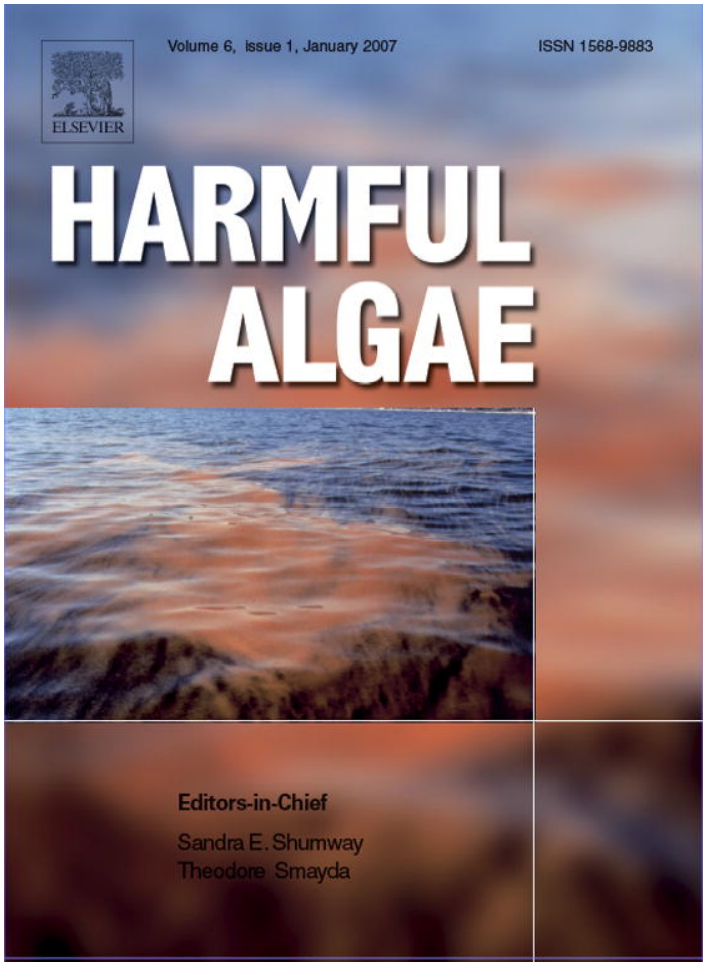

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A handheld NASBA analyzer for the field detection and quantification of *Karenia brevis*

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Received 13 September 2006; received in revised form 16 October 2006; accepted 1 November 2006

Abstract

Blooms of *Karenia brevis*, the red tide forming dinoflagellate in the Gulf of Mexico, cause a myriad of ecological and economic problems for coastal communities, including massive fish and mammal mortalities, and damage to tourism and fisheries/shellfish harvesting industries. There is a need for accurate detection and prediction of *K. brevis* blooms, including rapid and inexpensive monitoring of both water and shellfish meats to ensure the safety of shellfish harvested for human consumption. To address this issue, we have developed a protocol for easy field extraction of cellular RNA from water samples and coupled it with a handheld nucleic acid sequence-based amplification (NASBA) sensor that amplifies and detects target mRNA specific to the *rbcL* gene of *K. brevis*. This extraction protocol is a modified version of the Qiagen RNeasy Mini Kit spin protocol and requires no specialized equipment or training. Once extracted, the RNA is amplified and detected by NASBA in an in-house designed and produced handheld sensor that provides a real-time fluorescence plotting of the amplification. Both the field RNA extraction protocol and the handheld NASBA analyzer compared favorably to laboratory-based technologies. In duplicate reactions, the amplification curves generated with the handheld detector closely mirrored the curves generated with the bench top Nuclisens EasyQ NASBA analyzer and there was no difference in the sensitivity obtained using the handheld device versus the bench top models. This extraction protocol and detection sensor will be a valuable tool for rapidly monitoring *K. brevis* in field environments.

Keywords: Field detection; *Karenia brevis*; NASBA; Quantification

1. Introduction

Blooms of the Florida red tide dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*) have plagued the Gulf coasts since the Spanish conquests and continue to inflict damage to marine life, coastal resource users, fisheries, tourism, and human health. Red tides, a near yearly event along the Florida Gulf coast, are responsible for massive fish kills and marine mammal mortalities (Landsberg, 2002; Landsberg and Steidinger, 1998). Impacts on human health due to red tides include eye irritation and respiratory distress from inhalation of the aerosolized toxin, as well as paralytic shellfish poisoning via ingestion of contaminated shellfish. Economic costs due to red tides in Florida are difficult to estimate, but likely exceed US$ 20 million per year from losses in tourism alone (Anderson et al., 2000). The Florida shellfishing industry, which employs over 2500 workers and whose annual value is greater than US$ 20 million

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doi:10.1016/j.hal.2006.11.001

per year, is also negatively impacted by red tides when blooms result in the closure of shellfish harvesting beds (http://www.floridaaquaculture.com/SEAS/SEAS_intro.htm).

The rapid detection of harmful microorganisms in seawater is vital for monitoring the safety and health of coastal waters. Maintenance of healthy waters for shellfish harvesting, recreational water contact usage, and detection of harmful algal blooms all require rapid, sensitive, and specific assays for noxious microbial detection. Current monitoring of *K. brevis* relies on manual counting of cells, which is time consuming and requires trained personnel. Additionally, samples must be brought back to the laboratory prior to analysis resulting in a lag between sample collection and generation of information on blooms. Rapid, quantitative methods are needed to detect blooms in early stages, to provide information to resource managers for monitoring shellfish beds, and to understand the ecology of this organism in the field.

The overall goal of this research is to develop portable sensor technology for detection and enumeration of *K. brevis* in coastal water using a previously developed nucleic acid sequence-based amplification (NASBA) assay for the molecular detection of *K. brevis* (Casper et al., 2004; Patterson et al., 2005). NASBA is an isothermal method for the amplification of RNA (Compton, 1991). Coupled with molecular beacon technology, NASBA becomes a quantitative, target-specific detection method (Leone et al., 1998). We have developed a simple field RNA extraction protocol that does not require specialized laboratory equipment or training, to be used in conjunction with an in-house designed (Center for Ocean Technology, St. Petersburg, FL) handheld NASBA incubator and fluorescence analyzer for in-the-field, quantitative detection of *K. brevis*. Together, these technologies provide a portable assay that detects *K. brevis* in as little as 20 min. Furthermore, this device is an inexpensive alternative to existing laboratory instrumentation.

2. Materials and methods

2.1. K. brevis

K. brevis cultures (Piney Island and Charlotte Harbor strains) were obtained from The Fish and Wildlife Research Institute (FWRI) in St. Petersburg, FL. Cultures were incubated at 24 °C on a 12 h light:12 h dark cycle at 26 μmol photon^{-2}. Prior to RNA extraction, cell concentration was determined by filtering 1 ml of culture onto 0.22 μm pore size black polycarbonate membranes (Millipore Corp., Billerica, MA) and cells were counted under epifluorescence microscopy (Olympus BH-2 microscope) using blue excitation (Vernet et al., 1990). The appropriate volume of culture was then used for RNA extraction to generate standard curves for quantitative analysis.

2.2. RNA extraction

K. brevis RNA was extracted using either an RNeasy Mini Spin Kit (Qiagen Corp., Madison, WI) or the field RNA extraction protocol. The RNA field extraction protocol was developed by modifying the RNeasy Mini Spin Kit protocol and RNA extracted using this method was obtained as follows: a custom manufactured adapter (Center for Ocean Technology) was designed to connect the RNeasy spin column to a 60 ml syringe. Using the attached syringe, a 20–30 ml water sample was pushed through the spin column. The syringe and adapter were removed, and the column placed into a collection tube. RLT lysis buffer plus β-mercapto ethanol (10 μl β-me/ml RLT) was mixed with 100% ethanol in a ratio of 7:5. Seven hundred microliter of the RLT/EtOH mixture was applied to the RNeasy spin column and incubated at room temperature for 10 min. After the 10 min incubation, the spin column was removed from the collection tube and one end of a Value Plastics (Fort Collins, CO) female luer lug style coupler (part# FTLC-6) was attached to the bottom of the column. A 10 ml syringe was attached to the other end of the coupler, and the RLT/EtOH mixture was slowly drawn through the column by pulling the plunger. Leaving the syringe attached to the column, 700 μl of Qiagen's RWI buffer was added to the column and slowly drawn though with the syringe. Two washes of 500 μl RPE were applied to the column and pulled through as described above. After the second RPE wash, the syringe was removed from the column and capped with a Value Plastics female luer thread style cap (part# FTLLP-6) and stored for disposal. A clean 60 ml syringe, with the plunger pulled back, was attached directly to the spin column. The bottom of the spin column was allowed to rest on absorbent paper. While holding the syringe to the column, the plunger was depressed quickly to push air through the column and remove residual wash buffer. This was repeated at least five times or until no more liquid was expelled onto the paper. The syringe was removed and the spin column was placed in a collection tube. Fifty microliters of RNase free water was applied to the spin column. Using the same 60 ml syringe that was used to dry the column, the RNA was eluted from the column by pushing air through in the manner described above.

RNA was also extracted using the traditional RNeasy Spin protocol as a control for comparison to the field kit. The same volume of sample that was passed through the RNeasy column in the field kit was filtered onto a 25 mm, 0.45 μm HVLP (Millipore) filter via vacuum filtration. The filter was placed in 700 μl RLT plus β-me, and let sit at room temperature for 10 min. The sample was then vortexed, and 500 μl EtOH added. The sample was mixed, and applied to an RNeasy Mini column, and RNA extracted according to manufacturer's instructions (Qiagen).

2.3. Primers, beacons and IC-RNA

NASBA primers were designed to target an 87 bp region of the *K. brevis* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) mRNA as described in Casper et al. (2004) (Table 1). An internal control RNA (IC-RNA) molecule was synthesized as in Patterson et al. (2005), and was designed be identical to the 87 bp *K. brevis* target with the exception that the *rbcL* beacon binding site was removed and replaced with the beacon site from our enterovirus NASBA assay (Casper et al., 2005) (Table 1). IC-RNA was quantified with a Ribogreen RNA quantification kit (Invitrogen, Carlsbad, CA). Primers and beacons were obtained from Qiagen or IDT (Coralville, IA). The *K. brevis* molecular beacon was labeled with FAM (6-carboxyfluorescein) at the 5′ end and the quencher DABCYL at the 3′ end. The IC-beacon was labeled with 6-ROX (6-carboxy-rhodamine) at the 5′ end and DABCYL at the 3′ end.

2.4. NASBA assay

NASBA was performed using reagents and enzymes from the Nuclisens Basic Kit (bioMérieux Inc., Durham, NC) and the EasyQ NASBA Analyzer (bioMérieux), or the handheld NASBA analyzer. The IC-RNA was added to each reaction at either 10,000 or 1000 copies per reaction, depending on the range of the standard curve. Standard curves were generated using the ratio of the target time to positivity (TTP) to IC-RNA TTP (Casper et al., 2005; Patterson et al., 2005). Samples in the EasyQ were run in a 10 μl NASBA reaction (half the volume recommended by the bioMérieux protocol) consisting of 5 μl reagent–primer mix, 2.5 μl RNA or water, and 2.5 μl enzyme. Samples in the handheld analyzer and their EasyQ duplicates were run in the standard 20 μl NASBA reaction. Primers and beacons were diluted to final concentrations of 400 and 100 nM, respectively. The KCl concentration of each reaction was 80 mM.

2.5. Handheld NASBA reader

A portable NASBA incubator system with two-channel fluorescence detection was developed. The first channel was equipped with a high intensity blue light emitting diode (LED) (Stanley Electric, Irvine, CA) with a peak emission at 470 nm for the excitation of the molecular beacon used for detection of the target RNA. An optical band pass filter centered at 485 nm with a 22 nm band width (Omega Optical, Brattleboro, VT) was placed between the LED and the reaction tube to prevent the unwanted fraction of the spectrum, or optical noise, from entering the detection system (Fig. 1). The second channel's LED (peak emission at 578 nm) was optically filtered with a 585 nm low pass filter for excitation of the fluorophore used in detection of the IC-RNA. Both fluorescent channel detectors use low noise blue enhanced photo diodes (Advanced Photonics Waltham, MA) with a sensitivity of 0.23 A/W at 450 nm. To further minimize noise in channels 1 and 2, fluorescence readings were filtered with 530 nm (30 nm bandwidth) and 635 nm (20 nm bandwidth) band pass filters, respectively, at 90° angles. The photon-induced current was "sensed" with an operational amplifier situated on the electronics control board. To enhance the sensitivity, the signal was integrated over 1.0 s, averaged over 60

Table 1
Sequences of *K. brevis* primers, beacon, target RNA, and IC-RNA

Sequence name	Sequence (5′ to 3′)
K. brevis forward	ACGTTATTGGGTCTGTGTA
K. brevis reverse	AATTCTAATACGACTCACTATAGGGAGAAGGTACACACTTTCGTAAACTA
K. brevis molecular beacon	[6-FAM]-CGATCGCTTAGTCTCGGGTTATTTTTTCGATCG-[DABCYL]
IC-RNA molecular beacon	[6-ROX]-CGATCGTGGCTGCTTATGGTGACAATCGATCG-[DABCYL]
K. brevis target RNA	GAA**ACGTTATTGGGTCTGTGTA**CACGAATTAAC**CTTAGTCTCGGGTTATTTTTT**GGACAAGAATG-GGC**TAGTTTACGAAAGTGTGTACCT**
K. brevis IC-RNA	GAA**ACGTTATTGGGTCTGTGTA**CACGAATTAAC**TGGCTGCTTATGGTGACAAT**GGACAAGAATGG-GC**TAGTTTACGAAAGTGTGTACCT**

Bold text on the *K. brevis* target and IC-RNA sequences indicates primer and beacon binding sites.

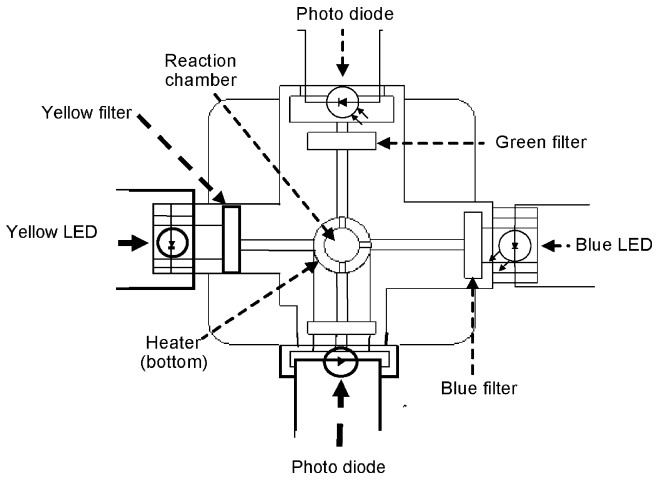

Fig. 1. Schematic diagram of the handheld NASBA analyzer.

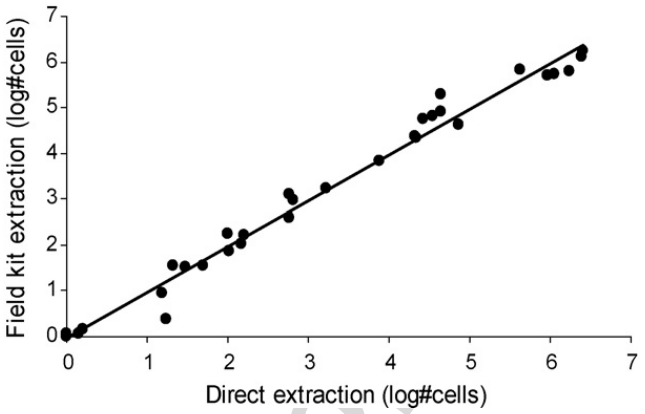

Fig. 2. Apparent cell counts generated by RNeasy RNA extraction compared to those generated by field kit RNA extraction ($r^2 = 0.98$).

readings, and recorded as one fluorescence reading every minute.

To maintain the 41 °C necessary for the NASBA reaction, an infrared light source (Ion Optics, Camarillo, CA) featuring a germanium window for infrared photon emission at 9.5 μm was coupled with a digital thermometer module (Melexis MLX90601 CKA, Concord, NH). The temperature control circuit in the NASBA reaction chamber is regulated through frequent temperature readings and heater adjustments by an in-house written C-code on a microcontroller (Motorola, Schaumburg, IL). The maximum deviation of the temperature control circuit is ±0.5 °C. All temperature calibrations were conducted in NASBA buffer using a digital thermometer and thermocouple probe (Fisher Scientific, Pittsburg, PA).

Readings of the fluorescence light intensity were captured using a 12-bit analog to digital converter (Texas Instruments, Dallas, TX), processed and routed to the handheld device's RS232 serial output (allows for connection with any computer). The numeric data was then processed using in-house developed software programmed in Lab View 6.0 (National Instruments Corp., Austin, TX) to provide a real time fluorescence plotting of the NASBA amplification.

Duplicate *K. brevis* reactions were run in the EasyQ Analyzer (bioMérieux) and the handheld NASBA detector. Raw fluorescence data plots from the handheld device were plotted with the calculated data plots from the EasyQ analysis software (bioMérieux) for comparison.

3. Results

Results from the field RNA extraction protocol were compared to those from direct RNeasy extraction (Fig. 2). All samples that were extracted using both the field and direct RNeasy protocols were analyzed simultaneously in the EasyQ Analyzer. A *t*-test was performed on the slope of the regression line, and results indicated the slope was not significantly different from 1.0 ($p > 0.05$). Additionally, the *y*-intercept of the regression line was compared to a 1:1 line that passed through the origin, and the two intercepts were not significantly different from each other ($p > 0.05$). A total of 31 samples (including both *K. brevis* bloom samples and cultured *K. brevis* cells) were extracted with both the methods, and cell numbers calculated and compared to each other. Of the 31, all but two values fell within an order of magnitude of each other. The two that fell outside an order of magnitude were both detecting approximately one *K. brevis* cell, and in the first case, the field method predicted 1.4 cells versus 16 cells for the direct method, and in the second case the field method predicted 0.17 cells versus 0.01 cells for the direct method. In both these cases, the field method was closer to the expected value than the direct extraction method. The remaining 29 samples all fell within a half order of magnitude of each other and the majority (24 of 29, 83%) agreed within a factor of two. Fourteen of the 31 total samples contained cultured cells ranging from 1 to 1000 cells, and the remaining 17 were bloom samples obtained from FWRI. The use of the cultured cells enables comparison of calculated cell abundance based upon NASBA (termed 'apparent cell counts') to the number of cells actually added, based upon cell counts and factoring in dilutions. Results of comparisons of the field extracted calculated cell numbers and RNeasy extracted calculated cell numbers (apparent cell counts) to expected values, rather than to each other are shown in Fig. 3.

Cell numbers for the 17 bloom samples were predicted based on cell standard curves, and results compared to

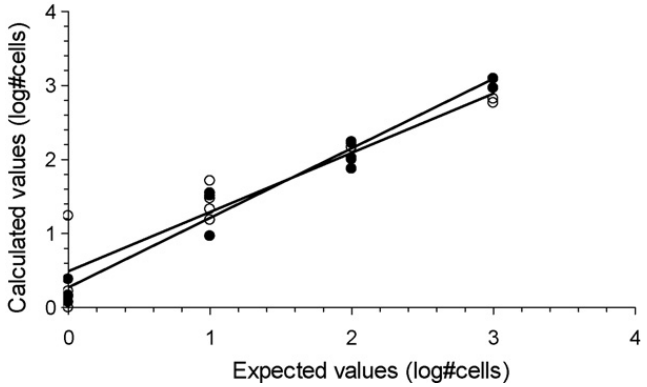

Fig. 3. Comparison of field kit and RNeasy apparent cell concentrations to expected values based upon dilution of cultures. Closed circles correspond to field kit values, and open circles correspond to RNeasy values. Field kit, $r^2 = 0.96$ and RNeasy, $r^2 = 0.88$.

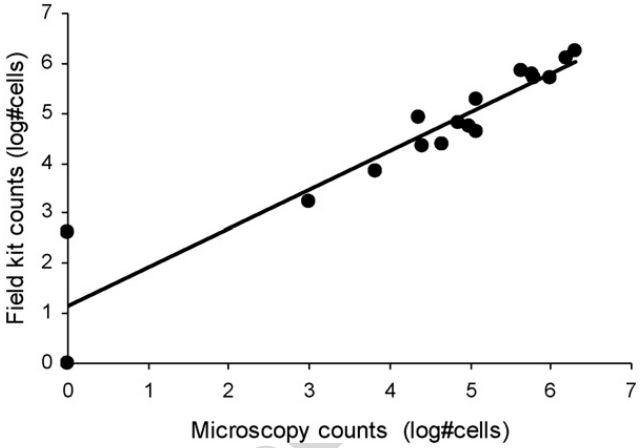

Fig. 4. Regression analysis of FWRI microscopy cell counts vs. the apparent cell counts obtained from the field extraction kit and NASBA analysis ($r^2 = 0.88$).

FWRI cell counts (Table 2; Fig. 4). A paired sample *t*-test of log transformed data indicated there was no significant difference between the NASBA cells counts and microscopy-based cell counts ($p > 0.05$), and regression analysis indicated the slope was not different from 1.0 ($p > 0.05$), and the intercept was not different from a 1:1 line that passed through the origin ($p > 0.05$). Except for one sample (MI) in which background levels of *K. brevis* were detected by NASBA while FWRI cell counting did not detect any cells, the apparent cell counts from NASBA were similar to FWRI microscopic counts within a factor of 3.5. Fourteen of the remaining 16 samples (87.5%) were within two-fold of each other. Quantified samples were classified using a modified version of FWRI's classification system (normal: 0–1000; very low: >1000–10,000; low: >10,000–<100,000; medium: 100,000–<1,000,000; high = ≥1,000,000). The very low classification can result in respiratory irritation, whereas fish kills are usually not noted until the medium level has been reached. High levels result in fish kills, respiratory irritation, and obvious water discoloration. Based on this system FWRI cell counts identified three normal samples, one very low sample, five low samples, five medium samples, and three high samples. As determined by NASBA, 14 of the 17 bloom samples (82%) fell into the same classification level selected by FWRI (Table 2). The remaining three agreed within one classification level.

Table 2
Comparison of the field extraction kit followed by NASBA analysis to FWRI cell counts

ID	Cell counts by NASBA field extraction (cells/l)	Microscopy by FWRI (cells/l)	Bloom classification	
			Field kit	Microscopy
WB	0	0	N	N
SS2	80,058	23,000	L	L
SS3	66,324	73,000	L	L
MI	408	0	N	N
PI	6,762	6,670	VL	VL
RP	188,899	117,000	M	M
RI	689,366	433,000	M	M
M22	527,847	633,000	M	M
BK	510,518	1,000,000	M	H
M57	1,687	1,000	VL	N
DK	21,264	26,000	L	L
CP	23,031	44,300	L	L
DK	55,124	94,700	L	L
RI	43,162	117,000	L	M
M8	624,701	600,000	M	M
RP	1,267,961	1,600,000	H	H
BGP	1,752,113	2,070,000	H	H

Classification levels are as follows—N, normal: 0–1000; VL, very low: >1000–10,000; L, low: >10,000–<100,000; M, medium: 100,000–<1,000,000; H, high: ≥1,000,000.

A comparison of amplification plots for two typical samples, one run in the handheld, one run in the EasyQ is shown in Fig. 5. Handheld amplification plots were

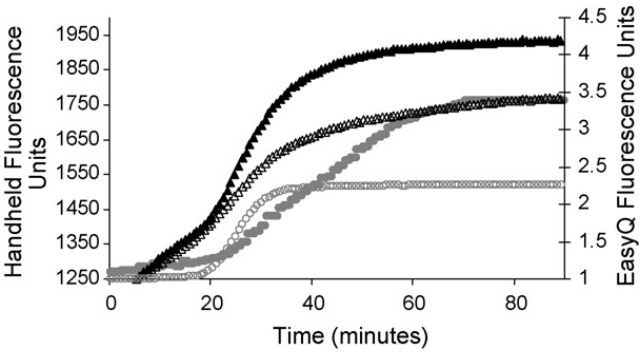

Fig. 5. Typical handheld amplification plotted alongside a corresponding EasyQ plot. Closed triangles, target RNA (EasyQ); open triangles, target RNA (handheld); gray, closed circles, IC-RNA (EasyQ); gray, open circles, IC-RNA (handheld).

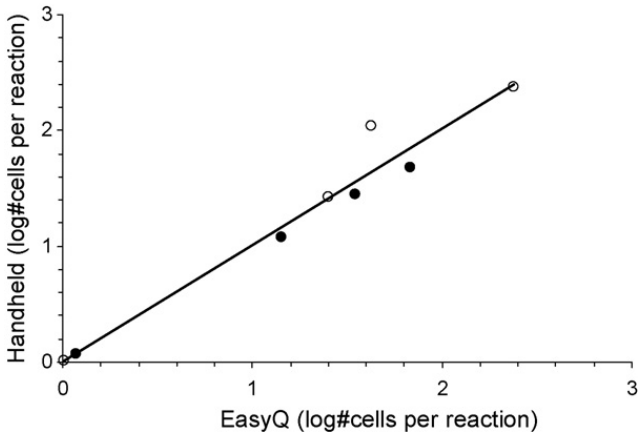

Fig. 6. EasyQ NASBA analysis vs. handheld NASBA analysis. Closed circles indicate cultured cells extracted with traditional RNeasy. Open circles indicate *K. brevis* bloom samples extracted with the field extraction kit ($r^2 = 0.96$).

similar to EasyQ amplification plots. In all, eight samples were run in the handheld NASBA analyzer with duplicate samples run in the EasyQ (Fig. 6). Four of these eight samples were *K. brevis* bloom samples obtained from FWRI and extracted with the field extraction method. The other four samples were from cultured samples and were extracted using the traditional RNeasy method. Cell numbers calculated from the handheld data fell within 2.7 times the cell numbers calculated by the EasyQ, and seven of the eight (88%) fell within two times of each other.

4. Discussion

Comparisons between the field RNA extraction method and traditional RNeasy RNA extraction and between the handheld NASBA analyzer and the EasyQ NASBA analyzer showed significant agreement in apparent cell counts. The field extraction method resulted in apparent cell counts that were generally within a half order of magnitude of RNeasy extracted apparent cell counts. The field extraction method sometimes resulted in values that were closer to the expected number of cells than the traditional RNeasy method, and the coefficient of determination (r^2) value for the linear relationship between the expected number of cells and apparent cell counts was actually higher for the field extraction protocol than the traditional RNeasy extraction ($r^2 = 0.96$ for field versus 0.88 for RNeasy). As each sample extracted using both the field and RNeasy methods was subsequently analyzed in the same NASBA run on the EasyQ, these results demonstrate that the field extraction protocol is at least as efficient an RNA extraction method for the quantification of *K. brevis* as the traditional RNeasy protocol.

The handheld NASBA analyzer amplification curves closely mirrored those from the bench-top EasyQ analyzer, and calculated cell concentrations showed excellent agreement between the two technologies. All samples analyzed in the handheld agreed within a factor of 2.7 with the EasyQ, and seven of the eight the samples agreed with the EasyQ within a factor of 2.0. These results indicate that the handheld NASBA device is an appropriate alternate to the traditional bench-top NASBA analyzer for easy and inexpensive use in field environments for real-time results.

The analysis of actual bloom samples indicated high agreement between calculated NASBA apparent cell counts and cell counts by microscopy performed by FWRI. Statistically, there was no difference in cell counts generated by NASBA as compared to microscopic counts. NASBA apparent cell counts resulted in bloom categories equivalent to those generated by microscopy counts for the majority (82%) of the time, and those that differed were only off by one classification level. The three bloom samples that fell into different categories as determined by microscopy counts were both within 3.0 times the microscopy value. Two of these samples fell right on the border between two different classification levels. Despite the three differences in bloom classification level, NASBA calculated cell concentrations were consistent with FWRI cell counts. Furthermore, we do not know what errors may exist between FWRI cell counts and actual *K. brevis* concentrations in the sample. One study indicated that consensus between determinations by different individuals may be as low as 43% (Culverhouse et al., 2003) which corresponds to 2.3 times. All but two of our NASBA analyzed samples agreed with FWRI cell counts within 2.3 times, indicating that our ability to quantify is generally as precise as microscopic analysis.

One bloom sample (MI, 7/13/2006) was determined by NASBA to have approximately 400 cells/l while no cells were detected by microscopy. Both samples were classified as normal, background levels, but this data point skewed the regression analysis, since the difference between the log transformed values for the two methods was relatively large. When that point was removed from the regression plot, the r^2 value increased from 0.88 to 0.98. The 400 cells/l calculated by NASBA is very close to the 333 cells/l detection limit of microscopy, and at these low concentrations of cells in the sample, small differences in calculated numbers can translate to larger differences in orders of magnitude. Since both cells counts fell into normal, background levels, however, the general conclusion about bloom conditions remains the same for the two detection methods. This highlights

the greater sensitivity of the NASBA amplification technique, and may enable detection of blooms in their formative stages.

Overall, both our field RNA extraction protocol and our handheld NASBA analyzer produced results that were similar to those produced by the traditional methods and more expensive laboratory instrumentation. Combined, the field RNA extraction protocol and handheld NASBA analyzer resulted in calculated cell concentrations that closely matched expected values.

5. Conclusion

Rapid detection of *K. brevis* blooms is critical to ensure the health of recreational water users and shellfish consumers by allowing for early notification of *K. brevis* blooms. A rapid, field-based method for the detection of *K. brevis* can therefore be an important tool to aid coastal managers in the timely closing of shellfish beds during blooms as well as to notify beachgoers of blooms. Our field RNA extraction kit, coupled with the handheld NASBA analyzer provides a simple means for a variety of users to quickly and easily detect *K. brevis* form marine waters. With little or no training, coastal managers and other interested parties can rapidly identify the presence of *K. brevis* using these technologies at a fraction of the cost required by the use of traditional NASBA analyzers.

Acknowledgements

This work was supported by NSF Biocomplexity Grant BES-0216305 and a grant from the Cooperative Institute for Coastal and Estuarine Environmental Technology (CICEET). Special thanks to Earnest Truby and Bill Richardson of the Florida Fish and Wildlife Research Institute for providing *K. brevis* cultures and bloom samples.[SES]

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