Lethal marine snow: Pathogen of bivalve mollusc concealed in marine aggregates

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Acknowledgements

We thank D. Leavitt, B. Walton, T. Marcotti, and D. Moran for field assistance and S. Roberts and C. Romano for laboratory assistance. We thank Drew Harvell and an anonymous reviewer for constructive comments on the manuscript. This work was funded by an NSF grant as part of the joint NSF-NIH Ecology of Infectious Disease program, by the Woods Hole Oceanographic Institution (WHOI) Sea Grant Program, under a grant from the National Oceanic and Atmospheric Administration, U.S. Department of Commerce, and a National Science Foundation Graduate Fellowship to M. Lyons.

Abstract

We evaluated marine aggregates as environmental reservoirs for a thraustochytrid pathogen, Quahog Parasite Unknown (QPX), of the northern quahog or hard clam, *Mercenaria mercenaria*. Positive results from in situ hybridization (ISH) and denaturing gradient gel electrophoresis (DGGE) confirm the presence of QPX in marine aggregates collected from coastal embayments in Cape Cod, Massachusetts where QPX outbreaks have occurred. In laboratory experiments, aggregates were observed and recorded entering a quahog's pallial cavity thereby delivering embedded particles from the water column to its benthic bivalve host. The occurrence of pathogen-laden aggregates in coastal areas experiencing repeated disease outbreaks suggests a means for the spread and survival of pathogens between epidemics and provides a specific target for environmental monitoring of those pathogens.

Introduction

Marine aggregates (i.e., marine snow, flocs, organic detritus) ranging in size from a few microns to more than a centimeter are common in coastal environments where large populations of benthic, suspension-feeding invertebrates thrive. Aggregation of living and non-living material is a natural process affected by a number of well-documented physical, chemical, and biological interactions (Alldredge and Silver 1988; Kiørboe 2001), and is recognized as an important mechanism for the transport of carbon, nutrients, and other materials to benthic ecosystems (Fowler and Knauer 1986; Alber and Valiela 1995). Although several studies have focused on the composition, formation, distribution, and fate of aggregates (reviewed by Simon et al. 2002) none have addressed the role of aggregates in the transmission of diseases of marine animals. Here we provide evidence of marine aggregates facilitating disease transmission by serving as an environmental reservoir for a thraustochytrid pathogen of a bivalve mollusc.

Marine pathogens cause extensive ecological and economical damage (Harvell et al. 2002). In order to fully understand the impacts of marine diseases on organisms, populations and ecosystems, it is essential to establish the modes of pathogen transmission (Harvell et al. 2004), including their reservoirs. Since the 1950s, northern quahogs, also known as hard clams and *Mercenaria mercenaria*, from the northeast coast of North America have suffered severe mortalities (30-100%) from a pathogen known as Quahog Parasite Unknown (QPX; Whyte et al. 1994; Smolowitz et al. 1998; Ragone-Calvo et al. 1998). QPX infections typically occur in the quahog mantle, gill, and siphon tissues indicating direct infection from seawater (Smolowitz et al. 1998). QPX is a small

(4-25 μm), round, non-motile thraustochytrid (Maas et al. 1999; Ragan et al 2000; Stokes et al. 2002). The thraustochytrids are a group of single-celled, fungal-like, marine protists associated with decaying vegetation, shells and detritus (Ragukumar 2002). Since some non-pathogenic thraustochytrids may survive in marine aggregates, we investigated the possibility that QPX may also be located in aggregates. The presence of pathogen-laden aggregates in areas subjected to disease outbreaks would suggest a method for the spread and survival of pathogens between epidemics. Aggregates may then provide a specific target for environmental monitoring of those pathogens. The purpose of this paper is to report the presence of a thraustochytrid pathogen (QPX) of an ecologically and economically important invertebrate species (*Mercenaria mercenaria*) concealed in marine snow.

Methods

Collection of marine aggregates

Marine aggregates were obtained from three locations in Barnstable Harbor, Massachusetts in June, July, and August of 2004 and from two locations in Pleasant Bay, Massachusetts in July and August of 2004. Aggregates were collected once each month during low tides (average depth of water 0.5-1 m), in sandy areas near quahog beds known to be infected with QPX. To obtain a sample, a 3-pronged Sheppard's Hook was assembled and pushed into the sand. Polycarbonate Imhoff settling cones were hung from each hook using a thick rubber band to keep the cone upright within its ring-rope harness. A 15-ml Falcon tube was secured to the bottom of each cone (Fig. 1). One liter

of seawater was gently collected from just below the surface with a wide-mouth, tri-corner beaker and transferred to the settling cone by tilting the cone and pouring as slow as practical. The cone was then returned to the vertical position and aggregated material was allowed to settle for 10 minutes. Triplicate samples were obtained for each location. Operationally defined, "aggregate-free" seawater was removed by siphoning from the top of the cone. The last 15 ml (volume of the Falcon tube) was settled overnight in the refrigerator. The following day, the top 13 ml was removed with a pipette and the final 2 ml was preserved in 6 to 8 ml of Tissue Storage Buffer (TSB: 0.25M EDTA, 20% DMSO, saturated NaCl) until analyzed by in situ hybridization (ISH; described below), or directly frozen (no TSB) until analyzed by Denaturing Gel Gradient Electrophoresis (DGGE; described below).

Generation of QPX-enriched marine aggregates

QPX-enriched marine aggregates were generated in the laboratory by adding 8 to 10 ml of cultured QPX (culture maintained at Marine Biological Laboratory, Woods Hole, MA) to 1-liter jars of unfiltered seawater (22 °C, salinity 30, pH = 8.1) collected from Avery Point, Connecticut in Long Island Sound, an area historically free of QPX mortalities. Jars were placed on a roller table (Shanks and Edmonson 1989) and rotated at room temperature for 2 to 4 days. After rolling, jars were removed from the roller table and visible aggregates were allowed to settle for 10 to 15 minutes. Operationally defined "aggregate-free" seawater was siphoned out of the top of the jar, leaving

approximately 150 aggregates suspended in a minimal amount of seawater (< 2 ml total). Aggregates were combined and aliquots were prepared for analysis as described below.

Analysis of marine aggregates for QPX

For in situ hybridization (ISH), aliquots of the aggregate mixture were dried according to a procedure for marine aggregate preparation for ISH (Grossart and Ploug 2001) and fixed according to a procedure for the detection of QPX in cell smears and paraffin-embedded bivalve tissue (Stokes et al. 2002). Aggregate aliquots were smeared onto Superfrost Plus slides, dried at 46°C for approximately 2 hours, and fixed with fresh 8% paraformaldehyde overnight in a humid chamber. Paraformaldehyde was then decanted and slides were rinsed three times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), air dried, and stored frozen until analysis. Similar slides, made with pure QPX culture, were simultaneously prepared for visual comparison of ISH results. The ISH procedure utilized a cocktail of two DNA oligonucleotide probes (QPX641 and QPX1318) targeting the small subunit ribosomal RNA molecules (Stokes et al. 2002). The hybridization solutions contained 4 ng/µl of each digoxigenin-labeled probe except for a negative control that was incubated in hybridization buffer without probe. The hybridizations were followed by color development with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP). TE buffer and water rinses were used to stop the color development. Aggregate samples provided enough natural background color and contrast such that no counterstaining with Bismarck Brown Y was necessary. Slides were cover-slipped with aqueous-based mounting solution, sealed with nail polish,

and examined using light microscopy (National DC3-163 Digital Microscope, 40x objective).

Total nucleic acids were recovered from marine aggregates using the hot detergent/bead beating method of Kuske et al. (1998) as described in Gast et al. (2004). Polymerase chain reaction (PCR) products for Denaturing Gradient Gel Electrophoresis (DGGE) were generated using the eukaryote-specific 18S ribosomal GC-clamped primer 960FGC (Gast et al. 2004), and the QPX-specific primer QPXR2 (Stokes et al. 2002) following touchdown PCR and precipitation conditions as previously described (Gast et al. 2004). Perpendicular gel analysis indicated that a denaturing gradient of 45 to 75% was most useful for the 960FGC/QPXR2 product (~200 base pairs in length). Five microliters of sample were loaded per lane, along with a sample of PCR product generated from the QPX organism in culture. The gel was run overnight at 100V, stained with ethidium bromide and photographed. Bands corresponding to material from field samples (marked with an 'o' in Fig. 3) were recovered from the gel, reamplified using the non-GC clamped 960F (Gast et al. 2004) and QPXR2 primers, and sequenced using the 960F primer and ABI BigDye™ Terminators (PE Applied Biosystems) on an ABI 377 DNA sequencer to assess their taxonomic affiliation.

Video-endoscope techniques

Endoscopy was performed according to methods described previously (Ward et al. 1991). The endoscope (Olympus K-17 series), with optical insertion tube (OIT) of 1.7 mm in diameter, was connected to an optical zoom-adapter and attached to a color, CCD

camera (Cohu, Inc.). The resolution of the video endoscope was approximately 3 μm at a maximum magnification of about 150x. Video signals were recorded on an 8-mm VCR (Sony Hi8). Marine aggregates were generated as described above but with the incorporation of 10 μm yellow-green fluorescent beads (instead of pathogens) as tracers for pathogens and fed to the bivalves. The OIT of the endoscope was maneuvered above the incurrent siphon of a quahog that was partially buried in a container of sand submerged in a small, aerated aquarium.

Results

We compared ISH results for pure QPX culture (dark spheres in Fig. 2A) to ISH results for aggregates generated in the laboratory with QPX culture (positive control; dark sphere in Fig. 2B) and without QPX culture (negative control; no dark spheres in Fig. 2C). These laboratory-generated, artificially-enriched aggregates were used as visual comparisons for evaluating the occurrence of QPX in natural marine aggregate samples. Positive ISH results revealed QPX thalli embedded in natural marine aggregates (Fig. 2D) from all samples collected near quahog beds known to be infected with QPX.

To confirm our discovery, representative aggregate samples were also processed for DGGE. These aggregate samples indicated the presence of QPX-specific DNA fragments (Fig. 3). Bands (accession numbers DQ083533 - DQ08538) were recovered from the gel and sequenced. All bands yielded sequences that were confirmed by Blast analysis and sequence alignment as having significant similarity to QPX ribosomal sequences from GenBank (Fig. 4). The bands at positions other than the QPX control

band in aggregate samples still have significant similarity to the QPX sequence (Fig. 4). The presence of these additional bands can result for several reasons. DGGE is able to separate fragments with a single base difference, so the multiple bands in the natural samples may represent either natural strain variation or variability within the ribosomal repeat unit. When multiple sequences that are very similar are the target for amplification, they can form heteroduplexes during the repeated rounds of PCR (Kanagawa 2003). These heteroduplexes occur as multiple bands in the same sample on DGGE, but show very little sequence variation. We were unable to confirm whether multiple strains, repeat unit variation, or PCR error was the source of the additional bands in the natural samples.

Chimeric sequences are another artifact that can occur in the PCR amplification. These occur through incompletely extended products acting as primers or through template switching during the extension phase (Kanagawa 2003). It seems unlikely that chimeric molecules would be generated on such a small fragment, especially since our extension times are long enough to ensure full-length amplification and our amplification cycle number is low, at least for the first two rounds of amplification. Unfortunately, it would likely be very difficult to identify chimeric sequences if they did occur because the target sequences are so similar to each other.

The additional bands in the QPX culture lane could also arise due to repeat unit variability, but we have not determined whether this is the case. We believe that much of the banding pattern here occurs due to PCR artifact and overloading of the sample on the

gel. Dilution of the sample has, of course, reduced the number of additional bands that are visible, as has reducing the number of amplification cycles.

Observations from the video endoscope revealed that all aggregates smaller than the diameter of the siphon entered the quahog's pallial cavity, thereby delivering embedded particles from the water column to the tissues of the quahog.

Discussion

Aggregates are ubiquitous in the marine environment (Alldredge and Silver 1988), but are often overlooked in field studies because they are destroyed by traditional sampling equipment such as plankton nets and other techniques that homogenize bulk water samples prior to analysis. Although the preferred method for aggregate collection is via SCUBA divers with wide bore syringes (Alldredge 1979), the shallow depth of water in tidally driven embayments prohibits the use of this method. Instead, the use of the settling cones with attached Falcon tubes provides a quick and reliable method to acquire sufficient material for testing for the presence or absence of pathogens. Although our method generally under-samples the microaggregates (aggregates <500 μm) that do not settle as quickly as the marine-snow size aggregates (aggregates >500 μm), it also limits sampling of individually suspended cells that settle slower than the larger aggregates. In addition, it is the larger, more rapidly sinking, aggregates that the benthic, suspension-feeding quahogs are more likely to encounter.

Marine aggregates are a relatively unexplored link between waterborne pathogens and their benthic hosts. To our knowledge, these results are the first documentation of

QPX in the environment outside of quahogs and the first report of a protistan (thraustochytrid) pathogen embedded in marine aggregates. The thraustochytrids are important ecological group in coastal environments but comparatively little is known about their taxonomy and phylogeny (Ragukumar 2002). Thus, the specificity of the amplification primers needs to be addressed. The FA2/RA3 and QPXF/QPXR2 primers were designed and tested by other researchers (Mo et al. 2002; Stokes et al. 2002). We re-examined their specificity relative to sequences in current databases through Blast (Altschul et al. 1997) searches of GenBank and Check Probe at the Ribosomal Database Project (Cole et al. 2005). Our searches found no strong matches for the QPX-specific primers besides the QPX organism, but the primers that were supposed to be thraustochytrid-specific did match a significant range of other organisms. It is likely that the first amplification would cross-react with an unknown organism in the environment, but the second and third rounds of amplification are more specific. To date all of the bands that we have recovered and sequenced from marine aggregate samples represent QPX-like organisms.

We focused on a thraustochytrid pathogen, but the concept of pathogens persisting in and being transported by marine aggregates applies to other pathogens including those infective to humans and those entering the marine ecosystem from the terrestrial environment. Aggregates have been reported to harbor human bacterial pathogens such as *Vibrio parahaemolyticus* (found in "sinking particles"; Venkateswaran et al. 1990) and *Vibrio cholerae* (found in "particulates > 20 μm"; Colwell et al. 2003). Aggregates are microscale ecosystems with relatively higher levels of productivity and

biomass than the surrounding seawater (Alldredge and Silver 1988; Simon et al. 2002), and would provide a means for pathogen survival and transport between epidemics by serving as environmental reservoirs. Pathogens with reservoirs in the environment have the potential to infect and kill all of their hosts since the pathogens would not be limited by the density of the host species (Harvell et al. 2004). Pathogens that exploit aggregates as reservoirs will be difficult to control because marine aggregates can not be eliminated from the ecosystem but the marine aggregates may provide an opportunity for targeted surveillance of those embedded pathogens.

Examples of marine diseases with vectored transmission are scarce compared to terrestrial diseases that rely on flying insect vectors for dispersal (Harvell et al. 2004). We hypothesize that marine aggregates also function as vectors (i.e., vehicles of transmission) linking pathogens in seawater to their benthic hosts. QPX is a non-motile, internal parasite which requires a means of transport to its quahog host. Observations by means of video endoscopy revealed that all aggregates, smaller than the diameter of the siphon, are drawn into the pallial cavity of the quahog. Suspension-feeding bivalves preferentially ingest and reject particles acquired from filtering seawater (Ward and Shumway 2004). For quahogs, those particles (including aggregates) that are not ingested are collected at the base of the incurrent siphon until periodically expelled as pseudofeces (Grizzle et al. 2001). Since the base of the incurrent siphon is also a location of localized inflammatory nodules in quahogs infected with QPX (Smolowitz et al 1998), we propose that marine aggregates facilitate direct infection of this tissue by delivering pathogens to this site.

To date, no studies have evaluated the role of marine aggregates in the uptake of pathogens by suspension feeding invertebrates, but a few have examined the role of marine aggregates as a food resource. In laboratory experiments, Alber and Valiela (1995, 1996) examined incorporation of nitrogen by bivalves fed aggregates produced from organic matter released by marine macrophytes. They found that scallops and mussels could gain some organic matter when in the form of aggregates and suggested that this might be an important pathway in the detrital food web. In field surveys, Graf et al. (1982) reported increases in glycogen and lipid resources of bivalves following the settling of aggregates formed after the spring phytoplankton bloom. These studies support our contention that suspension feeding bivalves come in contact with and process marine aggregates, and in doing so could be infected with embedded pathogens. Other marine invertebrates that utilize organic aggregates as a food resource (e.g., zooplankton; Dilling et al. 1998 and references therein) may also be susceptible to infections via this process.

As reports of marine disease epidemics continue to increase (Harvell et al. 2002, 2004; Ward and Lafferty 2004) the need for comprehensive surveillance programs will also increase. Many current surveillance programs monitor pathogens in seawater, sediments, and animals but fail to evaluate the role of marine aggregates. We believe that one path for the transmission of diseases to benthic, suspension-feeders flows through aggregated material. We utilize bivalves as our model organisms because they are both ecologically and economically important, but the concepts reported here apply to other

marine animals and demonstrate the need to evaluate the role of marine aggregates as reservoirs and vectors of disease.

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Figure 1. Apparatus for collecting marine aggregates in shallow coastal habitats with soft substrates. A 15 ml Falcon tube (arrow) was secured to the bottom of a 1-L settling cone which was hung from a Sheppard's hook using a ring-rope harness.

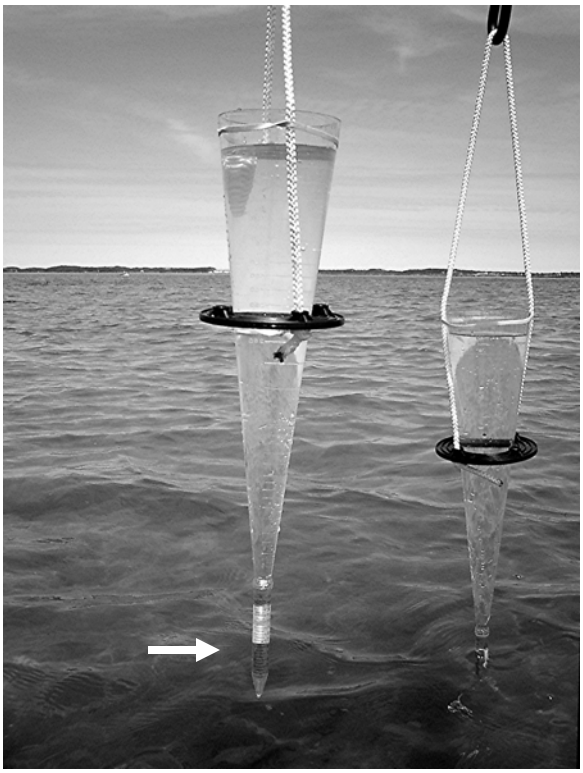
Figure 2. Examples of in situ hybridization results for Quahog Parasite Unknown (QPX). (A) Positive results for QPX culture. (B) Positive results for laboratory-generated aggregates made with seawater and QPX culture (positive control). (C) Negative results for laboratory-generated aggregates made with seawater with no culture added (negative control; no dark round spheres present). (D) Positive results for natural aggregates collected near quahog beds infected with QPX. Each darkly stained sphere within a red circle is one QPX thallus. All scale bars are 25 μm.

Figure 3. Denaturing Gradient Gel Electrophoresis (DGGE) results for marine aggregates. Lane 1 – QPX culture, lanes 2 – 4 are aggregate samples from Barnstable Harbor. The band indicated by the arrow corresponds to the QPX culture positive control band. Bands marked with an 'o' were recovered from the gel, reamplified, and confirmed by Blast analysis and sequence alignment as being QPX. Bands are identified as 102004.4, 102004.5, 102004.6, 102004.9, 102004.10 and 102004.13.

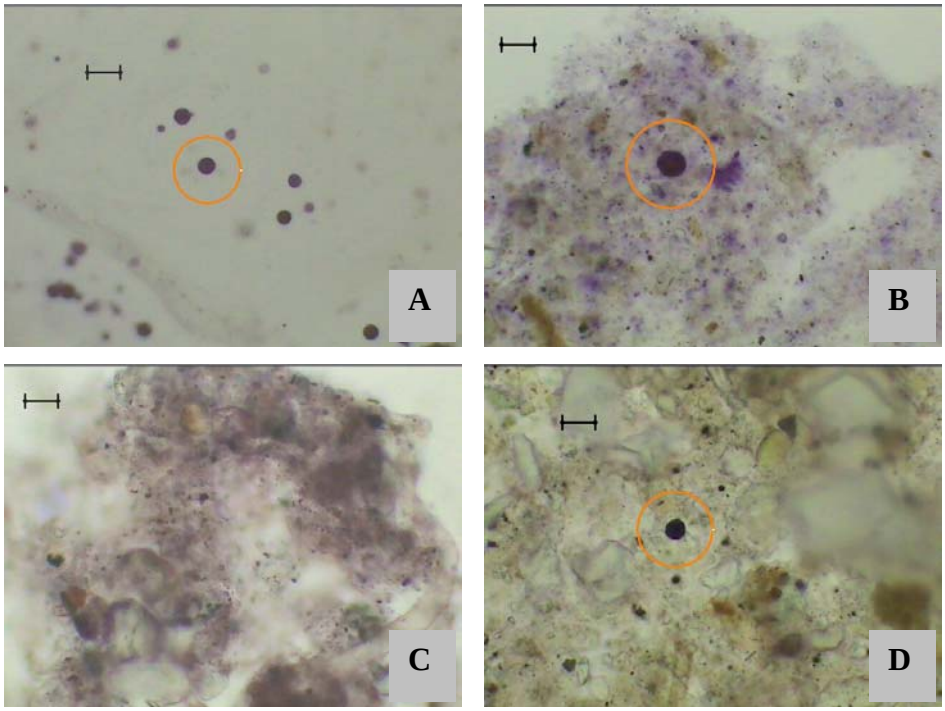
Figure 4. Alignment of sequences from aggregate bands with a portion of the QPX and thraustochytrid ribosomal genes from GenBank. The fragment corresponds to positions

1164-1331 in the QPX sequence. *T. sp* C9G (AF474172) is a thraustochytrid sequence recovered from a clam. *T. pachydermum* (AB022113) and *T. sp.* C9G are the most similar sequences to QPX. QPX Stokes (AY052644), QPX Ragan (AF261664) and QPX Maas (AF155209) represent sequences for the QPX organism isolated independently and from different locations. –'s indicate gaps, .'s indicate the same base as the QPX sequence, and letters indicate substitutions.

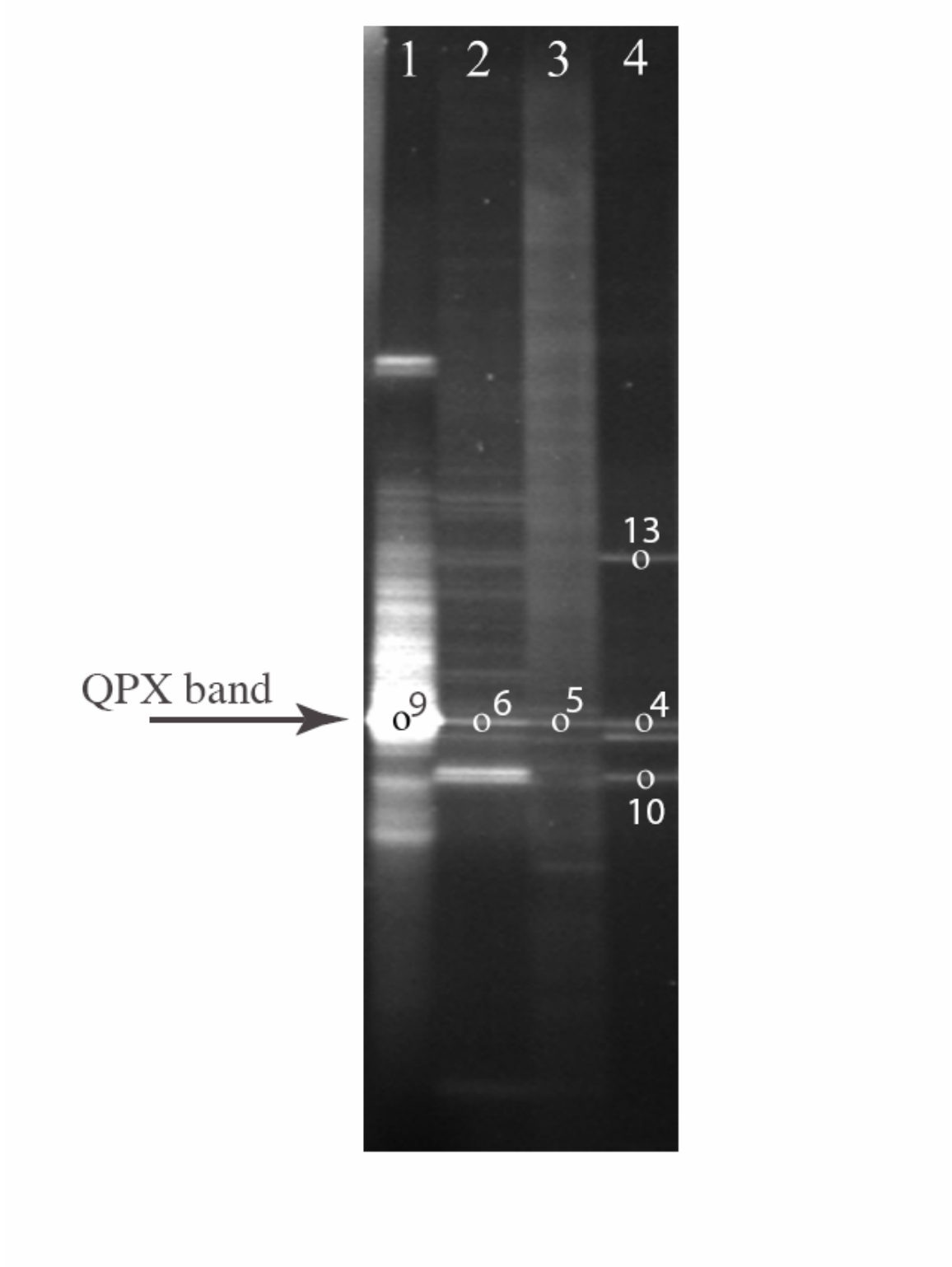
Lyons Figure 1



Lyons Figure 2



Lyons Figure 3



Lyons Figure 4