td>Mycteroperca bonaci</td>
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<td>1 2 2 +</td>
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<td>Tiger Grouper</td>
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<td>Yellowfin Grouper</td>
<td>NOAA MVEN001(^a)</td>
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<td>Cephalopholis cruentata</td>
<td>Graysby</td>
<td>KU 2424(^a)</td>
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Table 4. (Continued)

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<td>Chevron Tailed Grouper</td>
<td>KU 5554&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Sand Perch</td>
<td>KU 3980&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Mahi-mahi</td>
<td>BAMA no designation</td>
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</table>

<sup>a</sup> Denotes a vouchered specimen
<sup>b</sup> Denotes a non-target fish.
* Denotes molecular beacon mismatches when referencing non-target 16S rDNA sequences from same species entries in GenBank, or members of the same genus if species is unknown.
Fig. 3. Typical RT-NASBA amplification plots for four levels of molecular beacon heterogeneity. Panel (A) represents at least one beacon having no more than one nucleotide mismatch with the target sequence. Panel (B) represents no more than two mismatches; panel (C), no more than three mismatches; and panel (D), more than three mismatches. Thin, horizontal line is the detection threshold, set at 1.35.

Discussion

Given the high taxonomic diversity of fish that the FDA allows to be sold as grouper in the U.S., we provide evidence that the multiplex RT-NASBA assay described here targeting 16S rRNA can be useful in the accurate detection of most of these species, including all of groupers commonly caught in Florida waters. This is important in that many restaurants not only claim to
serve grouper, but also often claim to serve “Fresh from Florida Grouper.” Thus, applications of this assay may be optimally suited for grouper retail in Florida.

Of the 35 target grouper species we could obtain tissue from, we were able to successfully detect 29 of them using RT-NASBA. The six species that were undetectable had exceeded the threshold of nucleotide mismatch for our three molecular beacon variants. We have performed some research using a fourth beacon variant ([6-FAM]-CGATCGCGTTTACA-ACCAAGAGCTACCGATCG-[DABCYL]) that may better target some of the outlying Cephalopholis spp. (no more than two mismatches to any species). However, we noticed that the inclusion of four beacon variants into a single RT-NASBA master mix detrimentally increased the background fluorescence and also decreased the specificity of the overall assay (data not shown).

Unfortunately, two grouper species listed on the FDA Seafood List (Caprodon schlegelii and Cephalopholis spiloparaea) had no 16S rDNA sequence submissions in GenBank, nor were we able to obtain any tissue samples from these species. Thus, we are currently unable to make any assumptions about the potential for accurate detection of these species using the RT-NASBA assay. We were, however, able to obtain 16S rDNA sequence information from GenBank for the remaining 19 target grouper species for which we were unable to obtain tissues from. Fortunately, sequence alignments performed on entries from each of these species presented no more than two mismatches to any one beacon variant used in the RT-NASBA assay, most having less than two mismatches (data not shown). Thus, there is no evidence to suggest any of these 19 species would not be accurately detectable using the current multiplex RT-NASBA assay given that a maximum of two mismatches is within the beacon heterogeneity threshold of all other species tested. Moreover, we were able to support the specificity of the RT-NASBA assay by
testing 14 non-target species, often sold as impostor grouper, for cross-reactivity. None of the non-target species generated a positive detection over the 1.35 detection threshold set for the assay.

We also presented evidence that the RT-NASBA assay may have practical applications in the testing of cooked grouper samples served in restaurants. We were able to generate positive fluorescence detection on tissues from three fillets of Spineycheek Grouper (*E. diacanthus*) after being cooked at 375°F (191°C). It is important to note that these were the only full-sized fillets that could be obtained from a reputable seafood wholesaler that regularly tests their imported grouper using outsourced DNA analysis at the time of publication, and that this species has 100% homogeneity to one of the beacon variants. Moreover, there was a statistically significant increase in the TTPs for all replicate assays after the tissue was cooked. Thus, positive detection of cooked tissues may prove unreliable when testing species with higher beacon heterogeneity which already tend to have large TTPs when assaying frozen or preserved tissue. More research needs to be performed on cooked fillets from alternate grouper species to better define the limitations of the assay on species with greater beacon heterogeneity.

One significant advantage that RT-NASBA has over RT-PCR is the high potential for integration into hand-held sensor assay applications that can be performed in real-time on location, decreasing turnover time significantly. Due to the isothermal (41°C) nature of NASBA amplification, adaptability to remote sensing platforms is more practical than molecular assays requiring thermal cycling for amplification, as thermal cycler machines tend to be very bulky and have very high power demands. We have developed a prototype hand-held NASBA analyzer for the detection of harmful algal bloom-causing organisms (Casper et al., 2007), and are currently developing a next-generation hand-held NASBA analyzer that is being optimized for the
accurate identification of grouper species by employing the RT-NASBA assay described herein. This type of technology, coupled with an accurate and timely molecular assay such as RT-NASBA, has the potential for a myriad of commercial and regulatory applications in seafood forensics.

Acknowledgments

This article is the result of research funded by the Florida Sea Grant project R/LR-Q-33-Grouper Forensics for Seafood Quality Control awarded to JHP, as well as an endowment from the Guy Harvey Ocean Foundation and support from the Gulf and South Atlantic Fisheries Foundation, Inc. to USF.

We would like to express our gratitude to Trey Knott and members of the NOAA Center for Coastal Environmental Health and Biomolecular Research in Charleston, SC, Philip Hastings, H.J. Walker, and members of the Marine Vertebrates Collection at SCRIPPS, Edward Wiley, Andrew Bentley, and members of the Natural History Museum & Biodiversity Research Center, Division of Ichthyology at KU, members of the Mann Lab in the College of Marine Science at USF, as well as Fred Stengard at Bama Sea Products, Inc. for providing us with numerous tissue samples used in the validation of this assay.

References


Staff. (December 2011). *Mystery fish: The label said red snapper, the lab said baloney.* Yonkers, NY: Consumer Reports Magazine.


CHAPTER THREE:

A HANDHELD SENSOR ASSAY FOR THE IDENTIFICATION OF GROUPER AS A SAFEGUARD AGAINST SEAFOOD MISLABELING FRAUD

Note to Reader

This chapter has been submitted for review to Food Chemistry in its entirety.

Abstract

Increases in international trade and global seafood consumption, along with fluctuations in the supply of different seafood species, have resulted in fraudulent product mislabeling. Grouper species, due to their high demand and varied commercial availability, are common targets for mislabeling by exploiting inefficient inspection practices. Compounding this problem is the fact that there are currently 59 species of fish from eleven different genera allowed to be labelled “grouper” per U.S. Food and Drug Administration guidelines. This wide diversity makes it difficult for regulators to discern legally salable groupers from restricted species. To obviate taxonomic misidentification when relying on external phenotypic characteristics, regulatory agencies are now employing genetic authentication methods which typically offer species-level resolution. However, standard genetic methods such as DNA barcoding require technical expertise and long turnover times, and the required instrumentation is not amenable for on-site analysis of seafood. To obviate some of these limitations, we have developed a handheld genetic sensor that employs a real-time nucleic acid sequence-based amplification assay (RT-
NASBA) previously devised in our lab, for the analysis of fish tissue in the field. The base RT-NASBA assay was validated using a lab-based, benchtop RNA purification method as well as non-portable, commercial RT-NASBA analyzer. Described herein, is an uncomplicated method for purifying RNA from fish tissue in the field, which had similar efficiency to the benchtop method demonstrated through direct comparisons. We have also demonstrated that the field sensor is only slightly less sensitive than the benchtop instrument, and was able to discern 84% of the grouper species on the 2013 FDA Seafood List from potential impostors. The complete field assay requires fewer than 80 min for completion and can be performed outside of the lab in its entirety.

Introduction

Grouper are the third-most economically valuable seafood product in the state of Florida, with a dockside value estimated at over $24 million (Putnam, 2012). Due to supply limits caused by increased regulation on commercial quotas, as well as their high market value, grouper are often targets for fraudulent mislabeling. Presently, there are 59 finfish species in the family Serranidae, spanning eleven separate genera that can carry the acceptable market name “grouper” according to the U.S. Food and Drug Administration (FDA) in conjunction with the National Marine Fisheries Service (FDA, 2013). The majority of these species are included in three genera: Epinephelus (32 species), Cephalopholis (8 species), and Mycteroperca (9 species). The remaining species are included in 8 minor genera: Aethaloperca (1 species), Anyperodon (1 species), Caprodon (1 species), Dermatolepis (1 species), Diplectrum (1 species), Plectropomus (2 species), Variola (1 species), and Hyporthodus (2 species). To further complicate grouper identification, there have been some recent suggested taxonomic revisions to
the monophyletic classification of Serranidae as well as reclassification of some members of the *Cephalopholis*, *Epinephelus*, *Hyporthodus*, and *Mycteroperca* genera (Craig & Hastings, 2007). Fortunately, the FDA has yet to fully recognize these amendments. Due to the diversity of grouper species, accurate taxonomic identification can be difficult, particularly when the head and skin are removed to market them as fillets (Buck, 2010).

Oceana recently reported findings from one of the largest seafood fraud investigations in the world, performed over a two-year period to determine the prevalence of mislabeled fish sold by seafood retailers in the U.S. DNA analysis of over 1,200 seafood samples from 21 states revealed that approximately one-third were mislabeled with regard to FDA legal trade criteria (Warner, Timme, Lowell, & Hirshfield, 2013). Grouper were chosen as one of the finfish categories of interest and 26% were found to be mislabeled farmed Asian catfish (*Pangasius* spp.), freshwater perch, Weakfish, Bream, or King Mackerel. Asian catfish imported into the U.S. are primarily farmed in Vietnam along the Mekong River, a body of water that has become polluted in many areas due to increases in unregulated mining activity and anthropogenic run-off (Fu, Su, He, Lu, Song, & Huang, 2012; Ongley, 2009). Moreover, Vietnamese fish farming regulations are often less stringent than those of domestic aquaculture. Antimicrobial compounds such as malachite green and enrofloxacin (Baytril®) have been found in imported Pangasius catfish fillets, both of which are prohibited from use in U.S. food production per FDA criteria (Staff, 2011). King Mackerel surrogate fillets were also discovered which often contain high levels of mercury to the extent that the FDA advises against consumption by sensitive groups such as children and pregnant women (FDA, 2004).

The Oceana investigation also revealed that at least one fish sample, mislabeled as legally salable grouper, was actually Speckled Hind (*E. drummondhayi*) which is a species on the
International Union for Conservation of Nature and Natural Resources (IUNC) Red List as critically endangered (Chuen & Huntsman, 2013). This type of seafood fraud undermines conservation efforts put in place to prevent overfishing of at-risk species by making illegal fishing profitable. Furthermore, mislabeling can negatively affect estimates of fish stocks used in fisheries management by contradicting the true state of the fishery. The misidentification of species before commercial landings data are reported to regulatory agencies can cause a two-fold consequence by both inflating fisheries-dependent landings data of the more desired species as well as underestimating catches of less desirable, and possibly unmanaged species (Jacquet & Pauly, 2008; Marko, Lee, Rice, Gramling, Fitzhenry, McAlister, et al., 2004).

In 2009, the South Atlantic and Gulf of Mexico Region’s seafood industry generated approximately $13 billion in sales impacts and created 65,000 jobs for Florida, of which grouper accounted for 10 percent of all non-shellfish total landings revenue (NMFS, 2010). The demand for grouper in the U.S. is so strong that it cannot be met by the harvesting of domestic species alone. In 2012, over four thousand metric tons of foreign grouper, worth approximately $33.5 million, were imported into the U.S. (NOAA, 2012). This mass quantity of imported grouper creates numerous foreign outlets for the sale of fraudulently mislabeled fish. Recently, a group of U.S. seafood wholesalers were convicted of felony offenses for their roles in purchasing and selling farm-raised Asian catfish which were purposely mislabeled as grouper (Staff, 2011). In addition to misleading consumers into paying more for lesser-valued seafood species, fraudulent mislabeling negatively affects consumer confidence in domestic seafood, which can undercut the profits of local grouper fishermen (Ropicki, Larkin, & Adams, 2010). Seafood fraud is also detrimental to the national economy, as some importers may mislabel to avoid paying tariffs and
anti-dumping duties. In 2010, Vietnamese catfish were sold as grouper to evade over $63 million in tariffs that would have been owed to the U.S. government (FDA, 2010).

As of 2011, the sole standard method used by the FDA for forensic identification of seafood species was isoelectric focusing (IEF) on soluble muscle proteins, which is meant to produce species-specific electrophoretic banding patterns when referenced against a database consisting of vouchered specimens (AOAC, 1980). While this method has proved dependable when testing unprocessed seafood under cold storage, it has been described as unreliable when analyzing cooked tissues or fillets packaged with acidic sauces or marinades to increase value (Applewhite, 2012; Rasmussen & Morrissey, 2008). Furthermore, IEF procedures are tedious, often require several days, and interpretation of results can be subjective (Applewhite, 2012). To obviate some limitations of IEF, the FDA has recognized the need to transition towards DNA-based forensic analysis of seafood. Additionally, genetic analysis may offer higher species-level resolution than protein-based assays due to the variable degeneracy of some nucleotide sequences that are not translated into proteins between some closely related species (Rasmussen & Morrissey, 2008).

The FDA recently approved DNA barcoding as an acceptable method for seafood species identification (Hanner, 2011). This method involves the amplification of a specific gene locus by PCR, primarily the cytochrome c oxidase subunit I gene (COI), and performing nucleotide sequencing reactions on the amplicons. The unknown sequence is then referenced to a database consisting of sequence submissions generated from verified specimens of respective species (Yancy, Zemlak, Mason, Washington, Tenge, Nguyen, et al., 2008). The particular database utilized by the FDA is a product of the Fish Barcode of Life (FISH-BOL) Initiative, which is an international effort to accumulate standardized reference barcodes from global fish species using
highly qualified specimens (www.fishbol.org). Barcoding is advantageous in that it relies on validated standard methods and gene loci; however, the requirement of DNA sequencing machinery obligates the outsourcing of analysis to a select number of labs having this capacity which creates prolonged turnover times.

There is an emerging interest in genetic identification technologies for seafood that reduce processing time and eliminate the need for lab-based DNA sequencing. An alternate technology termed TwistFlow® Red Snapper (http://www.twistdx.co.uk) utilizes recombinase polymerase amplification (RPA), which allows for the field identification of red snapper (\textit{Lutjanus campechanus}) in conjunction with a portable real-time fluorometer. Due to the isothermal nature of RPA, conventional PCR thermal cycling is not required freeing the system from the necessity of large lab-based thermal cyclers with high power demands, making it amenable to field applications.

We have previously developed a real-time nucleic acid sequence-based amplification assay (RT-NASBA) that is able to differentiate most of the FDA allowable groupers from potential surrogate fish species by targeting a region of the mitochondrial 16S rDNA gene (Ulrich, John, Barton, Hendrick, Fries, & Paul, 2013). NASBA is an isothermal RNA amplification method that, when used in conjunction with fluorescently-labeled molecular beacons, provides real-time identification of specific nucleotide sequences (Compton, 1991; Tyagi & Kramer, 1996). The prior validation of this assay was performed using a commercial lab benchtop NASBA analyzer (bioMérieux, NucliSENS EasyQ®) which cannot be easily transported for on-site forensic analysis of fish tissues. Here we report the development of a portable grouper forensics test by integrating our RT-NASBA assay with an in-house designed handheld heated fluorometer dubbed QuadPyre, which is a refined evolution of a prototype
sensor developed by our group (Casper, Patterson, Bhanushali, Farmer, Smith, Fries, et al., 2007). We have also devised a simple and inexpensive method for the field purification of RNA from fish tissue allowing the entire analysis to be performed in the field in less than 80 min. We believe this technology will provide a useful on-site screening tool to aid seafood processors, distributors, retailers and restaurateurs in remaining compliant with compulsory FDA regulations on salable grouper species.

Materials and Methods

**Fish specimens**

Raw tissue samples were obtained from the National Oceanic and Atmospheric Administration (NOAA), Southeast Fisheries Science Center (Charleston, SC); Scripps Institution of Oceanography (SCRIPPS), Marine Vertebrates Collection (La Jolla, CA); University of Kansas (KU) Natural History Museum & Biodiversity Research Center, Division of Ichthyology (Lawrence, KS); University of South Florida (USF), College of Marine Science, David Mann or John Paul Labs; or Bama (BAMA) Sea Products, Inc. (St. Petersburg, FL). Sample designations and preservation conditions were as previously described (Ulrich et al., 2013). No fish tissues from any of the above collections were used for cooked species analysis as the samples where typically less than 1 g and would not accurately mimic restaurant-sized preparations.

Grouper samples designated as “market” sources were obtained from various seafood retail establishments and all species were confirmed by in-house 16S rDNA sequence analysis as previously described (Ulrich et al., 2013). When analyzing cooked fish tissues, the pan-frying protocol was performed as previously described (Ulrich et al., 2013). Briefly, whole fillets from
various market sources were breaded and cooked in canola oil heated to 191 °C (375 °F) for 2 min on each side.

**Field RNA purification**

RNA extraction and purification were performed using a modified RNeasy® Mini Kit (Qiagen, Valencia, CA) protocol adapted to obviate the requirement of a microcentrifuge, similar to a method devised by our lab for the field quantification of a harmful algal bloom-causing dinoflagellate (Casper, et al. 2007). Approximately 10 mg of muscle tissue was cored from each fish sample using a 2 mm sterile biopsy punch (Integra™ Milex®, York, PA, part number 33-31-P/25) and transferred into a 1.5 mL microcentrifuge tube containing 700 µl of lysis buffer (Buffer RLT including 30% ethanol). The tube was then shaken by hand for 30 sec and allowed to incubate at room temperature for 10 min. Approximately 500 µl of lysate was then transferred to an RNeasy® Mini Spin Column, taking care to exclude any pieces of fish tissue. The pneumatic purification assembly was constructed by screwing a sterile male-to-male luer adapter (Value Plastics, Inc., Fort Collins, CO, part number FTLC-6) into the male threaded sleeve of a 60 mL Luer-Lok™ syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, part number 309653). The narrow outlet of the RNeasy® column containing the lysate was then plugged into the opposing end of the luer adapter attached to the purification assembly. The entire assembly was then oriented upright with the column on top and (with the column lid open) the plunger of the syringe was partially drawn out, pulling the lysate through the silica capture membrane. The membrane was then washed with 700 µl of Buffer RW1 in the same manner by partially drawing the plunger out, capturing the waste buffer inside the syringe. This step was repeated with two separate washes of 500 µl RPE, and a subsequent wash of 700 µl HPLC-grade acetone. The column was then removed from the apparatus, and the syringe containing the flow-through was
stopped using a male luer cap (Value Plastics, Inc., Fort Collins, CO, part number FTLLP-6) for appropriate disposal of chemical reagents. To dry the capture membrane, the plunger of a fresh 60 mL syringe was drawn out to capacity and the male sleeve was snapped into the top portion of the column (no adaptor required). The full volume of air in the syringe was pushed through the membrane by depressing the plunger firmly, while suspending the column outlet over absorbent toweling to capture any residual acetone. The drying step was repeated nine times by un-attaching the syringe, drawing back the plunger, and re-attaching to the column.

Purified RNA was captured by adding 100 µl of nuclease-free water to the column, and passing it through the membrane using syringe force from the top of the column into a collection tube. This eluate was stored on ice until its use as RT-NASBA template (within 5 hrs).

**Multiplex RT-NASBA assay**

All RT-NASBA reactions were carried out in triplicate on purified RNA from each tissue core using NucliSENS EasyQ® Basic Kit chemistry per manufacturer’s instructions (bioMérieux, Durham, NC). Oligonucleotide primer and molecular beacon design was performed as previously described, and nucleotide sequences are presented in Table 2 (Ulrich et al., 2013). Synthesis of all oligonucleotides was performed by Eurofins MWG Operon (Huntsville, AL). For reactions analyzed using both the commercial NucliSENS EasyQ® analyzer and the handheld QuadPyre, primers were diluted to a final concentration of 400 nM, and the three molecular beacons were diluted to a final concentration of 100 nM each. The optimal KCl concentration was determined to be 80 mM (data not shown). To maximize the number of reactions per kit for the EasyQ® analyzer analysis, 10 µl reaction volumes were used (half the volume recommended by bioMérieux) containing 5 µl reagent mix (containing primers and beacons), 2.5 µl RNA template, and 2.5 µl enzyme mix. Due to minimum volume
restrictions of the QuadPyre reaction chambers, an increased reaction volume of 30 µl was used consisting of 15 µl reagent mix, 7.5 µl enzyme mix, and 7.5 µl RNA template. These RT-NASBA chemistry mixes have proven to be stable at room temperature for at least 12 hrs prior to amplification for purposes of controlled lab preparation prior to transporting for field analysis (data not shown). All RT-NASBA reactions were performed in 0.2 mL MicroAmp™ Optical Tubes and Caps (Life Technologies®, Carlsbad, CA) for both EasyQ® and QuadPyre analysis.

RT-NASBA amplification and fluorescence detection were carried out at 41°C for 60 min (minimum allowable run time) using the EasyQ® analyzer and 45 min using the QuadPyre. Fluorescence threshold values were chosen to establish positive or negative detection events for each analyzer. Typically, this value was 0.25 relative fluorescence units (rfu) above the final fluorescence generated from a negative control reaction (generally 1.35 rfu) for the EasyQ®. Due to variability in rfu outputs between the EasyQ® and QuadPyre analyzers, fluorescence values are not directly interchangeable; however, they are analogous when determining the time to positivity (TTP), which is the time at which target amplification curves reach the threshold level for each reaction. The threshold value for the QuadPyre analysis was typically 0.5 rfu above the final fluorescence of a negative control (generally 0.15 rfu). Average TTP were recorded from pseudo-replicates for each tissue core by performing RT-NASBA on three aliquots from same RNA eluate.

Evaluating performance of field RNA purification

We assessed the efficacy of the field RNA purification protocol by comparing it with the lab benchtop RNeasy® Mini Kit purification method as described in Ulrich et al. (2013). Both methods were used to extract RNA from fillets of Red Grouper (E. morio), Gag (M. microlepis), and Black Grouper (M. bonaci) obtained from market sources (sequences confirmed). All
samples were stored frozen at -20°C for a minimum of 24 hrs until the full suite of samples could be compiled for replicate analysis performed during a single day. When analyzing fish prior to cooking, tissue punches from three separate fillets of each species were processed for RNA purification by either the lab benchtop or field method. Each respective fillet was then allowed to thaw at room temperature for 3 hrs and then cooked to aforementioned specifications. Again, tissue punches were removed from each fillet after cooking and processed using either RNA purification method. RT-NASBA was performed on purified RNA from each fillet (generated from both methods) using the EasyQ® analyzer.

**Handheld RT-NASBA analyzer**

The QuadPyre RT-NASBA detection system is the product of engineering advancements from the prototype handheld analyzer which was limited to a single reaction chamber and rudimentary software (Casper, et al., 2007). A comprehensive description of the design and functionality of the QuadPyre sensor has been detailed in a manuscript recently submitted for review (Fries, Hendrick, Ulrich, Barton, Gregson, & Paul, in review). In summary, the QuadPyre was developed to capacitate four modular optical reaction chambers which increase the sampling throughput of the system while maintaining portable size dimensions (Fig. 4). The reaction chambers were comprised of high intensity light emitting diodes (LEDs) for fluorophore excitation, blue-enhanced photodiodes for emission detection, and a feedback-controlled infrared (IR) heater and detector for temperature modulation. Each chamber was fabricated to incorporate two separate LED and photodiode pairs for applications requiring concurrent, multi-spectral analysis of control reactions or internal calibrants. However, for applications described herein, only LEDs with peak emissions of 470 nm were required to excite the three carboxyfluorescein-labeled molecular beacons during NABSA amplification. The photodiodes
and LEDs were optically isolated from each other at 90° to prevent background excitation light from scattering into the detection system. To further reduce the incidence of optical noise, bandpass filters matching the beacon excitation and emission wavelengths were positioned in front of the LEDs and photodiodes, respectively. A constant NABSA amplification temperature of 41°C was maintained within the reaction tube using a pulse-width-modulated IR heater and thermopile-based digital thermometer (Fig. 4).

The control software for the QuadPyre consisted of a multi-window graphical display allowing for real-time observation of fluorescence intensity indicating RT-NASBA amplification. Fluorescence data generated from integrated readings of voltage across the photodetector was streamed to a laptop computer by means of an RS-232 serial-to-USB adapter where it was logged and processed using a novel set of analytical functions which were coded using the Python™ language, NumPy. Output of raw numerical data consisting of rfu and temperature readings are logged in a Microsoft® Excel worksheet-compatible format, as well as providing source data for the graphical display. The fluorescence threshold value used for positive/negative grouper determination is manually entered into the software prior to analysis and detection results are displayed by a graphical indicator within the window. This allows for a more user-friendly interface by removing the requirement of post-analysis TTP determination by manual manipulation of raw numerical fluorescence data.

**Statistical analysis**

Summary statistics including mean calculations, standard deviations, and significant relationships among means were computed for TTP using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA). Differences among average TTP between raw and cooked fish samples, as well as between samples analyzed using either the lab benchtop or field
assays were determined using paired t-tests. All means were considered significantly different when $P < 0.05$. Post hoc power analysis was performed to determine the necessary differences needed to detect a significant difference in the paired t-test analysis using GraphPad StatMate version 2.0 (GraphPad Software, San Diego, CA).

Results

Performance of field RNA purification

RT-NASBA was performed on raw and cooked fillets from three grouper species using RNA procured using both the field and lab purification methods. The average TTP generated from both protocols were compared to qualify potential inefficiencies in RNA extraction such as reduced yields or purity. Potential carryover of chemical inhibitors of NASBA amplification or inadequate RNA yields can be problematic and would be evident either by false negative results or a significant increase in TTP. All RT-NASBA reactions using RNA purified by the field method as template gave positive detection results for all three species (Table 5). Moreover, there were no significant increases in TTP for any non-cooked or cooked fillets when compared with the lab benchtop purification method (all $P$-values $> 0.05$). However, post hoc power analysis indicated differences ranging from 0.47 to 1.15 in the TTP values were necessary to detect significant differences with an acceptable 80% power (data not shown). Therefore, more samples would need to be analyzed to confidently conclude there is not a significant difference between the two methods.
Fig. 4. Schematic and photographic depiction of the QuadPyre handheld RT-NASBA sensor. A bottom and top view of the modular components comprising a single reaction chamber are illustrated in (A) and (B), respectively. Panel (C) is an overhead view of the QuadPyre with the protective cover removed exposing the four-chamber arrangement. Panel (D) represents the complete handheld detection system while performing four RT-NASBA reactions simultaneously.
Table 5. Testing field RNA purification efficacy on raw and cooked tissues using RT-NASBA.

<table>
<thead>
<tr>
<th>Species</th>
<th>Raw Average TTP (min ± STDEV)</th>
<th>Lab Benchtop RNeasy®</th>
<th>Field RNeasy®</th>
<th>P-value</th>
<th>Cooked Average TTP (min ± STDEV)</th>
<th>Lab Benchtop RNeasy®</th>
<th>Field RNeasy®</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Grouper</td>
<td>16.1 ± 0.3</td>
<td>16.2 ± 0.3</td>
<td></td>
<td>0.87</td>
<td>18.2 ± 0.3</td>
<td>18.4 ± 0.4</td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td>Gag</td>
<td>16.1 ± 0.6</td>
<td>16.3 ± 0.4</td>
<td></td>
<td>0.40</td>
<td>18.3 ± 0.3</td>
<td>18.4 ± 0.4</td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>Black Grouper</td>
<td>19.0 ± 0.4</td>
<td>19.1 ± 0.3</td>
<td></td>
<td>0.44</td>
<td>35.1 ± 0.5</td>
<td>35.4 ± 0.5</td>
<td></td>
<td>0.33</td>
</tr>
</tbody>
</table>

Means were considered significantly different when $P < 0.05$ using paired t-test analysis.

Evaluation of field QuadPyre RT-NASBA

When previously evaluating the multiplex RT-NASBA assay using lab benchtop RNA purification and the commercial EasyQ® analyzer, there were 56 species of salable grouper included in the 2011 FDA Seafood List (Ulrich et al., 2013). We provided evidence, both empirical and inferred from bioinformatics sequence analysis, that the assay can successfully differentiate approximately 86% of the FDA allowable grouper species from potential impostor fish. We determined that when the nucleotide sequence heterogeneity between target 16S rRNA and the molecular beacons increased, so did the TTP. Furthermore, we demonstrated that at least one of the molecular beacon variants could have no more than three nucleotide mismatches for a given grouper species to be detectable (Ulrich et al., 2013).

To evaluate the performance and sensitivity of the field RT-NASBA assay, we tested an array of grouper species with varying levels of beacon nucleotide mismatch. Using the field RNA purification method in conjunction with the QuadPyre sensor, we assayed species representing minimum nucleotide mismatches of zero, one, two, and $\geq$ three to at least one of the three beacons. We noticed an increase in TTP for each species analyzed using the QuadPyre
when compared with the lab benchtop EasyQ® analyzer. The average increase in TTP (min) for species with a minimum of zero mismatches was 3.5, 6.9 for one mismatch, and 10.0 for a single species with a minimum of two mismatches, yet all remained detectable in under 45 min (Table 6). However, we were unable to detect three grouper species having a minimum of three nucleotide mismatches with the QuadPyre, which were detectable using the EasyQ® analyzer (Table 6). Six grouper species having more than three mismatches to any beacon, as well as seven non-target fish, were undetectable using either the EasyQ® or QuadPyre (Table 6).

Evaluating the QuadPyre assay on cooked tissue

We have previously evaluated the lab benchtop RT-NASBA assay using the EasyQ® on cooked fillets of Spineycheek Grouper (E. diacanthus), which is a species having no more than one nucleotide mismatch to any beacon variant, and noticed a significant increase in TTP when compared with raw tissue from the same species (Ulrich et al., 2013). This phenomenon was also observed with three other species when evaluating the field RNA purification method (Table 5). Due to the reduced sensitivity to low levels of RT-NASBA fluorescence we believe inherent to the QuadPyre photodetection systems, we aimed to qualify the overall limitations of the sensor in detecting cooked tissue from species with higher beacon heterogeneity. We performed field RT-NASBA on cooked fillet replicates from species common to Florida waters where local market sources existed. All sixteen fillet replicates from species with 100% nucleotide complementarity to at least one of the beacon variants (Gag, Red Grouper, and Yellowmouth Grouper) were detectable when both raw and cooked (Table 7). However, two species tested having one or more nucleotide mismatch to any beacon only allowed for intermittent detection when testing cooked fillet replicates. Two out of five and three of seven cooked Yellowfin and
Black Grouper fillets where undetectable (respectively), and all five non-target fillets remained undetectable after pan-frying (Table 7).

**Discussion**

The chain of supply for global seafood commerce has become increasingly complex and is often lacking sufficient government inspection efforts to deter many from lucrative, duplicitous practices despite rising public awareness of mislabeling fraud. When federal inspections do occur they rarely transpire along various stops in the same seafood supply chain making it difficult to determine the source of the fraud whether it be at the vessel, processor, wholesaler, or retail venue. Moreover, where the DNA barcoding method recently being adopted by the FDA gains in specificity, it lacks in rapid turnover time making it difficult for regulators or concerned members of the seafood industry to perform timely screenings of bulk shipments before they change hands. It was our goal to develop a technology that would allow users to perform these front-end inspections on-location, and obtain reliable results in real time.

Our study demonstrates that the field QuadPyre RT-NASBA assay is capable of differentiating 47 of the 56 species (84%) we have sequence knowledge of, from common surrogates when testing raw tissues. Since performing validation studies on the lab benchtop RT-NASBA assay in 2011, there have been slight modifications to the Seafood List regarding species allowed to be labeled “grouper.” These include the reclassification of Yellowedge Grouper from *Epinephelus flavolimbatus* to *Hyporthodus flavolimbatus* and the addition of three more species; Bridled Grouper (*E. heniochus*), Scamp (*M. phenax*), and Star-studded Grouper (*H. niphobles*) (FDA, 2013).
Table 6. Field RT-NASBA assay testing un-cooked tissues from target and non-target species.

<table>
<thead>
<tr>
<th>Latin Binary Name</th>
<th>Common Name</th>
<th>Sample Designation</th>
<th>Molecular Beacon Nucleotide Mismatches</th>
<th>NucliSENS EasyQ® Lab Benchtop Detection TTP (min ± STDEV)</th>
<th>QuadPyre Field Detection TTP (min ± STDEV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Epinephelus diacanthus</em></td>
<td>Spinycheek Grouper</td>
<td>BAMA no designation</td>
<td>A 0 B 1 C 1</td>
<td>15.2 ± 0.6</td>
<td>19.3 ± 0.4</td>
</tr>
<tr>
<td><em>Epinephelus guttatus</em></td>
<td>Red Hind</td>
<td>NOAA ST159EGa</td>
<td>A 0 B 1 C 1</td>
<td>16.4 ± 0.9</td>
<td>18.7 ± 1.1</td>
</tr>
<tr>
<td><em>Epinephelus itajara</em></td>
<td>Goliath Grouper</td>
<td>NOAA 18-60-2a</td>
<td>A 1 B 2 C 0</td>
<td>16.1 ± 1.0</td>
<td>19.0 ± 0.9</td>
</tr>
<tr>
<td><em>Epinephelus morio</em></td>
<td>Red Grouper</td>
<td>USF no designation</td>
<td>A 0 B 1 C 1</td>
<td>16.3 ± 1.4</td>
<td>19.7 ± 1.8</td>
</tr>
<tr>
<td><em>Epinephelus nigritus</em></td>
<td>Warsaw Grouper</td>
<td>KU 5439a</td>
<td>A 0 B 1 C 1</td>
<td>15.7 ± 1.7</td>
<td>18.5 ± 0.2</td>
</tr>
<tr>
<td><em>Epinephelus niveatus</em></td>
<td>Snowy Grouper</td>
<td>KU 8347a</td>
<td>A 0 B 1 C 1</td>
<td>16.4 ± 0.8</td>
<td>20.1 ± 1.3</td>
</tr>
<tr>
<td><em>Hyporthodus flavolimbatus</em></td>
<td>Yellowedge Grouper</td>
<td>KU 1142a</td>
<td>A 0 B 1 C 1</td>
<td>14.8 ± 1.2</td>
<td>19.6 ± 0.4</td>
</tr>
<tr>
<td><em>Mycteroperca interstitialis</em></td>
<td>Yellowmouth Grouper</td>
<td>SCRIPPS 07-77</td>
<td>A 1 B 0 C 2</td>
<td>15.3 ± 0.7</td>
<td>18.9 ± 0.7</td>
</tr>
<tr>
<td><em>Mycteroperca jordani</em></td>
<td>Gulf Grouper</td>
<td>SCRIPPS 06-40</td>
<td>A 0 B 1 C 1</td>
<td>15.4 ± 1.6</td>
<td>19.9 ± 1.1</td>
</tr>
<tr>
<td><em>Mycteroperca microlepis</em></td>
<td>Gag</td>
<td>USF no designation</td>
<td>A 0 B 1 C 1</td>
<td>16.8 ± 0.9</td>
<td>20.0 ± 0.7</td>
</tr>
<tr>
<td><em>Mycteroperca phenax</em></td>
<td>Scamp</td>
<td>USF no designation</td>
<td>A 0 B 1 C 2</td>
<td>16.8 ± 1.7</td>
<td>19.9 ± 2.1</td>
</tr>
<tr>
<td><em>Epinephelus hexagonatus</em></td>
<td>Starspotted Grouper</td>
<td>KU 5729a</td>
<td>A 1 B 2 C 2</td>
<td>19.4 ± 0.4</td>
<td>27.3 ± 2.7</td>
</tr>
<tr>
<td><em>Epinephelus striatus</em></td>
<td>Nassau Grouper</td>
<td>KU 305a</td>
<td>A 2 B 3 C 1</td>
<td>22.8 ± 1.5</td>
<td>27.9 ± 2.0</td>
</tr>
<tr>
<td><em>Mycteroperca bonaci</em></td>
<td>Black Grouper</td>
<td>NOAA ST017MBa</td>
<td>A 1 B 2 C 2</td>
<td>19.0 ± 2.1</td>
<td>26.7 ± 1.7</td>
</tr>
<tr>
<td><em>Mycteroperca venenosa</em></td>
<td>Yellowfin Grouper</td>
<td>NOAA MVEN001a</td>
<td>A 1 B 2 C 2</td>
<td>21.2 ± 1.6</td>
<td>28.1 ± 2.9</td>
</tr>
<tr>
<td><em>Anyperodon leucogrammicus</em></td>
<td>Slender Grouper</td>
<td>KU 7002a</td>
<td>A 3 B 2 C 4</td>
<td>30.3 ± 1.8</td>
<td>40.3 ± 3.1</td>
</tr>
<tr>
<td><em>Cephalopholis cruentata</em></td>
<td>Graysby</td>
<td>KU 2424a</td>
<td>A 4 B 3 C 4</td>
<td>42.2 ± 2.3</td>
<td>–</td>
</tr>
<tr>
<td><em>Cephalopholis fulva</em></td>
<td>Coney</td>
<td>KU 219a</td>
<td>A 4 B 3 C 4</td>
<td>41.0 ± 2.8</td>
<td>–</td>
</tr>
<tr>
<td><em>Cephalopholis taeniops</em></td>
<td>Spotted Grouper</td>
<td>SCRIPPS 04-39</td>
<td>A 4 B 3 C 4</td>
<td>43.2 ± 2.6</td>
<td>–</td>
</tr>
<tr>
<td><em>Cephalopholis argus</em></td>
<td>Purplespotted Grouper</td>
<td>KU 5493a</td>
<td>A 5 B 4 C 5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Cephalopholis miniata</em></td>
<td>Coral Hind</td>
<td>KU 6806a</td>
<td>A 5 B 4 C 5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Cephalopholis urodeta</em></td>
<td>Chevron Tailed Grouper</td>
<td>KU 5554a</td>
<td>A 5 B 4 C 5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Diplerctrum formosum</em></td>
<td>Sand Perch</td>
<td>KU 3980a</td>
<td>A 6 B 5 C 6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Plectropomus areolatus</em></td>
<td>Squirrel Grouper</td>
<td>SCRIPPS 10-117</td>
<td>A 8 B 7 C 8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Variola louti</em></td>
<td>Yellow-edged Lyretail</td>
<td>KU 7159a</td>
<td>A 6 B 7 C 7</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 6. (Continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Market</th>
<th>Detection</th>
<th>Detection</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pangasius hypophthalmus</td>
<td>Swai Catfish</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Pangasius bocourti</td>
<td>Basa Catfish</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Lethrinus nebulosus</td>
<td>Emperor</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Oreochromis aureus</td>
<td>Blue Tilapia</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Coryphaena hippurus</td>
<td>Mahi-mahi</td>
<td>BAMA no designation</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Gadus morhua</td>
<td>Atlantic Cod</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Melanogrammus aeglefinus</td>
<td>Haddock</td>
<td>Market</td>
<td>&gt; 6</td>
<td>&gt; 6</td>
</tr>
</tbody>
</table>

NucliSENS EasyQ® lab benchtop detection results were taken from Ulrich et al. (2013).

a Denotes a vouchered specimen.
b Denotes a non-target fish.
Table 7. Testing cooked fillets using the field RT-NASBA assay.

<table>
<thead>
<tr>
<th>Latin Binary Name</th>
<th>Common Name</th>
<th>Raw Tissue Detection</th>
<th>Detection when Cooked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephelus morio</td>
<td>Red Grouper</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td>Mycteroperca interstitialis</td>
<td>Yellowmouth Grouper</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Mycteroperca microlepis</td>
<td>Gag</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Mycteroperca venenosa</td>
<td>Yellowfin Grouper</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Mycteroperca bonaci</td>
<td>Black Grouper</td>
<td>7/7</td>
<td>4/7</td>
</tr>
<tr>
<td>b Pangasius hypophthalmus</td>
<td>Swai Catfish</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>b Pangasius bocourti</td>
<td>Basa Catfish</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>b Oreochromis aureus</td>
<td>Blue Tilapia</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

Numbers represent number of replicate fillets detected per tested.
b Denotes a non-target fish.

We were able to successfully detect Scamp using the field RT-NASBA assay, and Star-studded Grouper has 100% complementarity to at least one beacon variant inferred from bioinformatics analysis. Thus, it is likely discernible from impostor species when employing the QuadPyre. Unfortunately, there are no 16S rDNA sequence submissions in GenBank for Bridled Grouper, nor have any been added for Sunrise Perch (Caprodon schlegelii) or Strawberry Hind (Cephalopholis spiloparaea) as previously reported (Ulrich et al., 2013). Therefore, we are unable to make any assumptions regarding detectability of these three species using the field RT-NASBA assay. Due to minor sensitivity limitations of the QuadPyre, we were unable to detect Graysby (Cephalopholis cruentata), Coney (Cephalopholis fulva), or Spotted Grouper (Cephalopholis taeniops) which were detectable using the lab benchtop EasyQ® RT-NASBA assay. However, from our own 16S rDNA analysis of obtainable tissues as well as bioinformatic database interrogation of the 59 allowable groupers, these were the only three species (along with the six previously undetectable) that could not meet the beacon complementarity criteria for detection using the field RT-NASBA assay. Moreover, all nine of the most commercially important grouper species in the state of Florida remained detectable when using the field RT-
NASBA assay including: Red Grouper (*E. morio*), Gag (*M. microlepis*), Yellowedge Grouper (*E. flavolimbatus*), Scamp (*M. phenax*), Snowy Grouper (*E. niveatus*), Black Grouper (*M. bonaci*), Warsaw Grouper (*E. nigritus*), Yellowfin Grouper (*M. venenosa*), and Yellowmouth Grouper (*M. interstitialis*) (FWC, 2012). Based on these results, the field RT-NASBA assay appears best suited for seafood commerce local to Florida and perhaps some contiguous coastal states.

We believe there is some utility for the field RT-NASBA assay to be able to diagnose grouper substitution on cooked tissue at the point of restaurant service where the fillets can be more readily masked with breading, crusting, or sauces. When evaluating the assay on breaded, pan-fried grouper fillets from several species commonly sold in Florida restaurant, we observed intermittent detection on replicates from two species which do not have full complementarity to any of the three molecular beacons used for the multiplex analysis. The evidence suggests that there is a deleterious aggregate reduction in the sensitivity of the field assay when testing cooked species with increased beacon heterogeneity. We also understand that there are numerous potential restaurant preparations of grouper with unstandardized temperature exposures and cooking durations which can negatively affect the integrity of target RNA making it unsuitable for molecular amplification. We do however pose evidence that due to increased sensitivity; the lab benchtop EasyQ® analyzer may be better suited for the analysis of cooked tissue from grouper fillets with slight nucleotide mismatch. We also provide evidence that the cooking process does not detrimentally affect the specificity of the field RT-NASBA assay as non-target fillets remained undetectable after pan-frying.

We believe there is heightened interest for a portable, uncomplicated technology such as the QuadPyre RT-NASBA assay that allows for rapid on-site screening of genetic material from
seafood similar to point-of-care testing devices used for rapid diagnosis in clinical settings (Ahmad & Hasham, 2012). What is more, the format of the handheld heated fluorometer allows for potential implementation of alternate RT-NASBA assays utilizing oligonucleotide sets to target other commercially important finfish groups such as snappers and tunas, which had the highest mislabeling rates according to the aforementioned Oceana investigation (Warner, Timme, Lowell, & Hirshfield, 2013). Presenting personnel in seafood purchasing and regulation of such commerce with rapid and portable forensic technologies such as the one presented here will help close inspection gaps to better combat seafood mislabeling fraud.

Acknowledgments

This article is the result of research funded by the Florida Sea Grant project R/LR-Q-33-Grouper Forensics for Seafood Quality Control awarded to JHP, as well as support from the Guy Harvey Ocean Foundation and the Gulf and South Atlantic Fisheries Foundation, Inc. to USF.

We would like to express our gratitude to Philip Hastings, H.J. Walker, and members of the Marine Vertebrates Collection at SCRIPPS, Trey Knott and members of the NOAA Center for Coastal Environmental Health and Biomolecular Research in Charleston, SC, Edward Wiley, Andrew Bentley, and members of the Natural History Museum & Biodiversity Research Center, Division of Ichthyology at KU, members of the Mann Lab and Dominik Lipinski at the College of Marine Science at USF, as well as Fred Stengard at Bama Sea Products, Inc. for providing us with numerous tissue samples used in the validation of this assay.
References


CHAPTER FOUR:
DETECTION AND QUANTIFICATION OF KARENIA MIKIMOTOI USING REAL-TIME NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION WITH INTERNAL CONTROL RNA (IC-NASBA)

Note to Reader

This chapter has been published in full (Ulrich, Casper, Campbell, Richardson, Fries, Heil, & Paul, 2010), and is included with permission from Elsevier®.

Abstract

Nucleic acid sequence-based amplification (NASBA) is an isothermal method used to amplify RNA and has been used for clinical, environmental, and food testing applications. Quantification of RNA by real-time NASBA occurs by comparing time to positive (TTP) fluorescence values, similar to threshold cycle (Ct) values in PCR, of unknown samples to a standard curve of known RNA titers. Incorporation of an internal control RNA molecule (IC-RNA) has been used to increase precision and accuracy of real-time NASBA and also serves as an indicator of NASBA inhibition. A real-time IC-NASBA assay was developed targeting the ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO) large-subunit gene (rbcL) of the harmful algal bloom (HAB) causing dinoflagellate Karenia mikimotoi. This assay is sensitive to one K. mikimotoi cell and $1 \times 10^3$ copies of in vitro transcript with a high degree of specificity against closely related organisms. Enumeration of K. mikimotoi from environmental samples by
IC-NASBA was not significantly different from microscopic cell counts \((P = 0.156, \alpha = 0.05)\) performed by the Fish and Wildlife Research Institute (FWRI, a division of the Fish and Wildlife Conservation Commission, St. Petersburg, FL), the agency responsible for monitoring red tide status throughout the state. In addition, the IC-NASBA enumeration had a good linear relationship \((r^2 = 0.887)\) with FWRI microscopic cell counts. IC-NASBA is an alternative method for the rapid and reliable detection and quantification of \(K. \text{mikimotoi}\) from marine waters.

**Introduction**

*Karenia mikimotoi* (Miyake et Kominami ex Oda) G. Hansen et Moestrup (formerly *Gymnodinium mikimotoi*) is a harmful algal bloom (HAB)-causing dinoflagellate that is found in temperate oceans worldwide including the coast of New Zealand (Seki, Satake, Mackenzie, Kaspar, & Yasumoto, 1995), Japan (Nakamura, Suzuki, & Hiromi, 1996), China (Lu & Hodgkiss, 2004), India (Godhe, Otta, Rehnstam-Holm, Karunasagar, & Karunasagar, 2001), Ireland (Raine, O'Boyle, O'Higgins, White, Patching, Cahill, et al., 2001), and in the Gulf of Mexico (K. A. Steidinger, C. Tomas, G. A. Vargo, and P.A. Tester 1998). \(K. \text{mikimotoi}\) has been linked to massive fish kills, including a devastating HAB occurring in China in 1998 that decimated fin-fish aquacultures, resulting in economic losses estimated at US$ 40 million (Lu & Hodgkiss, 2004). Exact mechanism of toxic effect to marine organisms remains unclear, however hemolytic and cytotoxic compounds produced by \(K. \text{mikimotoi}\) have been associated with icthyotoxicity (Neely & Campbell, 2006; Satake, Tanaka, Ishikura, Oshima, Naoki, & Yasumoto, 2005). \(K. \text{mikimotoi}\) is the second most common *Karenia* species (*K. brevis* is most common) in the Gulf of Mexico and can reach concentrations greater than 1,000,000 cells/liter.
Frequent occurrences of HABs along the Florida Gulf coast caused by *K. brevis* (often referred to as “red tides”) are responsible for enormous fish and marine mammal mortalities, and cost the state in excess of US$ 20 million in lost revenues from tourism (Anderson, 2000); however, it is still unclear to what extent *K. mikimotoi* may contribute to these events in the Gulf of Mexico.

Current red tide monitoring programs rely on microscopic detection and enumeration which can often be inaccurate due to morphological similarities between closely related species (Culverhouse, Williams, Reguera, Herry, & Gonzalez-Gil, 2003). Molecular assays targeting species specific genetic sequences have been developed allowing for accurate and rapid detection of nucleic acids from HAB species (Casper, Paul, Smith, & Gray, 2004; Gray, Wawrik, Paul, & Casper, 2003; Haywood, Scholin, Marin, Steidinger, Heil, & Ray, 2007; Penna, Bertozzini, Battocchi, Galluzzi, Giacobbe, Vila, et al., 2007; Touzet, Keady, Raine, & Maher, 2009; Tyrrell, Bergquist, Saul, MacKenzie, & Bergquist, 1997). One such molecular assay that allows for rapid and accurate quantification of species specific RNA is nucleic acid sequence-based amplification (NASBA) (Compton, 1991) incorporating real-time molecular beacon detection (Leone, van Schijndel, van Gemen, Kramer, & Schoen, 1998).

NASBA is an isothermal (41°C) method for the amplification of RNA that is catalyzed by an enzyme mix consisting of T7 RNA polymerase, avian myoblastosis virus reverse transcriptase, RNaseH, and two target-specific oligonucleotide primers. Real-time fluorescence detection of RNA amplicon generated from NASBA can be accomplished by the incorporation of molecular beacons (Tyagi & Kramer, 1996) first described by Leone et al. (1998). Quantification of RNA by real-time NASBA occurs by comparing time to positive (TTP) values (analogous to threshold cycle in real-time PCR) of fluorescence amplification plots to TTP.
values of a known standard curve. Due to variability in enzyme kinetics of the three enzymes involved in NASBA, TTP standard curves are less reproducible than those of quantitative PCR. To obviate this problem, Weusten et al. (2002) first described a method incorporating a relationship between the rate of synthesis of the target RNA to that of a synthetic calibrator RNA. This relationship was simplified by Patterson et al. (2005) in a study performed in our lab detecting *K. brevis*. IC-RNA is a synthetic RNA molecule designed with the exact sequence of the target RNA, only the wild type beacon binding site is replaced with an alternate site specific to an IC-beacon that contains a fluorophore that emits at a different wavelength of light than the target molecular beacon.

We have developed a novel IC-NASBA assay detecting a 94-base portion of the ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBisCO) large-subunit gene (*rbcL*) of *K. mikimotoi* that is specific, capable of detecting a single cell, and possesses a dynamic range over three orders of magnitude. A decrease in variability between inter-assay replicates was noticed with the IC-NASBA when compared with conventional NASBA lacking IC-RNA, thus validating increased precision of IC-NASBA. Due to high degradation rates of mRNA in the environment, positive detection events by this assay should represent only viable cells, allowing for a more accurate assessment of the growth stage of a potential bloom. The assay described herein enabled detection and quantification of *K. mikimotoi* in environmental samples collected from Florida coastal waters by comparing TTP ratios to standards of cultured cells and microscopic cell counts.
Materials and Methods

In vitro transcript

In vitro transcript of the targeted portion of *K. mikimotoi* was generated as described by Gray et al. (2003). Briefly, a 554-base region of the *rbcL* gene was amplified from a culture of *K. mikimotoi* (TAMU C22 isolate obtained from Texas A&M University) using degenerate primers (forward primer, 5’-GATGATGARAAYATTAACTC-3’; reverse primer, 5’-ATTGTCCCGCATTTGATTCCT-3’ [International Union of Pure and Applied Chemistry degeneracy symbols were used]) (Gray, Wawrik, Paul, & Casper, 2003). The resulting amplicon was TOPO® TA cloned into a pCR®II cloning vector (Invitrogen, Carlsbad, CA) per manufacturer’s instructions. Transformed colonies were grown in Luria broth with 50 µg of kanamycin per ml. Plasmids were extracted using Zyppy™ Plasmid Miniprep Kit (Zymo Research, Orange, CA). Plasmids containing inserts of correct orientation (confirmed by PCR) were sequenced at the DNA Sequencing Core at the University of Florida using bi-directional M13 priming sites. To produce positive control transcripts, plasmids were linearized with *HindIII* and run-off transcripts were generated using Riboprobe® In Vitro Transcription System (Promega Corp., Madison, WI) with T7 polymerase. Transcripts were purified using RNeasy® Mini Kit (Qiagen, Valencia, CA) and quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Confirmation of the desired transcript size was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Following quantification and size confirmation, RNA was stored (1:1) in RNA storage buffer (8M guanidinium isothiocyanate, 80mM Tris-HCl [pH 8.5], 24mM MgCl₂, and 140mM KCl) and frozen at -80°C until use.
Primer and molecular beacon design

*K. mikimotoi* and *K. brevis rbcL* sequences were obtained from GenBank and prior sequencing efforts in our lab (Gray, Wawrik, Paul, & Casper, 2003). Sequences were aligned using Clustal W 1.6 algorithm (Thompson, Higgins, & Gibson, 1994) and Omiga 2.0 (Accelrys, San Diego, CA). Primers were designed to target a 94-bp region internal to the *K. mikimotoi rbcL* gene that differed from *K. brevis rbcL* (Table 8). Primers were analyzed for self- and hetero-dimerization using Oligo analyzer 3.1 primer design tool (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) and synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The *K. mikimotoi* molecular beacon was designed internal of the two primers and labeled with 6-carboxyfluorescein (FAM) on the 5’ end and DABCYL on the 3’ end (Table 8). The IC-RNA beacon was labeled with 6-carboxy-X-rhodamine (ROX) on the 5’ end and DABCYL on the 3’ end. Both beacons were synthesized by Eurofins MWG Operon (formerly Operon, Huntsville, AL) (Table 8).

Synthesis of internal control (IC) RNA

The IC-RNA molecule was designed with the exact 94-base sequence as the target *rbcL* molecule, except that the *K. mikimotoi* beacon binding site was replaced with the beacon site from an enterovirus NASBA assay developed in our lab (Casper, Patterson, Smith, & Paul, 2005) (Table 8). Synthesis of the IC-RNA molecule was performed by modifying the protocol described by Patterson et al. (2005). Briefly, two IC-RNA generation oligonucleotides were designed to contain a 20-base reverse compliment overlap (Table 8) and totally span the IC-RNA sequence. A T7 RNA polymerase promoter region was added on the 5’ end of the forward oligo (Table 8). PCR was used to fully extend the overlapping oligos (100 pmol each), which served as both template and primers, under standard PCR conditions with a 55°C annealing step for 20
cycles. The resulting double stranded DNA (now with an active T7 promoter region) was purified using DNA Clean & Concentrator™ Kit (Zymo Research, Orange, CA) and used as template for in vitro transcription as described above. Resulting IC-RNA was quantified, analyzed for size confirmation, and stored as described above.

**Table 8.** Sequences of *K. mikimotoi* primers, beacons, target RNA, and IC-RNA.

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. mikimotoi</em> forward primer</td>
<td>AACCTAAAATGATTAAGGA</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> reverse primer</td>
<td>AATTCTAATACGACTCCTATATAGGGAGGAGACCCATTCTTGCGAAAAATAA</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> molecular beacon</td>
<td>[6-FAM]-CGATCGAAACAACATGATTTTTCGATCG-[DABCYL]</td>
</tr>
<tr>
<td>IC-RNA molecular beacon</td>
<td>[6-ROX]-CATGCGTGCTGTATATGTTGACAAATCGATG-[DABCYL]</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> target RNA</td>
<td>AACCUAAAUGAUAAAAAGGAUUUUUAAGACACUUCUGGAAUUACAAAC-</td>
</tr>
<tr>
<td></td>
<td>UAAAACAUUGAUUUUGCGUACGCUCUUAUUUUUCGCAAAGAUUGGG</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> IC-RNA</td>
<td>AACCUAAAUGAUAAAAAGGAUUUUUAAGACACUUCUGGAAUUUGGCUC-</td>
</tr>
<tr>
<td></td>
<td>GCUCUAUGGUGACACAAUCUACCGUCUUUAAUUGCGCAAAGAUUGGG</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> IC-RNA generation forward oligo</td>
<td>AATTCTAATACGACTCCTATATAGGGAGAAAACCTAAATGATTAAGGAT-</td>
</tr>
<tr>
<td></td>
<td>TTATAAAGACACTTCTGGATTTTGGCTGTTATGTTGACAAAT</td>
</tr>
<tr>
<td><em>K. mikimotoi</em> IC-RNA generation reverse oligo</td>
<td>CCCATTCTTGCGAAAAATAAAGACCGTAAGATTGTCACCATAGCAGCCA</td>
</tr>
</tbody>
</table>

Bold text for *K. mikimotoi* target and IC-RNA sequences indicates primer and beacon binding sites. Text highlighted in gray indicates T7 RNA polymerase promoter sites. Italicized text indicates reverse compliment overlap.

**Sensitivity and specificity**

Cultured *K. mikimotoi* cells (TAMU C22) were used to determine assay sensitivity. Cells were enumerated using epifluorescent microscopy by filtering cells onto black polycarbonate 0.22-µm-pore size filters (Osmonics, Inc. Minneapolis, MN) and then observing the filters using an Olympus BX-60 microscope and blue excitation (filter set U-MNIB) with either ×200 or ×400
magnification depending on cell density. Cells were then diluted to desired numbers per NASBA reaction in L1 medium (Provasoli-Guillard National Center for Culture of Marine Phytoplankton, West Boothbay Harbor, ME [CCMP]) and filtered onto 0.45-µm-pore size HV polyvinylidene difluoride filters (Millipore, Billerica, MA). RNA was extracted by incubating filters in 500 µl RLT lysis buffer (Qiagen, Valencia, CA) with 0.143 M β-mercaptoethanol for 10 min. Three hundred and fifty microliters of 100% ethanol were added and RNA from the resulting lysate was purified using RNeasy® Mini Kit (Qiagen, Valencia, CA) per manufacturer’s instructions. Final elution of RNA was carried out in 50 µl of nuclease-free water. To determine if competitive inhibition from closely related organisms affects the sensitivity of the assay, dilutions of *K. mikimotoi* were combined with *K. brevis* in several proportions, and RNA from the mixed cultures was extracted as described above.

Specificity of the primers and beacon were tested on four isolates of *K. mikimotoi* and various non-target organisms obtained from either FWRI or CCMP (Table 9). Fifty milliliters of each culture were filtered and RNA was extracted and purified via the above RNeasy® Mini Kit (Qiagen, Valencia, CA) protocol. Non-target strains were tested using 10 pg of RNA per NASBA reaction.

To test if *K. mikimotoi rbcL* mRNA expression is affected by various environmental stressors, cultures were exposed to low-nutrient conditions (incubated in sterile natural seawater with no L1 augmentation at 35-ppt), low-salinity (L1 medium at 25-ppt), low-light (3 µmol m⁻²s⁻¹), and high-light (200 µmol m⁻²s⁻¹). Low-light, high light, and low-nutrient cultures were maintained for 72 h prior to RNA extraction, while the low-salinity was maintained for 4 h prior to RNA extraction, similar to a study by Casper et al. (2004) performed on *K. brevis*. A control *K. mikimotoi* culture was maintained in L1 medium (35-ppt salinity) at 22°C in a 12 hr light/dark
cycle at 26 $\mu$mol m$^{-2}$s$^{-1}$ for 72 h prior to RNA extraction. Extracted RNA was compared to a standard curve of in vitro transcripts of known titers, and rbcL mRNA per cell was inferred from cell counts obtained from epifluorescent microscopy.

Table 9. Specificity of K. mikimotoi NASBA assay.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Detection result$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. mikimotoi</td>
<td>TAMU C22</td>
<td>+</td>
</tr>
<tr>
<td>K. mikimotoi</td>
<td>CCMP 429</td>
<td>+</td>
</tr>
<tr>
<td>K. mikimotoi</td>
<td>CCMP 430</td>
<td>+</td>
</tr>
<tr>
<td>K. mikimotoi</td>
<td>CCMP 2960</td>
<td>+</td>
</tr>
<tr>
<td><strong>Negative controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. brevis</td>
<td>CCFWC 263$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>K. brevis</td>
<td>CCFWC 261$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>K. brevis</td>
<td>CCFWC 257$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>K. brevis</td>
<td>CCFWC 259$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>K. brevis</td>
<td>CCFWC 268$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>Karlodinium micrum</td>
<td>CCFWC 114$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>Akashiwo sanguinea</td>
<td>NA$^c$</td>
<td>$-$</td>
</tr>
<tr>
<td>Chattonella subsalsa</td>
<td>NA$^c$</td>
<td>$-$</td>
</tr>
<tr>
<td>Pyrodinium bahamense</td>
<td>NA$^c$</td>
<td>$-$</td>
</tr>
<tr>
<td>Prorocentrum lima</td>
<td>NA$^c$</td>
<td>$-$</td>
</tr>
<tr>
<td>Alexandrium monilatum</td>
<td>CCFWC 350$^b$</td>
<td>$-$</td>
</tr>
<tr>
<td>Protoceratium reticulatum</td>
<td>NA$^c$</td>
<td>$-$</td>
</tr>
<tr>
<td>Synechococcus sp.</td>
<td>CCMP 836</td>
<td>$-$</td>
</tr>
<tr>
<td>Pseudo-nitzschia multiseries</td>
<td>CCMP 2708</td>
<td>$-$</td>
</tr>
<tr>
<td>Nitzschia ovalis</td>
<td>CCMP 1118</td>
<td>$-$</td>
</tr>
</tbody>
</table>

$^a$ +, detected; $-$, not detected.

$^b$ Culture Collection Fish and Wildlife Conservation Commission, provided by FWRI.

$^c$ NA, organisms provided by FWRI with unknown isolate designation.

**Environmental samples**

Environmental samples were collected as part of an ongoing harmful algal bloom-monitoring program by various agencies and were enumerated for K. mikimotoi within one day of sampling by members of FWRI using light microscopy. One hundred milliliters of sample
was filtered for extraction as described above the same day as enumeration. Filters were stored in 500 µl RLT+β-ME at -80°C until enough samples were acquired for a full NASBA assay, utilizing a single control *K. mikimotoi* cell standard curve (and a single NASBA chemistry master mix), for enumeration using regression analysis. RNA was extracted from filters using RNeasy® Mini Kit (Qiagen, Valencia, CA) protocol as described above. For environmental samples in which neither the wild type (FAM) fluorescence nor the IC-RNA (ROX) fluorescence reached the detection threshold, chemical inhibition of NASBA was assumed and either 1:10 or 1:100 dilutions of the resulting eluate were assayed.

**NASBA assay**

NucliSENS EasyQ® Basic Kit (bio-Mérieux, Durham, NC) chemistry was used for all NASBA assays per manufacturer’s instructions. Primers and beacons were diluted to 400 and 100 nM final concentrations, respectively, and the optimal final concentration of KCl was determined to be 80 mM (data not shown). Ten microliter final reaction volumes were used (half the volume recommended by bio-Mérieux) containing 5 µl reagent mix (containing primers, beacons, and IC-RNA), 2.5 µl RNA template, and 2.5 µl enzyme mix. The RNA template and reagent mix were added together and incubated at 65°C for 2 min prior to the addition of the enzyme mix. NASBA amplification and fluorescence detection were carried out at 41°C for 90 min using a NucliSENS EasyQ® analyzer (bio-Mérieux, Durham, NC). Optimal IC-RNA concentrations had to be determined as to not decrease assay sensitivity (competitive primer depletion) while still allowing for positive IC-RNA amplification (typically 0.25 fluorescence units above negative controls) over at least three orders of magnitude (data not shown). For all subsequent assays, 1×10^7 copies of IC-RNA per reaction were used. For assays testing increased precision of IC-NASBA over conventional NASBA, nuclease free water was used to replace IC-
RNA. TTP ratios were calculated for IC-NASBA by dividing the TTP of the wild type RNA by the TTP of the IC-RNA. Standard TTP comparisons of wild type RNA were used for conventional NASBA. The detection threshold for both TTP and TTP ratio calculations was 1.35 fluorescence units (Fig. 5).

Statistical analysis

When comparing linear relationships, the proportion of the variation in $Y$-variables as compared to the linear relationships with $X$-variables (Figs. 6-8) was determined by calculating coefficient of determination ($r^2$) values using Microsoft® Excel.

Summary statistics and significant relationships among means were computed for variables of interest using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA). Differences among the sensitivity of the $K. mikimotoi$ assay with increasing $K. brevis$ competition (Table 10) were determined using ANOVA. Differences among cellular $rbcL$ mRNA levels under various stress conditions (Table 11) were determined using paired $t$-tests. Differences among $K. mikimotoi$ microscopy counts and IC-NASBA (Table 12) were also determined using paired $t$-tests. All means were considered significantly different when $P < 0.05$.

Results

Sensitivity and specificity

To allow for detection of one $K. mikimotoi$ cell per reaction with a range of at least three orders of magnitude, optimal IC-RNA concentrations had to be determined due to competitive primer depletion. It was determined that $1 \times 10^7$ copies of IC-RNA per reaction were optimal for detecting RNA extracts from at least one cell (or $1 \times 10^3$ target transcript copies) over three orders
of magnitude. The IC-NASBA assay was unable to detect an order of magnitude greater than one hundred cells per reaction (or $1 \times 10^5$ transcript copies) with this IC-RNA concentration, as the wild type RNA would out-compete the IC-RNA for primer incorporation (data not shown). Increasing primer concentrations to 600 and 800 nM did not increase the range of the standard curve for either the culture cell, or in vitro transcript standard curves (data not shown).

![Graphical representation of typical NASBA amplification plots from 100, 10, 1 and 0 cells (A-D, respectively). The black line represents *K. mikimotoi* wild type RNA amplification. The gray line represents IC-RNA amplification. Thin, horizontal line is the detection threshold, set at 1.35.](image-url)

**Fig. 5.** Typical NASBA amplification plots from 100, 10, 1 and 0 cells (A-D, respectively). The black line represents *K. mikimotoi* wild type RNA amplification. The gray line represents IC-RNA amplification. Thin, horizontal line is the detection threshold, set at 1.35.
By increasing the IC-RNA to $1 \times 10^8$ copies per reaction, the assay was able to detect RNA extracts from one thousand cells (and $1 \times 10^6$ target transcript copies), however the sensitivity was decreased and was no longer able to detect one cell (or $1 \times 10^4$ transcript copies) (data not shown).

To validate the increased precision of IC-NASBA over conventional real-time NASBA, two sets of standard curves were compared with three replicates per titer. Standard curves generated from RNA extracts from cultured *K. mikimotoi* cells assayed both with and without IC-RNA were compared (Fig. 6) along with in vitro transcripts with and without IC-RNA (Fig. 7). Coefficient of determination ($r^2$) values describing the linear fit of the negative correlation between TTP vs. cell number (conventional) and TTP ratio vs. cell number (IC-NASBA) were calculated and found to be 0.848 and 0.995 respectively (Fig. 6). An increase in the $r^2$ value of the IC-NASBA standard curve generated from in vitro transcripts was also observed when compared to conventional NASBA ($r^2 = 0.919$ and $r^2 = 0.991$ respectively) (Fig. 7).

Since *K. mikimotoi* in the Gulf of Mexico often co-occurs with closely related species such as *K. brevis* (K. A. Steidinger, C. Tomas, G. A. Vargo, and P.A. Tester 1998), we tested RNA extracts from mixed cultures of *K. mikimotoi* and *K. brevis*. One, ten, and one hundred target *K. mikimotoi* cells were assayed with 1:1, 1:5, and 1:10 mixes of *K. brevis* RNA. No decrease in sensitivity was seen with mixed ratio assays for one hundred ($P = 0.063$), ten ($P = 0.197$), or one ($P = 0.322$) target cells (Table 10).

For assays testing the effects of culture stress on expression of *rbcL* mRNA from *K. mikimotoi*, all treatments resulted in equivalent levels in *rbcL* mRNA copy number per cell, except the high-light treatment which had a 1.8 fold increase in copy number per cell over the control treatment (Table 11). The IC-NASBA assay was positive for all strains of *K. mikimotoi* tested, while none of the non-target species were detected (Table 9).
Fig. 6. (A) Conventional NASBA TTP standard curve using RNA extract from 100 to 1 K. mikimotoi cells. (B) IC-NASBA standard curve using RNA extract from 100 to 1 K. mikimotoi cells and $1 \times 10^7$ copies of K. mikimotoi IC-RNA.
**Fig. 7.** (A) Conventional NASBA TTP standard curve using $1\times10^3$-$1\times10^5$ copies of target *K. mikimotoi* RNA transcript. (B) IC-RNA standard curve $1\times10^3$-$1\times10^5$ copies of target *K. mikimotoi* RNA transcript with $1\times10^7$ of *K. mikimotoi* IC-RNA.
Table 10. Sensitivity of *K. mikimotoi* NASBA assay with *K. brevis* competition.

<table>
<thead>
<tr>
<th><em>K. mikimotoi</em> cell number</th>
<th>No <em>K. brevis</em> (Control)</th>
<th>1:1 with <em>K. brevis</em></th>
<th>1:5 with <em>K. brevis</em></th>
<th>1:10 with <em>K. brevis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.533 ± 0.009</td>
<td>0.529 ± 0.008</td>
<td>0.522 ± 0.005</td>
<td>0.515 ± 0.006</td>
</tr>
<tr>
<td>10</td>
<td>0.856 ± 0.022</td>
<td>0.871 ± 0.016</td>
<td>0.858 ± 0.019</td>
<td>0.891 ± 0.020</td>
</tr>
<tr>
<td>1</td>
<td>1.211 ± 0.006</td>
<td>1.228 ± 0.023</td>
<td>1.211 ± 0.042</td>
<td>1.174 ± 0.047</td>
</tr>
</tbody>
</table>

Average TTP ratios ± SD of IC-RNA NASBA performed on RNA extract from mixed cultures of *K. mikimotoi* and *K. brevis*. *P*-values determined by ANOVA (α = 0.05) of treatments for 100, 10, and 1 target cells are *P* = 0.063, *P* = 0.197, and *P* = 0.322 respectively.

Table 11. Effect of stress on cellular *rbcL* mRNA levels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular <em>rbcL</em> mRNA levels ± SD (copies/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>3.50 × 10^2 ± 5.36 × 10^1</td>
</tr>
<tr>
<td>Low nutrient</td>
<td>3.35 × 10^2 ± 7.37 × 10^1</td>
</tr>
<tr>
<td>Low salinity</td>
<td>3.45 × 10^2 ± 7.04 × 10^1</td>
</tr>
<tr>
<td>Low light</td>
<td>2.66 × 10^2 ± 1.85 × 10^1</td>
</tr>
<tr>
<td>High light</td>
<td>6.34 × 10^2 ± 1.23 × 10^1^</td>
</tr>
</tbody>
</table>

^a Significantly different (*P* = 0.0009)

**Environmental samples**

*K. mikimotoi* was enumerated from samples collected from the southwest coast of Florida by members of FWRI using direct microscopic counts. IC-NASBA was performed on RNA extracted from the same samples within a few hours of microscopic analysis. TTP ratios from environmental samples were compared with a culture cell (TAMU C22) standard curve and regression analysis was used to infer *K. mikimotoi* cell numbers (Table 12). A strong correlation
was noticed between the two enumeration methods for the same samples when analyzed using a paired $t$-test ($P = 0.156, \alpha = 0.05$) as well as having a good linear relationship ($r^2 = 0.887$) (Fig. 8). Only one sample enumerated by FWRI at their limit of detection (333 cells/liter) did not have positive IC-NASBA detection (Table 12).

**Fig. 8.** Relationship between microscopic counts performed by FWRI and IC-NASBA inferred cell counts from environmental samples.

**Discussion**

IC-NASBA is a sensitive and specific method for the detection of $rbcL$ mRNA from as little as one *K. mikimotoi* cell in less than 90 minutes. We have been able to generate both cellular mRNA and in vitro transcript standard curves over three orders of magnitude with strong linear relationships ($r^2 = 0.995$ and $r^2 = 0.991$ respectively). Direct detection of a fourth order of magnitude (1000 cells or $1\times10^6$ copies of in vitro transcript) would be advantageous in screening environmental waters containing very high concentrations of *K. mikimotoi*. However, by simply filtering less initial volume or diluting extracts, wild type TTP ratios in the range of the standard curve can be obtained. A greater initial concentration of IC-RNA ($1\times10^8$ copies per reaction)
should increase the dynamic range to encompass higher concentrations of target. Incidentally, dilution of RNA eluate is occasionally required to decrease levels of NASBA inhibitors which can be determined by decreased IC-RNA amplification, as noted in this study when performing assays on some environmental samples.

Table 12. Comparison of FWRI environmental *K. mikimotoi* microscopic cell counts and IC-NASBA.

<table>
<thead>
<tr>
<th>Site</th>
<th>Date of collection (mo/day/yr)</th>
<th>FWRI cell count (cells/liter)</th>
<th>IC-NASBA (cells/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee County, South Seas Plantation Entrance</td>
<td>10/8/08</td>
<td>333&lt;sup&gt;d&lt;/sup&gt;</td>
<td>550&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lee County, Tarpon Road Beach</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Lighthouse Beach</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Lynn Hall Park</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Lovers Key State Park</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, South Seas Plantation Entrance</td>
<td>10/15/08</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Tarpon Road Beach</td>
<td></td>
<td>333&lt;sup&gt;d&lt;/sup&gt;</td>
<td>815&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lee County, Lighthouse Beach</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Lynn Hall Park</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lee County, Lovers Key State Park</td>
<td></td>
<td>333&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collier County, Barefoot Beach</td>
<td>10/20/08</td>
<td>333&lt;sup&gt;d&lt;/sup&gt;</td>
<td>735</td>
</tr>
<tr>
<td>Collier County, Clam Pass</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collier County, Naples Pier</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collier County, Marco Island, South Marco Beach</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collier County, Barefoot Beach</td>
<td>10/30/08</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collier County, Vanderbilt Beach</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collier County, Marco Island, South Marco Beach</td>
<td></td>
<td>1000</td>
<td>1735</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1:10 and <sup>b</sup> 1:100 dilution of RNA extract needed to overcome inhibition.  
<sup>c</sup> Positive detection of *K. mikimotoi* by microscopy with no IC-NASBA detection or inhibition.  
<sup>d</sup> FWRI microscopy limit of detection.
Application of various stresses on cultures of *K. mikimotoi* did not affect cellular *rbcL* mRNA levels except for high-light exposure which caused a 1.8-fold increase per cell, and has been similarly observed with *K. brevis* (Casper, Paul, Smith, & Gray, 2004). This increase is less than one order of magnitude and within the precision range of the IC-NASBA assay. Diel experiments (e.g. sampling over a 24 hr period) were not performed in this study. However, numerous standard curves generated from cultures sampled during both light and dark incubation cycles had no significant difference in TTP ratios (data not shown). This leads us to believe that there is no significant difference in cellular *rbcL* mRNA levels during normal diel periods, as has been shown for other dinoflagellates.

There is limited *rbcL* sequence information for *Karenia* sp. in GenBank from which we designed our specific primers and beacons. However, none of the non-target organisms chosen for comparison (including five isolates of closely related *K. brevis*) were detected. Additionally, no decrease in sensitivity of the IC-NASBA assay was observed in the presence of *K. brevis* RNA extracts at various concentrations.

Cell counts inferred from IC-NASBA on environmental samples did not differ significantly from microscopic cell counts performed by FWRI. All IC-NASBA positive samples (*n* = 4) were also positive by microscopy, and in twelve out of thirteen IC-NASBA negative samples, *K. mikimotoi* was not detected by microscopy. Incidence of error of microscopic identification by FWRI was not determined; however identification of 100% of *Karenia* in a given sample to the species level is difficult due to variations in cell orientation on the slide and morphological distortions caused by preservatives (Bill Richardson, personal communication). Culverhouse et al. (2003) suggest that consensus identification of dinoflagellates between individuals can be as low as 43%. Also, the limit of detection of FWRI
microscopic enumeration is no less than 333 cells/liter, which lacks the resolution needed to truly compare the two methods. A sandwich hybridization assay detecting rRNA from harmful dinoflagellates (including *K. mikimotoi*) was recently developed by Haywood et al. (2007). This assay is both rapid and specific, with a theoretical limit of detection is 1250 cells/liter (based on 250 ml of sample) and a possible reduced reactivity when assaying natural samples as opposed to pure cultures. Further validation of IC-NASBA needs to be performed on a greater number of environmental samples positive for *K. mikimotoi* to get a clearer picture of assay consistency, and how different inhibitory elements from various water bodies may affect the sensitivity of the assay.

Rapid and reliable identification of HAB forming species such as *K. mikimotoi* in low concentrations is critical for the early detection of bloom events that may make environmental waters unsafe for shellfish harvesting or recreational use. IC-NASBA is well suited for this type of monitoring due to low limits of detection and a high level of specificity. Also, being that NASBA is an isothermal process (41°C), adaptability to remote sensing platforms is more practical than molecular assays requiring thermal cycling for amplification. A handheld IC-NASBA analyzer assay to detect *K. brevis* has already been developed by our lab (Casper, Patterson, Bhanushali, Farmer, Smith, Fries, et al., 2007), and integration of the *K. mikimotoi* assay to this platform is intended by simple primer/beacon exchange. Diagnosis of inhibition levels of molecular amplification assays performed on environmental waters is necessary to reduce the incidence of false negatives, for which the internal control characteristics of the IC-RNA molecule is well suited. Furthermore, IC-NASBA may be useful in determining viable states of HABs due to the instability of mRNA targets, which may otherwise be difficult with assays targeting more stable DNA.
Acknowledgments

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We would like to thank Karen Steidinger and Earnest Truby of the Florida Marine Research Institute for providing us with cultures used in this study, as well as enumerated environmental samples.

References


CHAPTER FIVE:
CONCLUSIONS

This body of work was meant to illustrate several practical applications of RT-NASBA to provide solutions to marine science-related issues. The biological targets of these applications represent a wide range of taxonomic diversity, yet all play important roles, whether they are ecological, economical, or relevant to human health. Careful attention was made to describe the advantages of each RT-NASBA assay, as well as their potential limitations.

Chapter Two is a published article that describes the problem of seafood mislabeling as it relates to grouper, and how taxonomic diversity between numerous salable species limits conventional phenotypic identification. This chapter also discusses current molecular identification methods used by seafood regulators, including why they are practiced, as well as their limitations. The goal of developing the RT-NASBA assay detailed in this chapter was to obviate some of these limitations by providing a rapid and reliable method to discern grouper species on the FDA Seafood List from impostor fish, without the requirement of tedious DNA sequence analysis. Results from this study indicate that the assay is able to differentiate approximately 86% of the grouper species on this list from surrogates based on empirical data generated from experiments on tissues, as well as bioinformatic sequence analysis retrieved from online databases. Furthermore, the assay was able to amplify RNA from a single species of grouper after being cooked, possibly extending the market for this detection method to restaurant patrons.
The goal of Chapter Three was to adapt the grouper RT-NASBA, which was validated in Chapter Two using commercial benchtop instrumentation, into a handheld sensor format to be used in the field. Development of the sensor was performed by engineers at the USF College of Marine Science, and a manuscript prepared by these collaborators describing the technical aspects of the instrument was recently submitted for publication. The development of an inexpensive method for the field purification of RNA from fish tissue was also detailed in this section. When coupled with the handheld sensor, the RNA purification protocol enables the assay to be performed outside of the lab in its entirety. The complete field assay was validated against multiple grouper species, including many of those appearing on the recently updated FDA Seafood List. Due to a slightly lower sensitivity inherent to the handheld sensor, several species of grouper could not be detected which were detectable using the lab-based assay. However, all of the most commercially important species to the state of Florida were detectable, perhaps making this assay best suited for local commerce. When evaluating this field assay against cooked Florida grouper tissues, only intermittent detection was achieved for some species. More likely, benchtop instrumentation is better suited for assaying cooked tissue.

To my knowledge, Chapters Two and Three represent the most complete body of work documenting a technology for the intent of differentiating all grouper species listed on the FDA Seafood List from surrogates, in a single genetic assay. Work presented by Trotta et al. (2005) represents the most comparable approach by targeting grouper species using multiplex SYBR® Green I RT-PCR to detect mitochondrial 16S rDNA. Although comprehensive bioinformatic sequence analysis was performed, the study lacks adequate experimental assay validation, as only four tissue samples from *Epinephelus* and *Mycteroperca* species were tested (Trotta et al. 2005). The RT-NASBA assay was tested against 36 grouper species listed on the Seafood List.
with several levels of beacon heterogeneity requiring experimental evidence to determine
detectability. Perhaps the most valuable attribute of the grouper assay designed by Trotta et al.
(2005) was the incorporation of oligonucleotide primers specific for two species commonly
substituted for grouper, Nile perch (*Lates niloticus*) and wreck fish (*Polyprion americanus*). This allows for the simultaneous identification of impostor species within a grouper-negative
reaction, by performing dissociation curve analysis subsequent to SYBR® Green I RT-PCR.
Adapting the RT-NASBA detection technology to a similar format is worthy of future research.
Although the inclusion of additional oligonucleotides to a single multiplex RT-NASBA reaction
detrimentally decreased specificity (Chapter Two), designing alternate assays targeting popular
surrogates would provide the user with replacement species identification. These alternate
assays could be performed simultaneously with grouper RT-NASBA using numerous available
reaction wells inherent to the EasyQ® benchtop instrumentation, or as many as three available
with the field QuadPyre sensor. Moreover, the development of RT-NASBA assays targeting the
minor number of FDA grouper species currently undetectable should be investigated to augment
the assay in its current format.

Chapter Four is published work describing the development of an internally controlled
RT-NASBA assay for the detection and quantification of *K. mikimotoi* in marine waters.
Integration of IC-RNA into the RT-NASBA chemistry not only increases the precision for
quantification, but adds an internal control measure to each reaction for diagnosing inhibition.
The IC-NASBA assay was sensitive to one *K. mikimotoi* cell, and was specific against closely
related organisms, even when in mixed cultures. When compared with FWRI microscopic cell
counts on environmental samples, IC-NASBA quantification did not differ significantly,
although only a limited number of samples were positive for *K. mikimotoi.*
Two alternate molecular amplification techniques have recently been described for the detection and quantification of *K. mikimotoi* using both loop-mediated isothermal amplification (LAMP) (Zhang, Ma, Xu, Zheng, Shi, Lu, et al., 2009) and qPCR (Yuan, Mi, Zhen, & Yu, 2012), targeting ribosomal DNA internal transcribed spacer regions (ITS). LAMP is an isothermal (65°C) DNA amplification method that utilizes auto-cycling strand displacement synthesis that is performed by specialized polymerases that are able to displace double stranded DNA templates without the need for thermal cycling. Zhang et al. (2009) claim to have developed a LAMP method to detect low quantities of DNA; however the assay was validated using only a single environmental *K. mikimotoi* bloom sample and the cell number limit of detection is unclear, being reported as nanograms of template DNA. The qPCR assay described by Yuan et al. (2012) was also only validated using a single environmental isolate, yet the authors better define the limit of detection as five *K. mikimotoi* cells. The RT-NASBA assay described herein was sensitive to one cell and was validated using four separate *K. mikimotoi* isolates and several environmental samples taken on different dates from southwest Florida. Moreover, RT-NASBA targets mRNA which tends to be less stable outside of the cell than DNA, as described in previous sections. Thus, positive RT-NASBA detection events should represent only viable cells which may better predict the growth stage of potential blooms; however more research is needed to validate this claim.

The appendix following this section is unpublished preliminary research toward the development of an IC-NASBA assay targeting 23S rRNA for the detection and enumeration of *Enterococcus* as indicators of fecal pollution to natural waters. The specificity of the assay was evaluated against several control strains of *Enterococcus*, as well as a number of environmental isolates. Moreover, no cross-reactivity occurred for any non-target bacteria tested. A single
experiment was performed comparing the IC-NASBA assay with two EPA recommended methods for quantifying enterococci, in ambient marine and fresh recreational waters. Each of these methods was used to quantify enterococci in replicate sewage and tertiary-treated wastewater samples. The majority of these samples were not significantly different in enterococci enumeration when comparing IC-NASBA with each of the EPA methods.

Several PCR and qPCR assays have been developed to quantify Enterococcus spp. in environmental waters targeting ribosomal DNA genes and are mentioned in the appendix, including a culmination of research leading to the development of the newly recommended and highly validated EPA qPCR Method 1611 (EPA, 2012). Due to the labile nature of RNA and its sensitivity to ubiquitous endonucleases that are often difficult to remove from labware, many researchers are apprehensive to rely on it as a target for amplification assays. However, the benefit of increased sensitivity may outweigh difficulty in handling RNA, as there can be exponentially more rRNA copies per cell than rDNA gene copies (Arfvidsson & Wahlund, 2003). There are known to be only four large subunit rRNA gene copies per E. faecalis genome (EPA, 2012) which may limit the sensitivity of assays relying on DNA as targets. A study performed by Matsuda et al. (2007) compared rRNA-targeted RT-qPCR assays with DNA-targeted qPCR assays for quantifying human gut bacteria including E. faecalis, and found the former to be approximately 100- to 1000-fold more sensitive (Matsuda, Tsuji, Asahara, Kado, & Nomoto, 2007). More research is needed to ascertain the reproducible sensitivity of the RT-NASBA by testing a number of environmental water samples from various geographic locations. Natural waters used for recreation often range in chemical composition, and results presented below indicate that RT-NASBA may be slightly more sensitive to inhibitory substances than qPCR; however further verification is required.
References


APPENDIX A:

PRELIMINARY DEVELOPMENT OF AN IC-NASBA ASSAY FOR THE
DETECTION AND QUANTIFICATION OF ENTEROCOCCUS FOR
WATER QUALITY ASSESSMENT

Introduction

*Enterococcus* species are Gram-positive, aeroteolerant fermentive coccoid bacteria that are commonly found in the gastrointestinal tract of mammals and other warm-blooded animals. Some enterococci, particularly *E. faecalis* (Farrell, Morrissey, De Rubeis, Robbins, & Felmingham, 2003) and *E. faecium* (Das & Gray, 1998), may possess certain virulence factors and/or antibiotic resistance genes enabling them to become opportunistic human pathogens, however, this is only common with immunocompromised hosts (Rice, Carias, Rudin, Vael, Goossens, Konstabel, et al., 2003; Shankar, Lockatell, Baghdayan, Drachenberg, Gilmore, & Johnson, 2001). There is evidence that enterococci levels can correlate with gastrointestinal illness caused by exposure to recreational marine water that is impacted by point source pollution (Pruss, 1998; Wade, Calderon, Sams, Beach, Brenner, Williams, et al., 2006; Wade, Pai, Eisenberg, & Colford, 2003). The U.S. Environmental Protection Agency (EPA) has historically recognized members of the genus *Enterococcus* as bacterial indicators of water quality in both fresh and marine waters (EPA, 1986) and in 2004, enterococci replaced fecal coliforms (e.g. *Escherichia coli*) as the new federal standard for water quality at salt water beaches due to their higher tolerance for saline water (EPA, 2004). Currently in the state of
Florida, if the enterococci concentration exceeds 103 colony forming units (CFU) per 100 ml of beach water, a health advisory is implemented for that location.

The standard method employed by the EPA for monitoring enterococci levels in environmental waters is EPA Method 1600, which is a culture-based membrane filtration procedure that utilizes selective media (EPA, 2006). Briefly, environmental water samples are first filtered through gridded membranes having a 0.45 ± 0.02 μm pore size which allows for the retention of bacteria. This membrane filter is then placed on membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) and incubated for 24 h at 41°C. The mEI agar contains a chromogenic substrate that causes a distinctive blue halo to form around colonies of Enterococcus allowing for their enumeration and subsequent concentration determinations.

The primary limitation of culture-based methods for the detection of microbial indicators in natural water samples is the prolonged time needed for organisms to grow on culture media. In the time it takes for the lag between sample collection and test results, the water quality may have changed at that given site leading to inaccurate management decisions and the increased potential for pathogen exposure (Frick, Ge, & Zepp, 2008; Kim & Grant, 2004). Another limitation of culture-based methods is that some bacterial species, including some enterococci (Lleo, Bonato, Tafi, Signoretto, Boaretti, & Canepari, 2001; Signoretto, Burlacchini, Pruzzo, & Canepari, 2005), have been observed to adopt survival strategies when exposed to environmental stress or lack of nutrients (Barer & Harwood, 1999). Bacteria undergoing this state of defensive dormancy are termed viable but non-culturable (VBNC), allowing them to maintain their resuscitation capability until they encounter more favorable growth conditions. This confounds enumeration of indicator bacteria using culture-based methods by increasing the chance underestimating bacterial concentrations.
To obviate limitations of culture-based enumerations of enterococci, several molecular methods have been developed targeting DNA, primarily using conventional PCR (Cheng, McCleskey, Gress, Petroziello, Liu, Namdari, et al., 1997; Harwood, Delahoya, Ulrich, Kramer, Whitlock, Garey, et al., 2004) or quantitative PCR (qPCR) (Mohn, Ulvik, Jureen, Willems, Top, Leavis, et al., 2004; Ludwig & Schleifer, 2000; Ryu, Henson, Elk, Toledo-Hernandez, Griffith, Blackwood, et al., 2013; Santo Domingo, Siefring, & Haugland, 2003) formats that forgo the need to culture. Moreover, the EPA recently developed and evaluated a qPCR assay (EPA Method 1611) which it now recommends for the quantification of enterococci in recreational waters (EPA, 2012). However, there is evidence that exogenous DNA can persist outside the cell in environmental waters, which could lead to the overestimation of viable bacteria and inaccurate management decisions (Dejean, Valentini, Duparc, Pellier-Cuit, Pompanon, Taberlet, et al., 2011). Due to the increased susceptibility of RNA to degradation outside living organisms, molecular assays targeting RNA may allow for more accurate enumeration of only viable targets (Lleo, Pierobon, Tafi, Signoretto, & Canepari, 2000). Moreover, RNA detection technologies benefit from the often numerous copies of RNA targets per cell which allows for increased sensitivity, and in some instances, single cell detection (Casper, Paul, Smith, & Gray, 2004; Ulrich, Casper, Campbell, Richardson, Heil, & Paul, 2010). It was our goal to begin development of a sensitive and specific IC-NASBA assay for the detection and quantification of Enterococcus spp. from environmental waters.

This preliminary research was carried out in two phases; first to validate the specificity of the assay, and second, to test its performance against the two aforementioned EPA methods for quantifying enterococci. During the first phase, IC-NASBA was used to detect several Enterococcus spp. obtained from a commercial culture collection, as well as numerous
environmental isolates. Subsequently, several non-target bacteria were interrogated for cross-reactivity, including Gram-positives and negatives, as well as several species autochthonous to marine waters. The second phase was executed by quantifying enterococci in raw and treated wastewater for a collaborative experiment using the IC-NASBA assay, EPA Method 1600, and EPA Method 1611. The primary intention of this analysis was to evaluate a prototype electrocoagulation (EC) instrument by members of the Breitbart Lab at the College of Marine Science at USF. Briefly, the electrocoagulator utilizes electrolysis to alter the surface charge of unwanted particles within wastewater which then form suspended agglomerates to be subsequently removed by filtration. The detailed function and performance of this instrument is outside the scope of this dissertation, and thus will not be discussed in detail below.

Materials and Methods

Oligonucleotide design and IC-NABSA

IC-NASBA oligonucleotide primers and molecular beacons were designed to target a 136-bp region of large subunit ribosomal RNA gene (23S rRNA), specific to known species of enterococci important to water quality monitoring, and are listed in Table A1. Synthesis of the IC-RNA was performed as described in Ulrich et al. (2010), and the in vitro transcript generation oligonucleotides are provided in Table A1. The IC-NASBA chemistry composition and oligonucleotide concentrations used in all subsequent assays were as described in Ulrich et al. (2010); including the addition of 1×10⁷ IC-RNA copies per reaction. IC-NASBA was performed for 90 min at 41°C using a NucliSENS EasyQ® analyzer (bio-Mérieux, Durham, NC) for all of the following analysis. Whole-cell RNA extracts from American Type Culture Collection (ATCC®) *E. faecalis* 29212™ were used to generate standard curves for quantification of
enterococci in unknown samples. Standard curves were generated using DNA from $1 \times 10^5$, $1 \times 10^4$, $1 \times 10^3$, and $1 \times 10^2$ cells in triplicate (all $r^2$ from regressions $\geq 0.95$), and unknown cell concentrations were determined using the TTP ratio method detailed in Chapter 4. Enumeration of enterococci was reported as calculated cell number/ml.

### Table A1. *Enterococcus* IC-NASBA oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus</em> Forward Primer</td>
<td>GACCCGAAAACCATGTGATCTA</td>
</tr>
<tr>
<td><em>Enterococcus</em> Reverse Primer</td>
<td>AATTCTAATACGACTCACTATAGGGAGGAATATATCTCAAAGTCTTCGTTTGGA</td>
</tr>
<tr>
<td><em>Enterococcus</em> Target Molecular Beacon</td>
<td>[6-FAM]-CGATCGGATGGAGGTGTTGGTAGCGGACGATCG-[DABCYL]</td>
</tr>
<tr>
<td><em>Enterococcus</em> IC-RNA Molecular Beacon</td>
<td>[6-ROX]-CATGCGTGCGTCTTTATGGTAGCAATTCGATG-[DABCYL]</td>
</tr>
<tr>
<td><em>Enterococcus</em> IC-RNA generation forward oligo</td>
<td>AATTCTAATACGACTCACTATAGGGAGGACGGAAAACCATGTGATCT-</td>
</tr>
<tr>
<td><em>Enterococcus</em> IC-RNA generation reverse oligo</td>
<td>CCCATGCTCCAGGTTGGAAGGTGCGGTAACCGACTGAGGAACCGAACCC-</td>
</tr>
</tbody>
</table>

Text highlighted in gray indicates T7 RNA polymerase promoter site. Italicized text indicated reverse compliment overlap.

**IC-NASBA Specificity and sensitivity**

Target and non-target bacterial isolates were obtained from ATCC® or from various environmental sources isolated and confirmed by members of the Harwood (Department of Integrative Biology) and Paul Labs (College of Marine Science) at USF (Table A2). Bacteria were grown in overnight liquid cultures using recommended media and incubation conditions respective to each species’ requirements. RNA was extracted from liquid broths using RNeasy®
Mini Kit protocols (Qiagen, Valencia, CA) amended for either Gram-positive or Gram-negative bacteria.

Sensitivity of the IC-NASBA method was determined by assaying RNA extracts from *E. faecalis* 29212™. Cells were serially diluted in standard phosphate buffered saline (PBS) and quantified using standard CFU plate count enumeration on m-*Enterococcus* Agar (Difco™, Franklin Lakes, NJ) incubated at 41°C for 24 hr. RNA was extracted from parallel dilutions and used as template for IC-NASBA.

**IC-NASBA comparison with EPA quantification methods**

As stated above, the full suite of microbial and chemical manipulations and analyses performed in this experiment not relevant to *Enterococcus* will not be discussed here. However, a full presentation of this research is planned to be included in a peer-reviewed journal article prepared by Erin M. Symonds of the Breitbart Lab at the College of Marine Science at USF. To test the efficacy of the EC instrument in removing both microbial and chemical impurities, raw sewage influent and tertiary treated water from a wastewater treatment facility in St. Petersburg, FL were used as testing matrices. To ensure quantifiable numbers were achieved for each enumeration method, both the wastewater and treated water samples were augmented with known concentrations (data not shown) of control *E. faecalis* 29212™ prior to electrocoagulation. This was achieved by spiking each pre-electrocoagulation sample with a quantified overnight culture grown in Nutrient Broth (Difco™, Franklin Lakes, NJ) and inverting several times to homogenize. Four sequential EC treatments were performed on replicate samples of both wastewater and tertiary-treated samples, including intermittent sterilization measures between each run.
For both IC-NASBA and EPA Method 1611 qPCR analyses, three replicate 1 ml samples were taken prior to (pre-EC) and after (post-EC) each electrocoagulation event and filtered onto 0.45-µm-pore size HV polyvinylidene difluoride filters (Millipore, Billerica, MA). RNA and DNA were extracted from filters using either the RNeasy® Mini Kit or DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA) specific for Gram-positive bacteria, for IC-NASBA and qPCR, respectively. A truncated version of Method 1611 was used, utilizing only the portions specific to the base TaqMan® (Life Technologies®, Carlsbad, CA) qPCR Enterococcus assay. Aspects of Method 1611 pertaining to quality control and high-throughput analysis of natural water samples were not utilized in this analysis, such as matrix spikes, sample processing controls, calibrator samples, or comparative cycle threshold calculations (EPA, 2012).

TaqMan® qPCR Enterococcus reaction chemistry (25 µl total volume) consisted of 1 µM forward primer (5’-GAGAAATTCCAAACGAACTTG-3’), 1 µM reverse primer (5’-CAGTG-CTCTACCTCCATCATT-3’), 80 nM TaqMan® probe ([6-FAM]-5’-TGGTTCTCTCTCGAAATAGCTTTTAGGCTA-3’-[TAMRA]), 12.5 µl TaqMan® Universal master mix, 2.5 µl bovine serum albumin (2 mg/ml stock), and 2 µl DNA template eluted in nuclease-free water. Reactions were performed using an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies®, Carlsbad, CA). The thermal profile of the qPCR assay was as described in Method 1611 (EPA, 2012). A single standard curve generated from DNA extracts of E. faecalis 29212™ diluted to include four orders of magnitude (1×10^5 to 1×10^2 cells per reaction in triplicate) was used in linear regression analysis for unknown quantification. The r^2 value from the regression of this curve was 0.998, fitting the EPA recommended criteria of 0.99 or greater (EPA, 2012). Enumeration of enterococci were reported as calculated cell number/ml as with IC-NASBA.
EPA Method 1600 analysis was performed on triplicate pre-EC and post-EC samples for each of the four runs (EPA, 2006). Multiple dilutions of each replicate sample were filtered onto gridded 0.45-µm-pore size nitrocellulose filters (Millipore, Billerica, MA) and incubated on mEI agar for 24 hr at 41°C. Bacterial colonies exhibiting a blue halo were recorded as enterococci and reported as CFU/ml.

**Statistical analysis**

Calculating significant relationships of mean cell concentrations between IC-NASBA and the two EPA methods were computed using GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA). Differences among average cell concentrations were determined using paired t-tests. All means were considered significantly different when $P < 0.05$.

**Results**

**IC-NASBA specificity and sensitivity**

The IC-NASBA assay was able to detect five species of *Enterococcus*, including multiple strains of *E. faecalis*, *E. faecium*, and *E. avium* obtained from a commercial culture collection. Moreover, the assay was able to detect multiple environmental isolates obtained from several natural water bodies in Tampa, FL including Lake Carroll (freshwater), a brackish portion of the Hillsborough River (salinity, 17 ppt), and Ben T. Davis Beach located in an estuarine portion in Tampa Bay (salinity, 24 ppt) (Table A2). No cross-reactivity was observed when testing non-target organisms including several pathogenic, Gram-positive bacteria (*Listeria monocytogenes*, *Clostridium perfringens*, *Bifidobacterium dentium*, and *Lactococcus garvieae*) known to occur in marine waters (Lleò, Signoretto, & Canepari, 2005). The IC-NASBA assay was also non-
reactive with several *E. coli* isolates, as well as two pathogenic *Vibrio* spp. found naturally in estuarine waters (Table A2).

The limit of detection of the IC-NASBA assay was 24 ± 2 *E. faecalis* 29212™ CFU/ml diluted in PBS. Sensitivity analysis was not performed on any other *Enterococcus* spp. in pure culture. However, the assay was able to detect as few 114 ± 22 enterococci cells/ml in tertiary-treated wastewater (Fig. A2, NASBA Treat 3), and the mean concentration determinations using both EPA methods for the same sample were not significantly different from IC-NASBA (Tables A3 and A4).

**Table A2.** Specificity tests using the *Enterococcus* IC-NASBA assay.

<table>
<thead>
<tr>
<th>Strain/Isolate</th>
<th>Detection Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC® <em>E. faecalis</em> (29212™, 202014™)</td>
<td>2/2</td>
</tr>
<tr>
<td>ATCC® <em>E. faecium</em> (PTA-7478™, PTA-7480™)</td>
<td>2/2</td>
</tr>
<tr>
<td>ATCC® <em>E. avium</em> (49463™, 49465™)</td>
<td>1/1</td>
</tr>
<tr>
<td>ATCC® <em>E. casseliflavus</em> (25788™)</td>
<td>1/1</td>
</tr>
<tr>
<td>ATCC® <em>E. gallinarum</em> (49573™)</td>
<td>1/1</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp., Lake Carroll, Tampa (5 isolated from water)</td>
<td>5/5</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp., Lake Carroll, Tampa (7 isolated from sediment)</td>
<td>7/7</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp., Hillsborough River, Tampa (7 isolated from water)</td>
<td>7/7</td>
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<tr>
<td><em>Enterococcus</em> spp., Hillsborough River, Tampa (5 isolated from water)</td>
<td>5/5</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp., Ben T. Davis Beach, Tampa (6 isolated from water)</td>
<td>6/6</td>
</tr>
<tr>
<td><em>Enterococcus</em> spp., Ben T. Davis Beach, Tampa (4 isolated from sediment)</td>
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</tr>
<tr>
<td><em>ATCC® Listeria monocytogenes</em> (7644™)</td>
<td>0/1</td>
</tr>
<tr>
<td><em>ATCC® Clostridium perfringens</em> (10543™)</td>
<td>0/1</td>
</tr>
<tr>
<td><em>ATCC® Bifidobacterium dentium</em> (27680™)</td>
<td>0/1</td>
</tr>
<tr>
<td><em>Lactococcus garvieae</em> (1 environmental isolate of unknown origin)</td>
<td>0/1</td>
</tr>
<tr>
<td><em>ATCC® E. coli</em> (4 strains)</td>
<td>0/4</td>
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<tr>
<td><em>ATCC® Vibrio vulnificus</em> (27562™, 29307™, 33814™)</td>
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<tr>
<td><em>ATCC® V. parahaemolyticus</em> (17802™, 33157™)</td>
<td>0/2</td>
</tr>
<tr>
<td><em>E. coli</em> (4 environmental isolates of unknown origin)</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Non-target organism.
IC-NASBA comparison with EPA quantification methods

When comparing IC-NASBA with Method 1600 (mEI), no significant differences between mean cell concentrations were determined for any sewage or treated sample having enterococci levels above the detection limit (24 ± 2 CFU/ml), either before or after EC (Fig. A1 and A2; Table A3). Significantly different enterococci concentrations were calculated for two sewage samples when comparing IC-NASBA with Method 1611 (qPCR) (Fig A1; Table A4). However, no treated samples were significantly different in enterococci concentrations between IC-NASBA and qPCR methods.

![Fig. A1. IC-NASBA, Method 1600 (mEI), and Method 1611 (qPCR) quantification of enterococci in sewage (Sew) and tertiary-treated (Treat) samples prior to electrocoagulation. Cell concentrations are averages of three replicates for each assay ± STDEV. Numbers following each assay on the X-axis represent sequential EC events. Mean concentrations from assays indicated with an asterisk were significantly different from IC-NASBA for respective samples.](image-url)
Table A3. Comparison of mean Enterococcus concentrations generated using IC-NASBA and mEI quantification methods.

<table>
<thead>
<tr>
<th>Sample</th>
<th>P-value</th>
<th>Sample</th>
<th>P-value</th>
<th>Sample</th>
<th>P-value</th>
<th>Sample</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Sewage 1</td>
<td>0.077</td>
<td>Treated 1</td>
<td>0.137</td>
<td>Sewage 1</td>
<td>NA</td>
<td>Treated 1</td>
<td>NA</td>
</tr>
<tr>
<td>Sewage 2</td>
<td>0.429</td>
<td>Treated 2</td>
<td>0.804</td>
<td>Sewage 2</td>
<td>NA</td>
<td>Treated 2</td>
<td>NA</td>
</tr>
<tr>
<td>Sewage 3</td>
<td>0.964</td>
<td>Treated 3</td>
<td>0.122</td>
<td>Sewage 3</td>
<td>NA</td>
<td>Treated 3</td>
<td>0.110</td>
</tr>
<tr>
<td>Sewage 4</td>
<td>0.291</td>
<td>Treated 4</td>
<td>0.594</td>
<td>Sewage 4</td>
<td>NA</td>
<td>Treated 4</td>
<td>0.655</td>
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</table>

Means were considered significantly different when \( P < 0.05 \) using paired \( t \)-test. Samples below the enterococci detection limit of either or both methods are indicated with (NA).

Fig. A2. IC-NASBA, Method 1600 (mEI), and Method 1611 (qPCR) quantification of enterococci in sewage (Sew) and tertiary-treated (Treat) samples after electrocoagulation. Cell concentrations are averages of three replicates for each assay ± STDEV. Numbers following each assay on the X-axis represent sequential EC events.
Table A4. Comparison of mean *Enterococcus* concentrations generated using IC-NASBA and qPCR quantification methods.

<table>
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<th>Pre-electrocoagulation</th>
<th>Post-electrocoagulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td><strong>P-value</strong></td>
</tr>
<tr>
<td>Sewage 1</td>
<td>0.005</td>
</tr>
<tr>
<td>Sewage 2</td>
<td>0.161</td>
</tr>
<tr>
<td>Sewage 3</td>
<td>0.291</td>
</tr>
<tr>
<td>Sewage 4</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Means were considered significantly different when $P < 0.05$ using paired *t*-test. Samples below the enterococci detection limit of either or both methods are indicated with (NA).

**Discussion**

Preliminary results suggest that the IC-NASBA assay is a sensitive and specific method for the detection of 23S rRNA from *Enterococcus* spp. and provides results in less than 90 minutes. We provide evidence that the assay is specific against multiple control *Enterococcus* isolates, with little potential for cross-reactivity. Moreover, the EPA Method 1611 qPCR assay, which has been validated by multiple labs, is designed to target a similar portion of the 23S rDNA gene which further supports the specificity of the IC-NASBA assay (EPA, 2012).

The mEI and IC-NASBA quantification methods agreed over several orders of magnitude. Although there were no significant differences in mean enterococci concentration between IC-NASBA and mEI quantification for any sample above the IC-NASBA detection limit, three of four pre-EC sewage samples had slightly lower IC-NASBA cell numbers than mEI. Inversely, all tertiary-treated samples were quantified slightly higher using IC-NABA than with mEI. This may be attributed to higher concentrations of chemical inhibitors of NASBA in sewage than with tertiary-treated wastewater. All sewage and tertiary-treaty samples were either significantly, or slightly higher in enterococci using qPCR than with IC-NASBA, including one post-EC sewage sample that was below the detection limit of IC-NASBA. This may indicate
that Method 1611 is less sensitive to inhibitory substances found in these samples, or perhaps the qPCR assay is amplifying exogenous enterococci DNA persisting in the samples.

Further studies are required to validate IC-NASBA performance on wastewater, as it is difficult to make assumptions using data from a single trial. Moreover, a single sensitivity experiment targeting one control Enterococcus isolate in sterile PBS is not adequate to determine the true limit of detection of an assay intended to be performed on natural water samples. Additional analysis is required using IC-NASBA to target diluted sewage samples from various sources to better ascertain the sensitivity of the assay for its proposed implementation. Also, the prevalence of inhibitory substances often found in natural waters needs to be evaluated, and mitigation measures such as alternate methods for RNA purification should be explored.

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