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Real-time PCR assay for *Aquimarina macrocephali* subsp. *homaria* and its distribution in shell disease lesions of *Homarus americanus,* Milne-Edwards, 1837, and environmental samples

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ABSTRACT

Epizootic shell disease (ESD) is causing major losses to the lobster fishery in southern New England. Potential pathogens have been identified in lesion communities, but there are currently no efficient means of detecting and quantifying their presence. A qPCR assay was developed for a key potential pathogen, *Aquimarina macrocephali* subsp. *homaria* found to be ubiquitous in ESD lesions but not the unaffected integument. Application of the assay to various samples demonstrated that *A. macrocephali* subsp. *homaria* is ubiquitous and abundant in lobster lesions, commonly associated with healthy surfaces of crabs and is scarce in water and sediment samples from southern New England suggesting the affinity of this microorganism to the Arthropod integument. The qPCR assay developed here can be applied in future in vivo and in vitro studies to better understand the ecology and role of *A. macrocephali* subsp.*homaria*. in shell disease.

1. Introduction

Epizootic shell disease (ESD) is a major threat to the sustainability of the American lobster (*Homarus americanus*) fishery in southern New England (Cobb and Castro, 2006). The disease presents as lesions of the exocuticle caused by microbes that degrade the shell from the outside in (Smolowitz et al., 2005). Most lobsters are able to survive, by molting out of the disease, but if lesions penetrate the entire exoskeleton animals can succumb, due to an incomplete molt (Stevens, 2009). Pathogens of shell disease have been difficult to identify, because the disease is polymicrobial and does not have a directly transmissible infectious nature, however, bacteria have been implicated as primary agents of lesion formation. Chistoserdov et al. (2005) cultured a species of *Aquimarina* (originally proposed name *Aquimarina 'homaria'*) from the lesions of a high number of ESD lobsters and subsequently verified its ubiquity in ESD lesions as the most commonly detected bacterium in the polymicrobial community (Chistoserdov et al., 2012). The presence of *Aquimarina* sp. in high abundance in lobster lesions has been verified in other studies (Meres et al., 2012). While all forms of shell disease of *H. americanus* have been shown to have *Aquimarina macrocephali* subsp. *homaria* in their lesions, it was found to be inconsistently present on healthy carapaces (Chistoserdov et al., 2012). Furthermore, the application of this bacterium to compromised cuticles of healthy lobsters has demonstrated its pathogenic nature (Quinn et al., 2012), and therefore, this bacterium was suspected to be one of the primary pathogens of the ESD polymicrobial infection.

There are currently eight described species of *Aquimarina* (*Flavobacteriaceae*): *A. muelleri* (Nedashkovskaya et al., 2005), *A. intermedia*, *A. latercula*, *A. brevivitae* (Nedashkovskaya et al., 2006), *A. litoralis* (Oh et al., 2010), *A. macrocephali* (Miyazaki et al., 2010), *A. spongiae* (Yoon et al., 2011) and the most recently described species *A. addita* (Yi and Chun, 2011). All species have been isolated from marine environments, but their sources of isolation are diverse. *A. muelleri, A. latercula, A. litoralis* and *A. addita* were isolated from sea water around Japan and Korea, *A. latercula* from aquarium outflow in California, USA (the only isolate from North America), *A. spongiae* was isolated from the marine sponge *Halichondria oshoro*, *A. brevivitae* from tidal flats and *A. macrocephali* in sediment next to a sperm whale carcass. *A. macrocephali* subsp. *homaria* and *A. muelleri* are the only known isolates associated with crustaceans and both were able to degrade crude chitin, which is most analogous to crustacean shells (Chistoserdov et al., 2005). Little is known about their ecology, but phenotypically the genus is characterized as dark-yellow to brownish colored, gliding bacteria that produce antibacterial flexirubin-like pigments (Mojib et al., 2010; Nedashkovskaya et al., 2006),

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but not exclusively (Yi and Chun, 2011). Lesions of lobster shell disease have often been described as having a 'yellow' color, which may be due to the presence of Flavobacteria producing these pigments. These bacteria, and *A. macrocephali* subsp. *homaria* in particular, appear to play major roles in the shell disease polymicrobial infection.

Little is known about *A. 'homaria's* ecology or presence in the environment. The bacterium has only ever been detected on the surface of *H. americanus*. Therefore, there is a need to develop a better means of its detection and enumeration. The bacterium can be cultured on marine agar (Chistoserdov et al., 2005), but is easily outgrown by less fastidious marine bacteria. Here, we developed a qPCR assay for detection and enumeration of *A. macrocephali* subsp. *homaria* in natural samples and applied it to detect the bacterium on the surface of crustaceans and in marine water and sediment samples.

2. Materials and methods

2.1. Primer design

Specific olignucleotides were designed based on sequences of the *A. macrocephali* subsp. *homaria* I32.4 full length 16S rDNA sequence. Multiple sequence alignments were constructed using MegAlign (DNASTAR, Madison WI) with sequences of all Proteobacteria and Bacteroidetes from lobster lesions (Chistoserdov et al., 2012; GenBank sequences JF904894-JF904934) and other Bacteriodetes species closely related to *A. macrocephali* according to a BLASTn search (Altschul et al., 1997). Unique oligonucleotides were selected and tested for a specificity against the GenBank database using BLASTn tool (Altschul et al., 1997), the Ribosome Database Project database (RDP; http://rdp.cme.msu.edu/) and forty bacterial sequences detected in ESD lesions (Chistoserdov et al., 2012). The specific nucleotides were designed to be present at the 3′ ends of both forward and reverse primers to assure specificity. The *A. macrocephali* subsp. *homaria* specific primers are Ahom190F (5′TAGTATCMAAAGACAGCMTTGTTTTATG3′) and Ahom-470R (5′CCTTATTCGTAGAGTACCGTCAGAGTAT3′), which generated a 302 bp amplicon (Fig. 1). This primer set was submitted to the NCBI Probe database.

2.2. Standard curve and qPCR optimization

A. macrocephali subsp. *homaria* I32.4, *A. macrocephali* JAMB N27^T and *A. muelleri* I33.1 were grown in marine broth at room temperature and *Alcaligenes faecalis* in LB at 37 °C and DNA was extracted using a phenol/chloroform procedure and then was purified using PowerClean® DNA CleanUp Kit. (MO BIO Carlsbad, CA). To obtain a known concentration of *A. macrocephali* subsp. *homaria* 16S rDNA copies, ribotyping was done using a DIG DNA Labeling and Detection Kit (Roche, Indianapolis, IN).

All qPCR reactions were run on an Applied Biosystems (Beverly, MA) StepOnePlus™ real-time PCR system. Reaction components consisted of 1 × GoTaq® qPCR Master Mix (Promega), 0.2 µl (50 µM) of both primers, 1 µl of template and 0.1 µl of 100 × CXR reference dye (Promega) in a 10 µl reaction volume. Reactions were carried out in 96 well MicroAmp® (Applied Biosystems) reaction plates. The thermocycling parameters were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s and a read at 80 °C for 30 s (fluorescence read at this step). Following amplification, a melting curve analysis was performed over a temperature range of 60 to 95 °C reading fluorescence at increasing 0.3 °C increments. Two negative controls were used, one with only water and another with 10^6 *A. muelleri* I33.1 gDNA. All samples qPCR standards and negative controls were amplified in triplicate and gene copy numbers were averaged. Any positive environmental samples were re-tested in a separate plate to ensure positive amplification was not due to cross contamination from the standard curve.

To verify the specificity of the amplification, the PCR product

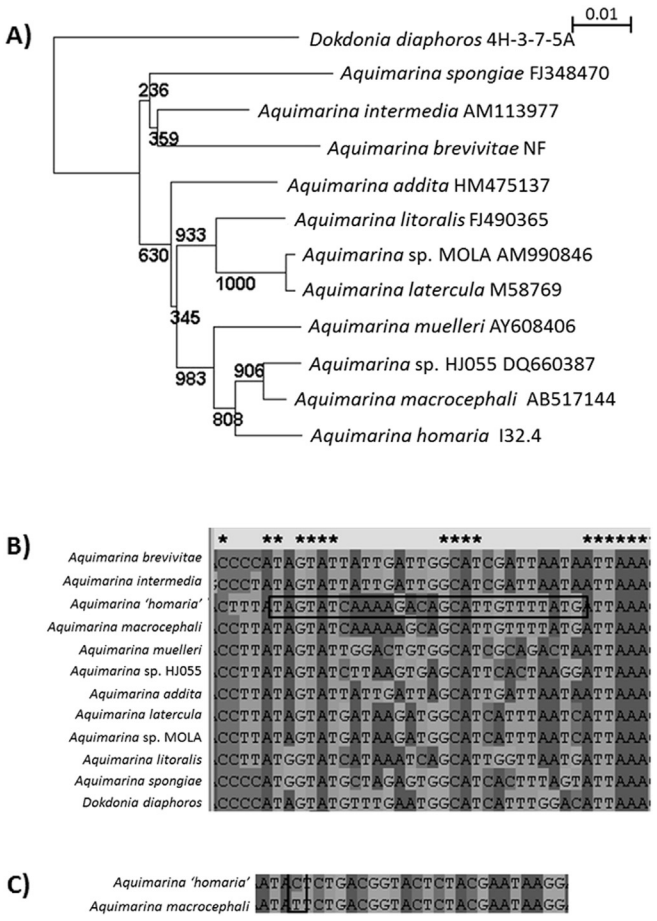

Fig. 1. A) Neighbor joining tree and alignment from ClustalX multiple sequence alignment of *Aquimarina* sp. and closest relative. Boostrap values of branch nodes are shown. Multiple sequence alignments showing the sequence of B) Ahom190F (highlighted with box) and a C) 3′ terminal SNP between *A. macrocephali* subsp. *homaria* and *A. macrocephali* in the Ahom470R primer.

generated with the Ahom190F and Ahom470R from pure culture and environmental templates (DNA isolated from shell disease lesion, normal carapace, sediment and was directly sequenced. In all cases the sequence obtained was that of *A. macrocephali* subsp. *homaria* 16S rDNA

To test the effect that a plasmid standard would have on the kinetics of qPCR, an *A. macrocephali* subsp. *homaria* 16S rDNA PCR product was amplified using Ahom190F and a *Bacteroidetes* specific reverse primer 721R (Chistoserdov et al., 2012). The product was then purified with the MO BIO UltraClean™ PCR CleanUp kit and ligated into the multiple cloning site of the linearized pGEM-T vector (Promega, Madison, WI). This construct was then transformed into *Escherichia coli* JM109 competent cells (Promega) and blue-white colony screening was used to verify correct transformants. The intact plasmid, called pGEM-T-Ahom, was then purified from *E. coli* JM109- using the MO BIO UltraCLean™ plasmid purification kit and a subsequent round of PCR with Ahom190F-721R was used to verify the presence of the insert. The plasmid was linearized with *Eco*RI, purified, and then diluted to 10^5 copies/µl for use as a qPCR standard for comparison to *A. macrocephali* subsp. *homaria* genomic DNA. To test for the effect of background DNA on plasmid qPCR kinetics, an equal amount of *A. faecalis* gDNA was added to 10^5 copies of pGEMT-Ahom. Furthermore, to investigate the cause of the difference in kinetics between gDNA and plasmid DNA as templates in qPCR, genomic DNA of *A. macrocephali* subsp. *homaria* I.32.4 was sheared to sizes of approximately 3 kb using a Hydroshear® machine (Gene Machines Inc., San Carlos, CA) and then equilibrated to 10^5 copies of 16S rDNA template for comparison to native gDNA and

plasmid preparations at the same gene copy number. This gDNA shearing was meant to simulate a plasmid like background of DNA. The comparisons of the various template preparations were done by dilution from 10^5 copies to extinction and comparison to the plasmid DNA standard.

2.3. Environmental sample collection and DNA extraction

Twenty-seven lobster with shell disease and twenty lobsters without signs of shell disease were collected and DNA was extracted from lesions and unaffected claw, carapace and tail integument as described in Chistoserdov et al. (2012). Briefly, lesions scrapings were added to DNA extraction buffer. Egg white lysozyme (Amresco®, Solon, OH) was added and then incubated at 37 °C for 30 min and then proteinase K (Fisher Bioreagents®, Fair Lawn, N.J.) and sodium dodecyl suphate were added to a final concentration of 1 mg/ml and 2% respectively, incubated at 50 °C, followed by three cycles of a freeze/thaw procedure. DNA was extracted using phenol/chloroform and then purified for PCR (if required) using the PowerClean® DNA Clean-Up kit (MO BIO, Carlsbad, CA). Lesions of arthropods with shell disease and integument samples from arthropods unaffected by shell disease were sampled from the spider crab (*Libinia emarginata*, diseased $n = 9$, unaffected $n = 5$), green crab (*Carcinus maenus*, diseased $n = 7$, unaffected $n = 3$), Jonah crab (*Cancer borealis*, diseased $n = 7$, unaffected $n = 7$) and the Atlantic horseshoe crab (*Limulus polyphemus*, diseased $n = 3$, unaffected $n = 3$). The arthropods were collected by the Marine Biological laboratory in the vicinity of Woods Hole (MA). DNA isolation from lesions and unaffected carapaces of crabs was carried using the same techniques as for the lobsters (Chistoserdov et al., 2012). The area of the lobster lesions scraped for DNA extraction was determined using an estimate from photographs taken of each animal (contained a size reference) and their known carapace length at the time of sampling and recorded in cm^2; a 1 cm^2 area of healthy carapace was sampled. Lesions on crabs were not accurately quantifiable due to their small size, thus, quantification of crab samples was determined per μg of DNA extracted from healthy carapaces or lesions.

Eighteen water column and twenty-eight sediments samples were collected on three cruises on a lobster fishing boat (collection sites and their description are summarized in Table 1) and processed as described in Chistoserdov et al. (2005). Briefly seawater samples were collected in an 8 L GO-FLO Water Sampler (General Oceanics, Miami, FL) and filtered consecutively through 5 μM and 0.2 μM filters onboard of the vessels and the filters were used to isolate DNA. Sediment samples were collected by an Ekman Grab (WildCO, Yulee, FL). All samples were immediately refrigerated on ice upon collection and frozen upon arrival to the laboratory.

3. Results

3.1. PCR primer specificity

The primers Ahom190F and Ahom480R were determined to be specific to the 16S rDNA gene of *A. macrocephali* subsp. *homaria* in silico using the GenBank database, RDP and sequences of all bacteria detected in ESD lesions (Chistoserdov et al., 2012). The specificity was confirmed experimentally – sequences of amplicons generated by the Ahom190F and Ahom480R from variety of templates (i.e., derived from shell disease lesions, unaffected integument, water column and sediment) were that of 16S rRNA gene from *A. macrocephali* subsp. *homaria*. *A. macrocephali* subsp. *homaria* is a sub-species of *A. macrocephali*. (Chistoserdov & Hazra, in preparation), therefore, the primer set amplified the 16S rRNA gene from the original isolate *A. macrocephali* JAMB N27^T despite that the reverse primer was designed with a polymorphism between these two subspecies sequences in its 3′ terminus (Fig. 1). These primers did not amplify the DNA from the closest related species, *A. muelleri* I33.1, in up to 40 cycles of PCR in any of the assays.

3.2. Standard curve development and discovery of plasmid inaccuracy

The ribotyping indicated that *A. macrocephali* subsp. *homaria* I32.4 has 3 *rrn* gene copies and this number was used for all subsequent gene copy calculations (data not shown). A standard curve using a predetermined 10^6 *A. macrocephali* subsp. *homaria* 16S rDNA copies diluted 10-fold to extinction was effectively and reproducibly made from genomic DNA (Fig. 2). The standard curves had correlation coefficient values (R^2) were 0.99 for all assays. The standard curve remained linear in the range of 10^6–10^1 gene copies per reaction, but below 10 template copies the qPCR was unreliable due to a high variability of Ct values for higher dilutions. Thus, on purified genomic DNA (or plasmid) the sensitivity of the test is 10 copies/reaction, which equates to at least three *A. macrocephali* subsp. *homaria* I32.4 cells (3 copies of *rrn*). Only one DNA fragment of expected size (i.e., 302 bp) was detected in agarose gels following electrophoresis. This fragment generated from several template DNAs was sequenced and shown to be an expected portion of 16S rRNA gene of *A.* '*homaria*'. Surprisingly, the melting of PCR products after completion of PCR reaction contained two peaks in the melting profile: the major peak with 85 °C melting temperature and a second smaller peak at 79.4 °C was also observed. The presence of the second peak does not create a problem for *A. macrocephali* subsp. *homaria* quantification, since the total fluorescence (i.e., from both peaks) signal linearly responded to the number of 16S rDNA copies of *A* '*homaria*' added in the reaction.

An assay was done to test the accuracy of a plasmid template usage

Table 1
Sampling locations and sample types obtained in this study.

Sample #	Trip 1			Trip 2			Trip 3		
	Latitutde/Longitude	Sediment	Water	Latitutde/Longitude	Sediment	Water	Latitutde/Longitude	Sediment	Water
1	41°474.117/70°950.394	Mud	None	41°36.374/71°54.588	Sand/Mud	None	41°21.846/71°34.668	Sand	9 m
2	41°472.573/70°981.293	Trap Biofilm	9 m			None	41°20.099/71°38.814	Sand/Mud	15 m
3	41°456.622/70°978.546	Mud	15 m	41°38.040/71°42.921	Mud	None	41°18.237/71°39.122	Sand/Mud	27 m
4	41°444.271/70°990.906	None	15 m	41°13.040/71°61.255	Sand/Rock	None	41°15.770/71°36.913	Sand	None
5	41°442.726/71°009.445	Mud	18 m			None	41°14.656/71°38.116	Mud	30
6	41°416.985/70°986.099	Sand/Rock	9 m	41°18.040/71°54.588	Sand	None	41°21.320/71°27.600	Gravel	None
7	41°399.990/70°983.353	Mud	18 m			None	41°21.709/71°27.139	mud	12 m
8	41°391.234/71°000.519	Mud	6 m	41°31.374/71°51.255	Sand/Rock	None	41°22.457/71°25.476	mud	24 m
9	41°379.385/70°999.832	Mud	18 m	41°38.040/71°42.255	Gravel	None	41°22.964/71°23.485	mud	18 m
10	41°382.991/70°965.500	Sand	6 m	Harbor (not recorded)	Mud	None	41°23.140/71°27.792	Sand/Rock	6 m
11	41°387.113/70°934.601	Mud	12 m				41°22.151/71°30.529	Sand	3 m
12	41°374.232/70°902.328	Trap Biofilm	None						
13	41°374.748/70°887.222	Sand	12 m						

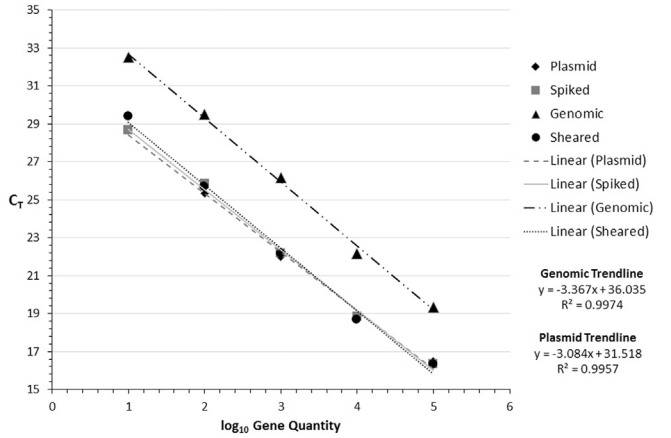

Fig. 2. Mean Ct values of log 16S rDNA gene copies of *A. macrocephali* subsp. *homaria* in different template formulations. All formulations have an equal number of template copies. The linear trendline of the plasmid standard and the genomic DNA is shown along with the equation of the lines. Plasmid is purified pGEMT-Ahom, spiked DNA is a background of *Alcaligines faecalis* genomic DNA spiked with a known concentration of pGEMT-Ahom, sheared DNA is *A. macrocephali* subsp. *homaria* gDNA sheared into 3 kb fragments and genomic DNA is a quantified amount of *A. macrocephali* subsp. *homaria* genomic DNA.

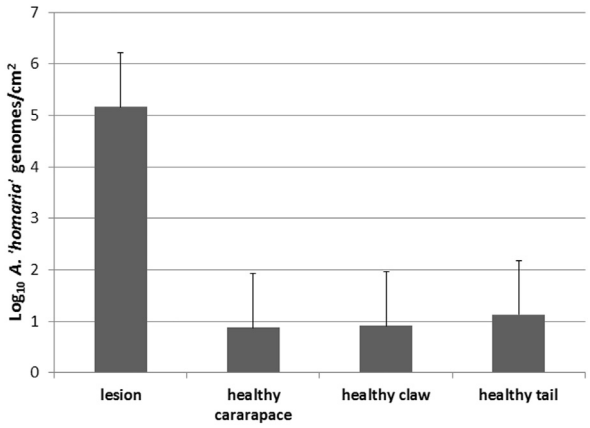

Fig. 3. Amount of *A. macrocephali* subsp. *homaria* in lesions and healthy carapaces of American lobsters with ESD and the standard deviations of the means.

for qPCR by comparison to the natural template, genomic DNA. Linearized pGEMT-Ahom was quantified, diluted to 10^5/μl and directly compared in the qPCR assay with 16S rDNA copy number of *A. macrocephali* subsp. *homaria* gDNA, pGEMT-Ahom DNA spiked into a genomic DNA background (*Alcaligenes faecalis*) and sheared to ~3 kb *A. macrocephali* subsp. *homaria* gDNA (Fig. 3). All three preparations were diluted to extinction and tested with qPCR for equal amplification kinetics. Intact *A. macrocephali* subsp. *homaria* genomic DNA had consistently higher mean C_T values than plasmid DNA template with or without background DNA or sheared DNA from *A. macrocephali* subsp. *homaria* (Fig. 2). Using these values, plasmid DNA with or without non-homologous DNA background, sheared DNA overestimated the gene copy number by 13.1 fold compared to intact genomic DNA of *A. macrocephali* subsp. *homaria* (Fig. 2). Equal kinetics of the sheared gDNA and plasmid DNA preparations indicated that this effect was due to the large molecular weight of intact gDNA, and not inhibitors of PCR as both came from the same gDNA preparation and were purified. Thus, using plasmid DNA as a standard for our qPCR overestimated the template abundance compared to gDNA, in the natural samples, by 13-fold (Fig. 2). Natural sample templates are always gDNA extracts from microbial communities. Moreover, shearing does not work reproducibly with diluted DNA preparations (such as DNA from environmental sources) and/or laborious for multiple samples. Thus, a genomic template must be used as a qPCR standard to most accurately reflect the PCR kinetics of natural samples and this was done herein for all samples tested with our qPCR assay.

3.3. Efficiency of DNA isolation from various environmental media

Environmental DNA used for quantification of *A. macrocephali* subsp. *homaria* may come from various environmental media and incomplete DNA recovery may affect the accuracy of any quantitative method. In relation of *A. macrocephali* subsp. *homaria* to the shell disease epidemiology, the most important environmental media appear to be seawater, marine sediments and lobster integument. The efficiency of isolation of *A. macrocephali* subsp. *homaria* DNA from all these three environmental media was verified by spiking them with known quantities of *A. macrocephali* subsp. *homaria* cells (10^6). The cells were mixed in unaffected carapace scrapings ($n = 6$), collected seawater ($n = 17$) and marine sediment ($n = 24$) samples that did not show any presence of *A. macrocephali* DNA by PCR. DNA was isolated from these samples using exactly the same DNA isolation protocols as used for the isolation of environmental DNA. The quantity of *A. macrocephali* subsp. *homaria* DNA recovered from the samples was quantified using qPCR and compared with the original quantities of *A. macrocephali* subsp. *homaria* added (i.e., 3×10^6 copies of the 16S rRNA gene). Recovery of DNA from the seawater column and chitin suspensions was comparable and close to 100%, whereas, in comparison, only approximately 70% of DNA was isolated from marine sediments.

3.4. Detection and quantification of A. macrocephali subsp. homaria on Crustacean surfaces

A. macrocephali subsp. *homaria* was detected in the lesions of all ESD lobsters tested ($n = 27$) in this study, but only in 45% of carapaces from unaffected animals ($n = 20$). There were over 5.0×10^6 cells/cm^2 (standard deviation ± 1.54 log) of the bacterium in ESD lesions and 10^1 cells/cm^2 on the unaffected carapace samples that were positive for *A. macrocephali* subsp. *homaria* (Fig. 3). The amount of *A. macrocephali* subsp. *homaria* was highest in lobster lesions followed by lesions of spider crabs, green crabs, horseshoe crab and Jonah crabs, respectively (Fig. 4). The amount of *A. macrocephali* subsp. *homaria* in lesions was significantly higher (Student's *t*-test) than in healthy carapaces for lobsters and spider crabs ($p < 0.01$ and $p < 0.05$, respectively), but not for the other species. The difference between the levels of *A. macrocephali* subsp. *homaria* in lesions of lobsters versus unaffected carapaces was approximately four orders of magnitude, whereas for

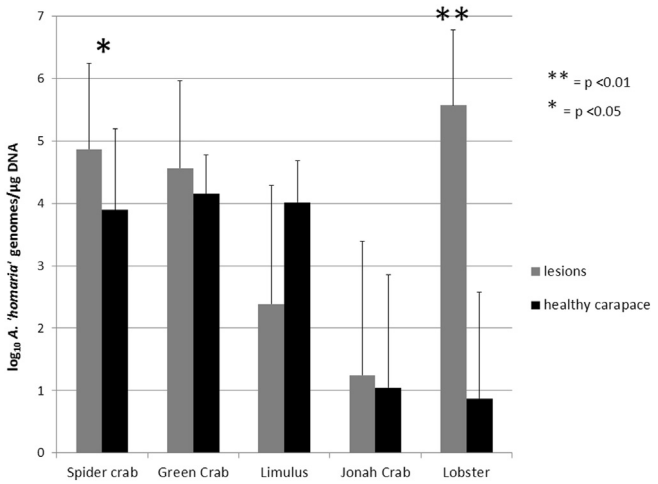

Fig. 4. Amounts of *A. macrocephali* subsp. *homaria* 16S rDNA copies per μg DNA extracted from lesions and healthy carapaces of crabs and lobsters and the standard deviations of their means.

Table 2
Results of quantitative PCR assay for the detection of *A. homaria* in environmental samples from the three cruises.

Sampling site #	Cruise 1			Cruise 2			Cruise 3
	Sediment	5 μm	0.2 μm	Sediment	5 μm	0.2 μm	Sediment
1	< 10	< 10	< 10	< 10	< 10	< 10	2.64×10^3
2	< 10	< 10	< 10	–	–	–	< 10
3	< 10	< 10	< 10	< 10	< 10	< 10	< 10
4	< 10	< 10	< 10	< 10	< 10	< 10	4.20×10^2
5	< 10	< 10	< 10	–	–	–	< 10
6	< 10	< 10	< 10	< 10	< 10	< 10	< 10
7	< 10	< 10	< 10	–	–	–	< 10
8	< 10	< 10	< 10	3.50×10^3	< 10	< 10	< 10
9	< 10	3.27×10^3	< 10				< 10
10	2.91×10^7	< 10	< 10				< 10
11	< 10	< 10	< 10				
12	< 10	< 10	< 10				
13	< 10	< 10	< 10				
Skate mucus	3.1×10^4						
Haddock mucus	5.3×10^3						

spider crabs this difference was only about ten times. Levels of *A. macrocephali* subsp. *homaria* were higher on the lesions and healthy carapaces of crabs than that of lobsters, but due to the large discrepancy in sample size (i.e., twenty-seven versus nine), this difference was not statistically significant.

3.5. Detection of *A. macrocephali subsp. homaria* in environmental samples

Our qPCR assay detected *A. macrocephali* subsp. *homaria* in the 5 μm fraction of 1 of 18 water samples at a depth of 60 ft. at 3.27×10^3 cells/l, south of Cuttyhunk Island and directly west of Martha's Vineyard, MA (Table 1; Table 2). The bacterium was detected in 4 of 28 sediment samples three of which were of sandy bottom type and one mud sample. One sand sample from cruise 1 was especially high with over 10^7 cells/g, the other positive samples were no higher than 10^3 cells/g. The bacterium was not detected on trap biofilm samples; however, both samples of fish bait used by the Rhode Island fishermen were positive for the bacterium (Table 2).

4. Discussion

Specific pathogens have been suggested to be present in ESD lesions of *H. americanus* (Chistoserdov et al., 2012); however, there is no means of rapid detection and enumeration of their presence. Little is known about the ecology of any of the principle pathogens detected in ESD lesions, as these bacteria have only ever been detected on the surface of lobsters. Furthermore, there are discrepancies between studies concerning the presence and abundance of these pathogens on healthy lobster surfaces (Meres et al., 2012; Chistoserdov et al., 2012) thus; a better method of detecting them is required. In this study we developed a sensitive and specific qPCR assay for the detection of a principle pathogen of the ESD polymicrobial infection *A. macrocephali*, to allow many of these questions to be addressed. The MIQE (*M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments) guidelines were followed during development of the *A 'homaria'* qPCR assay (Bustin et al., 2009). The guidelines consider 58 essential pieces of information divided into eight major groups (experimental design, sample [preparation], nucleic acid extraction, qPCR target information, qPCR oligonucleotides, qPCR protocol, qPCR validation and data analyses) with one groups pertaining only RT-qPCR, which were adhered to in the *A. macrocephali* subsp. *homaria* assay.

A major problem, which any SybrGreen- based PCR assay faces is the specificity. Other species of *Aquimarina* have been described and some strains have been detected on the surface of lobsters (Quinn et al., 2012), thus, it was particularly important that this qPCR assay was specific and could distinguish *A. macrocephali* subsp. *homaria* from *A. muelleri* and other species (*A. latercula* and other more distantly related Bacteroidetes) present on the arthropod integument. Primers Ahom190F and Ahom470R were determined to be specific in silico using BLASTn and the RDP database, and experimentally, there was no amplification of *A. muelleri* DNA up to 40 cycles. 16S rRNAs of *A. macrocephali* subsp. *homaria* and A. *macrocephali* are 98% identical, which suggests that they likely belong to the same species, although the type strain of *A. macrocephali* has never been encountered on lobster surfaces. Furthermore, using sequences available in GenBank from Chistoserdov et al. (2012; accession# JF904894-JF904934) the qPCR primers did not match any other sequence identified in the ESD lesions of numerous lobster sampled over a 10 year period. In vitro, the assay has a sensitivity of four *A. macrocephali* subsp. *homaria* cells per reaction, however, the ability to match this lowest detection limit in natural samples will depend on the sample integrity and DNA purity.

A major goal of this study was to enumerate the abundance of *A. macrocephali* subsp. *homaria* present in an ESD lesion compared to a healthy carapace. To date only non-quantitative (PCR-DGGE; Chistoserdov et al., 2012) or semi-quantitative methods have been applied (second generation sequencing; Meres et al., 2012) for detection of *Aquimarina* spp. These methods have shown that *Aquimarina* spp. was the most abundant bacterial genus in the lesions of ESD lobsters, but they did not provide actual cell counts or specifically detect *A. 'homaria'*. Using our assay, the bacterium was detected in lesions of all 27 ESD lobsters and found to be highly abundant at approximately 5.0×10^6 cells/cm^2 lesion; however, there was a large standard deviation around this mean (Fig. 3), indicating the levels of the bacterium can vary greatly. This is not surprising considering the dynamic polymicrobial community that exists in ESD lesions (Quinn et al., 2009; Chistoserdov et al., 2012; Meres et al., 2012). It is likely that the length of time, during which a lesion has developed, and the specific microbial community members present within it, have a great effect on the levels of *A. macrocephali* subsp. *homaria*. It has been shown that *A. macrocephali* subsp. *homaria* colonizes early developing lesions and can persist in the community over time (Quinn et al., 2012b), thus, even though its levels may fluctuate it appears to remain steadfast in the lesions. In contrast, the bacterium was only detected in 9 out of 20 unaffected carapace samples, and if present, its levels were extremely low (approximately 10^1 cells/cm^2). This is a particularly important finding in the context of ESD as it demonstrates that *A. macrocephali* subsp. *homaria* multiplies to high densities in lesions and appears to thrive in that environment, whereas it merely colonizes a healthy carapace and is not well adapted to that niche. It is possible that *A. macrocephali* subsp. *homaria* existing on a healthy carapace is in a physiologically dormant or slow growing state and that quorum signals from itself or other bacterial cells trigger its virulence and attack on the

lobster shell. This is similar to the properties of other bacterial pathogens such as *Pseudomonas aeruginosa* where virulence and biofilm formation is triggered by an accumulation of quorum sensing signals from like cells (Pearson et al., 2000; Costerton et al., 1999). This phenomenon has been observed in other flavobacterial pathogens including the closely related *Tenacibaculum maritimum* (Romero et al., 2010). Another possibility for the discrepancy between lesions and healthy carapace abundance is that the bacterium attaches to healthy carapace and only begins to multiply to high levels when it adheres to a compromised area of shell where the lobster innate immune defenses are not able to limit its growth.

During development and optimization of the assay for *A. macrocephali* subsp. *homaria* plasmid DNA template was compared to genomic template and it was found that at the same gene copy number, the amplification kinetics were not identical. We found that our genomic DNA template was underestimating the number of gene copies in a reaction by 13-fold. It was possible that the reason for this was due to inhibitors in the DNA sample, but this possibility is discounted due to the linearity of the PCR throughout dilution to extinction of the gDNA template, the fact that the preparation was purified with a DNA clean up kit and that sheared DNA of the same sample had equivalent PCR kinetics to plasmid DNA (Fig. 2). The genome size of *A. macrocephali* subsp. *homaria* was only estimated based on that of its closest sequenced relative, but the 13-fold difference in gene abundance cannot be explained by differences in genome size (i.e., an inaccurate calculation). Moreover, coincidence of amplification kinatics for plasmid and sheared gDNA of *A. macrocephali* subsp. *homaria* indicate that its genome size was guessed very accurately. Since its invention (Syvanen et al., 1988), and to date (Dang et al., 2011; Zweilehner et al., 2011; Shu et al., 2012; Shanks et al., 2012), plasmid DNA has been the template of choice as a standard for qPCR assays, and it has recently been determined that linearized plasmid DNA must be used to ensure accuracy (Huo et al., 2010). However, our study found that when applying qPCR for enumeration of genes from whole chromosomal extracts or environmental sample DNA extracts, plasmid DNA is not an accurate qPCR standard. We determined this by comparing the amplification kinetics of plasmid DNA template to various other template preparations (Fig. 2). It was determined that only intact genomic DNA itself is adequate for comparison to natural sample templates, because plasmid DNA had more efficient qPCR kinetics, as did plasmid DNA in a heterologous DNA background and sheared homologous genomic DNA. The fact that sheared genomic DNA had the same amplification kinetics as plasmid DNA indicates that the lag in amplification observed with gDNA is due to the high molecular weight of the DNA fragments. We hypothesize that the DNA melting stage of PCR does not equate between small plasmid DNA and large gDNA. Due to the millions of bp of DNA present around the target template, the genomic DNA does not melt as quickly and efficiently for primer binding as occurs for plasmid DNA templates. However, it is not strictly due to the large amount of background DNA, because plasmid DNA spiked with gDNA had the same kinetics (Fig. 2). Thus, because the application of qPCR to environmental samples is done on genomic DNA extracts consisting of whole genomes (as is the case here), plasmid DNA cannot be used as an accurate qPCR standard. This is especially pertinent to the detection of uncultured bacteria where only clones of DNA fragments exist. In order to ensure accuracy for detection of uncultured bacteria, either the gene target must be inserted by transposon mutagenesis or other means into a chromosomal background, or the environmental samples must be sheared to the size of the plasmid template.

A. macrocephali subsp. *homaria* had previously only ever been detected on lobster surfaces, therefore, we were particularly interested to determine if it was present on other marine arthropods, including plankton and large crabs common to southern New England. The bacterium was only detected in one 5 μm water sample, which contains the planktonic crustaceans, but was detected on all species of crabs (and the chelicerate L. *polyphemus*). The levels of the bacteria on crab lesions were generally higher than on healthy carapaces, but were also highly abundant on healthy crab surfaces. Therefore, *A. macrocephali* subsp. *homaria* is highly associated with the surface of large marine arthropods, including lobsters, and may naturally reside as an epibiont on the surfaces of crabs. Further research into the incidence of this bacterium on healthy crab surfaces is required. Furthermore, in the two samples of lobster fish bait mucus tested with our qPCR assay, both were found to harbor *A. macrocephali* subsp. *homaria*. The bacterium may also reside on the surface of fish, as many marine Flavobacteria are known to be associated with fish mucus (Madetoja et al., 2002; Wilson et al., 2008; Klesius et al., 2008), but broader sampling of fish bait used by fisherman is also required.

A. macrocephali subsp. *homaria* DNA was only rarely detected in the environmental samples. Its DNA was amplified from 1 of 18 water samples and 4 of 28 sediment samples, 3 of which were sand sediment samples. Thus, the bacterium is rare in the marine environment, but may be particularly associated with sandy sediments in southern New England. It is also possible that all environmental samples positive for *A. macrocephali* subsp. *homaria* contained small arthropods with *A. macrocephali* subsp. *homaria* residing on their integument.

The qPCR assay developed here for the detection of *A. macrocephali* subsp. *homaria* is specific for this bacterium and sensitive enough to detect 4 bacterial cells in a pure DNA template. This qPCR assay can be particularly useful for enumerating levels of the bacterium in lobster lesions and healthy surfaces for future in vitro and in vivo studies. We demonstrated that the bacterium is highly abundant in ESD lesions and only sparse on healthy surfaces, if present at all. Future monitoring of the presence of this bacterium in lobsters with ESD and in the environment will allow for a better understanding of the role this bacterium plays in ESD and how lobsters acquire it on their cuticle.

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