The 2009 Bermuda Reef Fish Dieoff: Evidence for the Role of an Infectious Disease Process

A Final Report Submitted to

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EXECUTIVE SUMMARY

Title: The 2009 Bermuda Reef Fish Dieoff: Evidence for the Role of Infectious Disease

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During August and September of 2009, finfishes inhabiting the fringing coral reefs of the British overseas territory of Bermuda experienced a significant dieoff. Many of these fish exhibited prominent skin ulcers, a common clinical sign of microbial infection. This generated concern among fisheries scientists, environmental managers, seafood merchants and Bermudian citizens about the health of the coral reefs and the safety of local seafood resources. Bermudian scientists suggested several possible causes for the fish-kill event, primary among them being infectious disease, harmful algal blooms, chemical pollution and stress associated with changes in physical/chemical water quality parameters.

We initiated a study in late September 2009, to determine the underlying causes of this fish kill event. To investigate possible involvement of an infectious disease agent(s), we conducted detailed necropsies of 26 dead or moribund (dying) reef fishes belonging to 10 species and evaluated their health status using basic biomedical methods including bacteriology, molecular biology, parasitology and histopathology. We cultured bacteria from the tissues (skin ulcers and visceral organs) of 11 of these fishes (42%) and obtained 31 bacterial isolates. Phenotypic characterization using the Biolog Gene III System definitively identified 14 of these isolates as *Vibrio harveyi* (SI > 0.5), whereas 10 others very closely resembled *V. harveyi* but exhibited low SI values (< 0.5). Seven other isolates were identified as different species, most of them also vibrios. 16S rRNA sequencing of the 31 bacterial isolates produced 29 usable sequences, 15 of which displayed 100% sequence identity with *V. harveyi,* while 5 more were >99% identical with *V. harveyi*. Six other bacterial isolates were identified as different species, most of them other vibrios. Parasitological and histopathological analyses identified moderate to severe gill pathology in 17 of 22 (77%) fishes. In 11 (50%) of these, we identified a highly pathogenic ciliate protozoan parasite called *Brooklynella* sp. Several of these infections were severe. Additionally we observed significant pathology of the liver, exocrine pancreas and alimentary tract in many (86%) of the specimens. Some of this pathology may have been associated with systemic bacterial infection, however, moderate to severe hepatitis and pancreatitis was observed in 10 of the 12 (83%) seargent majors (*Abudefduf saxatilis*), only two of which exhibited *V. harveyi* infection. Thus, it is possible that some third disease process was active in this species.

No single infectious disease process was identified as the underlying cause of the 2009 Bermuda fish kill event. Consistent isolation and identification of *V. harveyi* by two methods from many of the fish and the high prevalence and severity of *Brooklynella* infections associated with severe gill pathology implicate these two organisms as causative agents playing some role in the 2009 Bermuda reef fish dieoff. However, neither infection was consistently observed in every affected fish and the cause of pancreatitis in seargent major remains unknown. This suggests that

other presently unidentified environmental factors were involved in modulating the expression of disease and mortality, a primary suspect being elevated water temperature. Our findings indicate that concerns for human health expressed at the time of the die-off are unfounded, since neither *V. harve*yi nor *Brooklynella* sp. has been reported to infect humans. Both are known to infect and cause disease only in marine organisms and have no demonstrated zoonotic potential. It is recommended however that marine species exhibiting external disease signs and fish that are dead or dying should not be handled by people and should also not be consumed.

We recommend that the Bermudian government 1) initiate a limited, cost-effective citizen-based fish health monitoring program on a few selected coral reef communities around Bermuda, 2) incorporate regular and specific finfish tissue collections and physicochemical water quality parameter measurements into current existing local environmental monitoring programs, 3) fund a Bermudian citizen to pursue advanced training (Masters or Ph D degree) in aquatic animal health issues to bring this specific scientific expertise to Bermuda and limit future reliance on outside experts, and 4) set aside funding in support of near-term future investigations should another reef fish dieoff occur in Bermuda waters.

Background

During August and September of 2009, finfishes inhabiting the fringing coral reefs of the British overseas territory of Bermuda experienced a significant dieoff. Numerous dead and moribund (dying) reef fishes were observed floating on the surface and washing up on the beaches in various locations around the island. The largest numbers of fish were observed on the west end of the island near Dockyard and Scotts Bay. Other locations where significant numbers of dead fish were observed included Tobacco Bay, Tucker's Point in St. George's and points along the south shore beaches. Many of these animals exhibited severe skin ulceration and other skin lesions, generating concern among fisheries scientists, environmental managers, the seafood industry and Bermudian citizens about the health of the coral reefs and the safety of local seafood resources. Media outlets reported that fishermen had difficulty selling their catch because restaurants were not purchasing local reef fishes. At the time, pelagic species were considered safe for human consumption, as mortalities and skin lesions had only been observed in fishes inhabiting the coral reefs. Bermudian scientists suggested several possible causes for the fish-kill event including infectious disease, harmful algal blooms, stress associated with changes in physical/chemical water quality parameters including temperature, dissolved oxygen concentrations, increased nutrient levels, and the unusually large quantities of coral spawn observed at the time. Notably, the summer of 2009 was characterized by unusually high water temperatures and still waters. In late September, one of us (WKV) was contacted by Dr. Frederick W. Ming, Director of the Bermuda Dept. of Environmental Protection and invited to initiate an investigation of the underlying causes of the reef fish die-off. Because of the common occurrence of skin lesions in the affected fishes, the study we initiated focused specifically on the possible involvement of an infectious disease agent. Other lines of investigation initiated by the Bermudian government at the time included a search for harmful algal blooms and evaluation of selected physicochemical water parameters on the fringing reefs.

Vogelbein visited Bermuda from 25 Sept. to 2 Oct., 2009 and initiated the investigation that currently involves participation of several investigators at the Bermuda Institute of Ocean Sciences (BOIS) and specialists at the Virginia Institute of Marine Science (VIMS) (fish pathology, microbiology) and Old Dominion University (ODU) (molecular biology). Vogelbein has served as the Principal Investigator of this study and was responsible for its oversight, collection of the necessary biological samples in Bermuda (e. g., fish necropsies, parasitology evaluations, and microbiological isolations of fish tissues, fixation of fish tissues), conducting the histopathological analyses and submission of reports. Martha Rhodes and Howard Kator (VIMS) conducted the bacteriological analyses and David Gauthier (ODU) conducted the molecular analyses for specific identification of the predominant microbial infectious agents isolated in this study.

This Final Report details the findings of our initial investigation into potential causes of the 2009 Bermuda reef fish kill. The overall aim of our investigation was to determine what, if

any, infectious disease agents were associated with the reef fish die-off.   This first investigation was not envisioned to provide definitive answers as to the underlying environmental factors that might be modulating outbreak of infectious disease, although we initially hypothesized, and continue to hypothesize the presence of such underlying environmental stressors. Rather, our investigaion represents a preliminary study designed to identify the presence of any potential infectious etiologic agents that may be playing a central role in the die-off.

MATERIALS AND METHODS

From Sept. 25 through Oct 2, 2009, while in Bermuda, one of us (WKV) conducted necropsies of 26 fishes that had died in the field or that were moribund and exhibited skin lesions at the time of collection. These fish were collected by Dr. Tim Noyes and his team at BIOS. Fish were collected by netting them from the surface of the water, with an effort made to find and obtain moribund individuals exhibiting external clinical signs of disease (skin ulcers). Because they had observed fishes exhibiting skin lesion on certain fringing reefs, Noyes and his crew switched strategy and conducted numerous reef dives to collect affected live fish by spear fishing for them. Fish exhibiting external signs of disease were targeted specifically. Collected fish were immediately placed on ice and brought back to BIOS for examination. Throughout the week-long effort in Bermuda, fish were held on ice for varying lengths of time prior to necropsy. This was especially true for the first two days of necropsy, as fish had been collected and stored over several days before WKV arrived in Bermuda. After the first few days however, fish were processed as quickly as possible after collection, usually within 24-36 hrs (see **Table 1**).

Selected fish underwent a thorough gross and parasitological examination. They were measured (total length and fork length) and photographed. A skin scraping was taken and a wet mount was made and examined microscopically for parasites. A gill clip was taken and examined microscopically for parasites. The fish then underwent aseptic necropsy in a BSL-2 cabinet and tissue samples for microbiological culture were collected. Tissues collected for culture included small samples of brain, heart, liver, spleen, kidney and gonad. These samples were streaked onto two types of bacteriological media, a blood agar (BHI: brain-heart-infusion agar with sheep red blood cells) and a tryptic soy agar (TSA) with 2% salt added. A small block (1 mm^3) of spleen tissue was also collected aseptically and placed into 95% ethanol for preservation and possible workup by molecular methods. Culture of the bacteriological plates was conducted at room temperature in an incubator. Bacterial growth was recorded in most instances within 24 hours, colony morphology was noted and specific bacterial colonies were re-isolated onto nutrient media slants (TSA with 2% salt) for shipment to VIMS. Following collection of microbiological and molecular samples, the skin, gills, brain, eyes, liver heart, spleen, alimentary tract, kidney and gonad were visually inspected for gross signs of disease after which they were chemically preserved in fixative (seawater-buffered 10% formalin) for histopathological processing and evaluation. All samples that were collected and processed by

Vogelbein during Sept 25-Oct 2, 2009 were then packed and shipped from Bermuda to the Virginia Institute of Marine Science for additional analysis.

Bacteriology: Within 48 hours of arrival at VIMS, bacteriological slants were re-isolated onto blood agar. Multiple isolates based on colony morphology were selected from primary plates if colony variation was evident. All isolates were gram negative rods and all but one, were oxidase positive. Thirty-one purified isolates from 11 reef fish were cultured in brain heart infusion broth containing NaCl (20 ppt), incubated overnight at 30 ºC, and archived in 20% glycerol at -80ºC. Prior to characterization using the Biolog Gene III System (Biolog, Inc. Hayward, CA) isolates were grown twice on BUG agar with 5% sheep blood (Biolog Inc.) with overnight incubation at 30 ºC. Inocula for filling microplates containing 71 carbon substrate utilization assays and 23 chemical sensitivity assays were prepared per manufacturer's instructions. Initially all inocula were adjusted to the recommended cell density using the default inoculating fluid (IF) A (Biolog, Inc.) which is reportedly appropriate for the majority of species. Selected isolates which did not yield a definitive identification were reanalyzed using IF B (see **Table 2** below), recommended for some strongly reducing strains, including some vibrios. Remaining microplates were insufficient in number to analyze all isolates with ambiguous results.

Histology: Tissue samples were processed by routine methods for paraffin histology (Prophet et al., 1972). Briefly, tissues containing bone were decalcified overnight and all tissues were then rinsed overnight in running tap water to wash out the fixative and/or decalcifying solution. Tissues were then trimmed with a single edge razor blade, placed in plastic cassettes and transferred to 70% ethanol. They were then placed into an automatic tissue processor (Shandon Hypercenter), dehydrated by passage through a graded series of ethanols (70-100%), cleared, and infiltrated in warm melted paraffin wax. Tissues were then embedded on a tissue embedding center, sectioned at 5µm on a rotary microtome, and mounted on glass microscope slides. Slides were subsequently stained with hematoxylin and eosin (H&E) in an automatic slide stainer (Shandon Varistain) and coverslipped. All slides were examined and selected slides were photographed on an Olympus AX-70 photomicroscope.

Genetic Analysis: Bacterial cultures from the 2009 Bermuda fish kill were submitted to the laboratory of Dr. David Gauthier at Old Dominion University for genetic analysis. Subcultures were made from these cultures on Tryptic Soy Agar with 2.5% NaCl, and DNA was extracted from cell pellets after centrifugation. The 5' end of the 16S rRNA gene (462 bp) was amplified with universal bacterial 16S primers, reactions were purified with Exo-SAP (USB Scientific), and direct fluorescence cycle sequencing was performed on an ABI 3130*xl* sequencer (Applied Biosystems). Each isolate was sequenced at least in forward and reverse directions from two separate PCR products. Sequence traces were aligned with Geneious software (Drummond et al., 2009) and compared to NCBI database using BLAST.

For simplicity, the highest BLAST hit in terms of % sequence identity is presented in **Table 2**. Where the highest BLAST hit was an unnamed species, the named species returning the highest sequence identity is presented.

RESULTS

Necropsy Specimen Summary Data: Summary data for the fishes that were collected during Sept. 25 – Oct. 2, 2009 and processed at BIOS are provided in **Table 1**. Twenty-six fish specimens belonging to 10 different species were processed for analysis. These included fish that were collected floating at the surface and fish that were obtained by spear fishing on different reefs. An emphasis was placed on obtaining moribund fish and fish with skin lesions. This collection contains fish that appeared healthy at the time of collection as well as fish that exhibited gross external signs of disease.

Identification of Bacterial Isolates: Isolations from the tissues of 11 of the 26 fish processed for bacteriology yielded significant and largely pure, bacterial growth on nutrient agar media. Thirty-one distinct isolates were obtained and identified by traditional bacteriology (Biolog) and by molecular methods. Bacteriological analysis yielded isolates identified as *Vibrio harveyi* (Similarity Index > 0.5: **Table 2**) from ten of these 11 positive fishes. There was no consistency with respect to organ or tissue of isolation; positive isolations were obtained from both skin lesions and from several different visceral organs, including spleen, liver, heart, gonad and kidney. Note also that other isolates from many of these fish were similar to *V. harveyi* but had low Biolog Similarity Indices (**Table 2**). When three of these were re-analyzed using Biolog IF B, two isolates were identified as *V. harvey*i and one as *V. splendidus*.

Of the 31 isolates submitted to the Gauthier lab, 29 yielded usable 16S rRNA sequences. The named species with highest identity was *Vibrio harveyi* in 20 of these isolates. Fifteen isolates displayed 100% sequence identity with *Vibrio harveyi,* whereas the remainder were >99% identical. Biolog analysis and 16S sequencing agreed in calling isolates *V. harveyi* in 19 instances. In three instances, Biolog identified isolates as *V. harveyi*, whereas 16S rRNA sequence identified them as *V. hepatarius*, *V. communis*, and *V. rotiferianus* (% similarity 98.7, 100%, 99.8%, respectively).

Other species identified by 16S rRNA sequencing were: *Photobacterium damselae* (100% identity, Biolog ID same); *Enterovibrio coralii* (99.2% identity, Biolog ID *P. damselae*); *V. coralliilyticus* (99.6% identity, Biolog ID *V. fluvialis*); *V. ponticus* (100% identity, Biolog ID *V. splendidus*); *V. sinaloensis* (2 isolates, 99.8 and 99.6% identity, Biolog IDs *V. cholerae* and *Listonella pelagia*, respectively).

A striking finding of this investigation is the consistent identification of *V. harveyi*, largely in agreement with Biolog phenotypic identification. Genetic identification of 20 of the 29 isolates as *V. harveyi* supports and extends the results of the phenotypic bacteriological identifications.

Sample #	Coll Date	Necrop Date	Common Name	Scientific Name	Location	GPS Coordinates	Condition	FL	TL
BFK-09-02	9/22/2009	9/25/2009	Bermuda angelfish	*Holocanthus bermudensis*	41		dead @ surface		297
BFK-09-06	9/22/2009	9/25/2009	Cardinal fish	Uncertain ID	46		alive @ surface	101	119
BFK-09-14	9/25/2009	9/26/2009	French grunt	*Haemulon flavolineatum*	56	N32 19 44.2 W64 40 30.0	alive @ surface	218	243
BFK-09-15	9/25/2009	9/30/2009	dusky squirrelfish	*Sargocentron vexillarium*	59	N32 19 44.7 W64 41 01.1	dead @ surface	140	162
BFK-09-17	9/25/2009	9/26/2009	Blue tang	*Acanthurus cueruleus*	61	N32 19 26.6 W64 41 50.1	alive @ surface	203	235
BFK-09-19	9/25/2009	9/26/2009	dusky squirrelfish	*Sargocentron vexillarium*		N32 19 16.4 W64 41 11.7	alive @ surface	123	152
BFK-09-22	9/28/2009	9/28/2009	beaugregory	*Stegastes leucostictus*	68	N32 19 50.8 W64 40 28.8	spear: diseased	121	133
BFK-09-29	9/28/2009	9/28/2009	seargent major	*Abudefduf saxatilis*	68	N32 19 50.8 W64 40 28.8	spear: diseased	154	196
BFK-09-40	9/25/2009	9/30/2009	Bermuda angelfish	*Holocanthus bermudensis*	64	N32 18 41.6 W64 42 42.9	dead @ surface		273
BFK-09-42	9/25/2009	9/26/2009	Yellow damselfish	*Microspathodon chrysurus*	M. Celeste	N32 14 30.0 W64 49 54.0	died: gray tub	152	168
BFK-09-43	9/25/2009	9/26/2009	Yellow damselfish	*Microspathodon chrysurus*	M. Celeste	N32 14 30.0 W64 49 54.0	moribund: tub	175	195
BFK-09-44	9/26/2009	9/26/2009	Squirrelfish	*Holocentrus adscensionis*	66	N32 20 21.1 W64 40 17.8	alive @ surface	218	281
BFK-09-45	9/26/2009	9/26/2009	Squirrelfish	*Holocentrus adscensionis*	66	N32 20 21.1 W64 40 17.8	spear: healthy	233	290
BFK-09-50	9/28/2009	9/28/2009	seargent major	*Abudefduf saxatilis*	68	N32 19 50.8 W64 40 28.8	spear: diseased	172	199
BFK-09-52	9/26/2009	9/27/2009	Queen Parrotfish	*Scarus vetula*	Dive 9/26 65	N32 19 45.7 W64 40 28.7	spear: healthy	355	418
BFK-09-55	9/26/2009	9/27/2009	seargent major	*Abudefduf saxatilis*	Dive 9/26 65	N32 19 45.7 W64 40 28.7	spear: lesions	166	202
BFK-09-56	9/26/2009	9/27/2009	Queen Parrotfish	*Scarus vetula*	Dive 9/26 66	N32 20 21.1 W64 40 17.8	speared: alive	364	429
BFK-09-59	9/26/2009	9/27/2009	seargent major	*Abudefduf saxatilis*	Dive 9/26 65	N32 19 45.7 W64 40 28.7	speared: alive	161	196
BFK-09-62	9/26/2009	9/27/2009	seargent major	*Abudefduf saxatilis*	Dive 9/26 65	N32 19 45.7 W64 40 28.7	speared: alive	164	196
BFK-09-71	9/28/2009	9/28/2009	seargent major	*Abudefduf saxatilis*	68	N32 19 50.8 W64 40 28.8	speared: alive	153	183
BFK09-76	9/28/2009	9/28/2009	seargent major	*Abudefduf saxatilis*	68	N32 19 50.8 W64 40 28.8	speared: alive	173	208
BFK09-80	9/28/2009	9/28/2009	seargent major	*Abudefduf saxatilis*	68	N32 19 50.8 W64 40 28.8	speared: alive	152	197
BFK09-102	9/29/2009	9/30/2009	seargent major	*Abudefduf saxatilis*	69	N32 19 39.9 W64 41 08.8	speared: alive	179	196
BFK09-111	9/29/2009	9/30/2009	seargent major	*Abudefduf saxatilis*	69	N32 19 39.9 W64 41 08.8	speared: alive	146	199
BFK09-113	9/29/2009	9/30/2009	seargent major	*Abudefduf saxatilis*	69	N32 19 39.9 W64 41 08.8	speared: alive	178	195
BFK09-114	9/29/2009	9/30/2009	seargent major	*Abudefduf saxatilis*	69	N32 19 39.9 W64 41 08.8	speared: alive	150	179

Table 1. Summary data from 26 fishes necropsied during the period of 25 Sept. to 2 Oct., 2009 at BIOS, Bermuda.

Sample	Isolate	Tissue	Biolog ID[a]	Sim Index[b]	IP (hrs)[d]	16S Sequence ID	% Identity
BFK09-02	A	skin ulcer	*Vibrio harveyi*	0.616	18	*V. harveyi*	100
	B	skin ulcer	*Vibrio harveyi*	0.614	18	*V. harveyi*	100
	C	heart	*Photobacterium damselae*	0.803	24	*P. damselae*	100
BFK09-17	A	gonad	*Vibrio harveyi*	0.52	47	*Vibrio sp.* CF6-6, *V. harveyi*	100, 99.8
	B	skin ulcer	*Vibrio harveyi*	0.26	47	*V. harveyi*	100
	C-1	spleen	*Vibrio harveyi*	0.235	24	*V. harveyi*	100
	C-2	spleen	*Vibrio harveyi*	0.612	24	*V. harveyi*	100
BFK09-19	A	skin ulcer	*Vibrio harveyi*	0.635	24	*V. harveyi*	100
BFK09-22	A	skin ulcer	*Vibrio harveyi*	0.645	24	*V. harveyi*	100
	B	skin ulcer	*Vibrio harveyi*	0.607	24	*V. harveyi*	100
BFK09-42	A	skin ulcer	*Vibrio harveyi*	0.236	30	*V. harveyi*	100
	B	spleen	*Photobacterium damselae*	0.151	30	*Enterovibrio coralii*	99.2
	C	spleen	*Vibrio harveyi*	0.357	24	*V. harveyi*	100
	D-1	liver	*Aeromonas hydrophila* (DNA gr 1)	0.492	24	*V. harveyi*	99.1
	D-2	liver	*Vibrio harveyi*	0.373	48	*V. harveyi*	99
	E-1	kidney	*Vibrio harveyi*	0.616	48	*V. harveyi*	100
	E-2	kidney	*Vibrio harveyi*	0.644	48	N/D	N/D
BFK09-43	A	skin ulcer	*Vibrio harveyi*	0.63 (B)[c]	31	*V. harveyi*	99.9
	B	spleen	*Vibrio harveyi*	0.259	43	*V. harveyi*	99.9
	C	kidney	*Vibrio harveyi*	0.331	43	*V. harveyi*	100
BFK09-44	A	skin ulcer	*Vibrio fluvialis*	0.173	24	*Vibrio sp.* N05IS15, *V. coralliilyticus*	100, 99.6
	B a	skin ulcer	*Vibrio splendidus*	0.508 (B)	42	*V. ponticus*	100
	2A a	skin ulcer	*Vibrio harveyi*	0.191	24	*V. sp.* ME1-02	98.7
	B b	spleen	*Vibrio harveyi*	0.56	43	N/D	N/D
	2A b	spleen	*Vibrio cholerae*	0.184	43	*V. sinaloensis*	99.8
BFK09-50	A	liver	*Vibrio harveyi*	0.685	24	*V. harveyi*	100
BFK09-55	A	spleen	*Vibrio harveyi*	0.198	24	*V. communis*	100
BFK09-56	A	skin ulcer	*Vibrio harveyi*	0.68	43	*V. harveyi*	100
BFK09-71	A	skin ulcer	*Listonella pelagia*	0.213	24	*V. sinaloensis*	99.6
	B	skin ulcer	*Vibrio harveyi*	0.747 (B)	42	*V. harveyi*	100
	C	skin ulcer	*Vibrio harveyi*	0.265	43	*Vibrio sp.* S2685, *V. rotiferianus*	100, 99.8

Table 2. Biolog[a] phenotype and 16S rRNA sequence-based identification of bacterial isolates obtained for Bermuda reef fishes during Sept. 2009. ([a]Identification based on Biolog Gen III, Biolog Inc., Hayward, CA. [b]Highest Similarity Index < 0.5 = no identification, may occur with atypical strains according to Biolog. [c]Identification of isolates labeled "B" obtained using Biolog Protocol B. [d]Culture incubation period.)

These findings suggest a significant role for this bacterium in pathology and mortality of fishes involved in the 2009 Bermuda reef fish die-off. The identification of other bacteria, largely also members of the Vibrionaceae, is also noteworthy. However, these microbes apparently exhibited no predilection for specific tissues or organs, being cultured in many instances from both the skin lesions and internal organs.

Table 3 summarizes the relationship between specific gross external clinical signs (skin pathology) and bacterial infection in the 26 fishes examined. Of these, 16 (62%) exhibited moderate to severe gross clinical signs of disease, largely skin ulceration (**Figure 1)** and other epidermal lesions, six (23%) exhibited mild skin pathology, and four (15%) appeared healthy externally. Of the 16 fish exhibiting moderate to severe skin pathologies, 10 (63%) harbored *Vibrio harveyi* infections (4 of these fish [25%] also exhibited co-infections, predominantly by other vibrios). No bacterial organisms (0%) were cultured from the 6 mildly affected or from the 4 externally healthy-appearing fishes. In fishes, skin ulceration is a non-specific clinical sign, but one often associated with microbial infection. Histologically, skin lesions of Bermuda reef fishes exhibited loss of the epidermis and scales with bacterial colonization of the underlying dermis, but little or no inflammation and minimal underlying tissue damage.

INFECTION	SKIN PATHOLOGY		
	Mod/Severe	**Mild**	**None**
Vibrio harveyi	10		
Other infections	1		
None	5	6	4

Table 3. Relationship between skin pathology and bacterial infection in 26 reef fishes necropsied during the period of 25 Sept. to 2 Oct., 2009 in Bermuda.
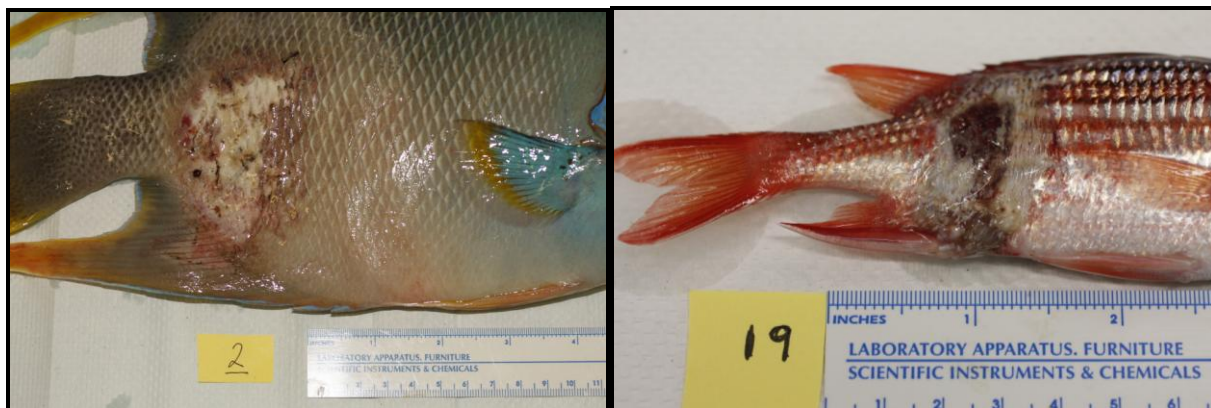

Figure 1: Skin ulceration in Bermuda angelfish (BFK09-02) and dusky squirrelfish (BFK09-19) infected with *Vibrio harveyi.*

GENERAL INFO		GROSS OBSERVATIONS			BACTERIOLOGY			GENETICS		HISTOPATHOLOGY		PARASITOL	
Specimen	Name	Collection	On Ice (d)	Skin	Tissue	Biolog ID	Biolog SI	16S-1 ID	% ID	Gill	Viscera	Gill	Other
BFK-09-02	Bermuda angel	dead @ surf	3	1	ulcer	*V. harveyi*	0.616	*V. harveyi*	100	autolys	liver	0	
					ulcer	*V. harveyi*	0.614	*V. harveyi*	100				
					spleen								
					liver								
BFK-09-06	Cardinal fish	alive @ surf	4	0	heart	*P. damselae*	0.803	*P. damselae*	100	autolys	autolys	0	
					spleen								
					liver								
					kidney								
					brain								
BFK-09-14	French grunt	alive @ surf	1	0	spleen					severe	liver/pancr	?	
					liver								
					hd kidney								
					trk kidney								
BFK-09-15	Dusky squirrel	dead @ surf	5	1	ulcer					autolys	autolys	1	dig/hrt
					spleen								
					liver								
					kidney								
BFK-09-17	Blue tang	alive @ surf	1	1	gonad	*V. harveyi*	0.52	*V. harveyi*	99.8	severe	liver	?	
					ulcer	*V. harveyi*	0.26	*V. harveyi*	100				
					liver								
					spleen	*V. harveyi*	0.235	*V. harveyi*	100				
					spleen	*V. harveyi*	0.612	*V. harveyi*	100				
					heart								
BFK-09-19	Dusky squirrel	alive @ surf	1	1	ulcer	*V. harveyi*	0.635	*V. harveyi*	100	severe	liver/stom	0	
					spleen								
					liver								
					kidney								
BFK-09-22	Beaugregory	speared/dis	<1	1	ulcer	*V. harveyi*	0.645	*V. harveyi*	100	severe	liver	1	
					ulcer	*V. harveyi*	0.607	*V. harveyi*	100				
					spleen								
					kidney								
					liver								

Table 4. A summary of all gross, bacteriological, genetic, histologic and parasitological data obtained from 26 Bermuda fishes.

GENERAL INFO		GROSS OBSERVATIONS			BACTERIOLOGY			GENETICS		HISTOPATHOLOGY		PARASITOL	
Specimen	Name	Collection	On Ice (d)	Skin	tissue	Biolog ID	Biolog SI	16S-1 ID	% ID	Gill	Viscera	Gill	Other
BFK-09-29	Seargent major	speared/dis	<1	1 mild	ulcer					severe	pancr	?	
					spleen								
BFK-09-40	Bermuda angel	dead @ surf	5	1	ulcer					autolys	autolys	0	
					spleen								
					kidney								
BFK-09-42	Yellow damsel	died @ lab	1	1	ulcer	*V. harveyi*	0.236	*V. harveyi*	100	severe	liver/intest	?	
					spleen	*P. damselae*	0.151	*Enterovibrio coralii*	99.2				
					spleen	*V. harveyi*	0.357	*V. harveyi*	100				
					liver	*A. hydroph*	0.492	*V. harveyi*	99.1				
					liver	*V. harveyi*	0.373	*V. harveyi*	99				
					kidney	*V. harveyi*	0.616	*V. harveyi*	100				
					kidney	*V. harveyi*	0.644	*N/D*					
BFK-09-43	Yellow damsel	morib @ lab	1	1	ulcer	*V. harveyi*	0.63 (B)	*V. harveyi*	99.9	severe	liver/intest	1	nem/nad
					spleen	*V. harveyi*	0.259	*V. harveyi*	99.9				
					kidney	*V. harveyi*	0.331	*V. harveyi*	100				
					liver								
BFK-09-44	Squirrelfish	alive @ surf	<1	1	ulcer	*V. fluviatilis*	0.173	*V. coralliilyticus*	99.6	mild	liver/stom	1	nem/mes
					ulcer	*V. harveyi*	0.191	*V. sp. ME1-02*	98.7				
					ulcer	*V. cholerae*	0.184	*V. sinaloensis*	99.6				
					spleen	*V. splendid*	0.508 (B)	*V. ponticus*	100				
					spleen	*V. harveyi*	0.56	*N/D*					
					kidney								
					liver								
BFK-09-45	Squirrelfish	speared/health	<1	0	spleen					normal	normal	0	
					kidney								
BFK-09-50	Seargent major	speared/dis	<1	1	ulcer					severe	pancr/brain	0	dig/hrt
					spleen								
					liver	*V. harveyi*	0.685	*V. harveyi*	100				
					kidney								
BFK-09-52	Queen Parrot	speared/health	1	0	kidney					mod	normal	0	
BFK-09-55	Seargent major	speared/dis	1	1	spleen	*V. harveyi*	0.198	*V. communis*	100	severe	pancr	1	
					kidney								

Table 4 contd.

GENERAL INFO		GROSS OBSERVATIONS			BACTERIOLOGY			GENETICS		HISTOPATHOLOGY		PARASITOL	
Specimen	Name	Collection	On Ice (d)	Skin	tissue	Biolog ID	Biolog SI	16S-1 ID	% ID	Gill	Viscera	Gill	Other
BFK-09-56	Queen Parrot	speared	1	1	ulcer spleen liver	*V. harveyi*	0.68	*V. harveyi*	100	mod	pancr	0	
BFK-09-59	Seargent major	speared	1	1, mild	spleen liver ulcer kidney					severe	liver/pancr	0	
BFK-09-62	Seargent major	speared	1	1, mild	ulcer kidney liver					severe	liver/pancr	1	dig/intes
BFK-09-71	Seargent major	speared	<1	1	ulcer ulcer ulcer spleen liver	*L. pelagia* *V. harveyi* *V. harveyi*	0.213 0.747 (B) 0.265	*V. sinaloensis* *V. harveyi* *V. rotiferianus*	99.6 100 99.8	severe	liver/pancr	1	
BFK09-76	Seargent major	speared	<1	1, mild	ulcer Spleen kidney					severe	no liver	1	
BFK09-80	Seargent major	Speared	<1	1, mild	ulcer liver kidney					severe	normal	1	
BFK09-102	Seargent major	speared	1	1	ulcer spleen kidney					mod	gut/pancr	1	
BFK09-111	Seargent major	speared	1	1	ulcer spleen kidney					severe	gut/pancr	1	
BFK09-113	Seargent major	speared	1	1	ulcer spleen kidney					severe	liver/pancr	0	
BFK09-114	Seargent major	speared	1	1, mild	spleen kidney					severe	pancr	0	

Table 4 contd.

Table 4 summarizes all of the data obtained from our gross pathological observations, phenotypic bacteriological characterization, 16S rRNA sequence-based bacteriological identifications, histopathological analysis and parasitological observations. Of the 26 fishes examined, 4 had been held on ice longer than 24 hours prior to necropsy (BFK09-02, 3 days; BFK09-06, 4 days; BFK09-15 and BFK09-40 both 5 days). Three of these fishes were dead at the time of collection (BFK09-02, BFK09-15, BFK09-40). Histologically, their tissues exhibited extensive autolysis (post-mortem tissue degeneration) and they are therefore omitted from the histopathological evaluations. Bacteria (*V. harveyi, P. damselae*) were isolated from only one of these specimens (BFK09-02).

The other 22 specimens were processed within 24-36 hours of their return to the laboratory and thus were considered to be of sufficient quality for histopathological evaluation. An almost ubiquitous histologic observation in these fishes was the occurrence of gill pathology. Seventeen fishes (77%) exhibited moderate to severe pathology including extensive gill necrosis, hemorrhage and sloughing of the respiratory and osmoregulatory epithelia. This was often associated with extensive inflammation and colonization of the sloughing necrotic tissues by bacteria. Additionally in some fishes there was widespread gill hyperplasia resulting in lamellar fusion. In others there was extensive chloride cell hyperplasia and intense inflammation accompanied by bacterial colonization of compromised gill filament tissues. Eleven of these 22 fishes (50%) harbored a highly pathogenic ciliate parasite of the gills tentatively identified as *Brooklynella* sp. An additional 3 specimens (total prevalence of 64%) likely also had the infection although the histologic evidence for definitive identification of the parasite was not clear. In many of the fishes exhibiting gill pathology, recognizable ciliates were not observed; instead large granular nucleated bodies were often present that may have been degenerating ciliates. Living ciliate parasites were observed in squash preparations of gill tissues only in fish that were freshly dead (on ice <4-5 hrs) (**Figure 1**). Histologically, the ciliates often remained recognizable in gill tissues of fishes kept on ice for longer time periods (up to 24 hrs and longer in some cases).
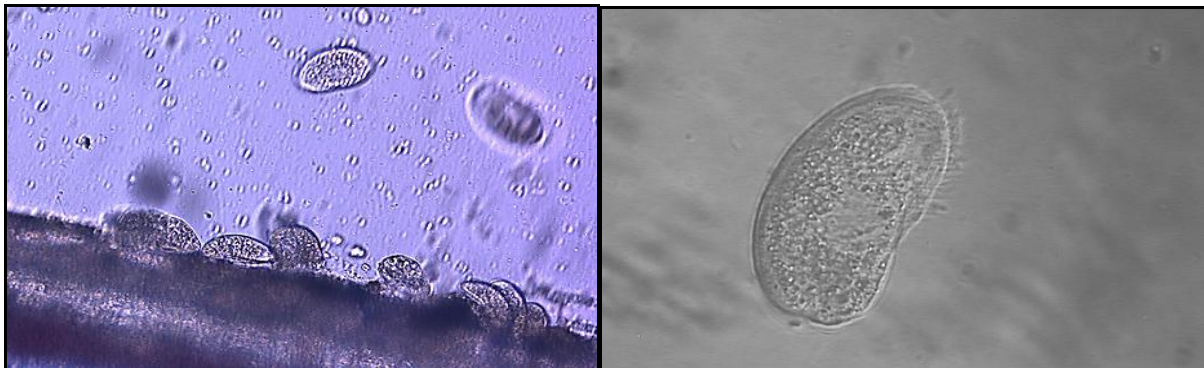

Figure 1. *Brooklynella* **sp. in a wet mount of the gills of the beaugregory,** *Stegastes leukostictus.*

However, we believe that ciliates rapidly degrade once fish are placed on ice and that we likely are under-estimating both the prevalence and severity of this infection. Two of the fishes exhibited very high infection levels, with numerous ciliates observed in the gill tissues. **Figure 2** illustrates the extensive gill damage associated with the histophagous parasites in a heavy infection. Thus the actual prevalence and perhaps also the high severity of this ciliate infection

may not be accurately reflected by our data. **Figure 3** illustrates portions of two healthy fish gill filaments with their structurally intact secondary gill lamellae for comparison. The thin flattened single cell layer covering the surfaces of healthy gill lamellae represents the delicate respiratory epithelium, whereas the cells located at the base of, and between two lamellae where they attach to the gill filament, constitute the osmoregulatory epithelium. Based on the high prevalence of this parasite infection (50-63%: based on histology) and the extensive associated damage to the gills, this organism, along with the commonly occurring *Vibrio harveyi* infection, warrants further study as one of two infectious agents that may be playing an important role in the observed fish mortalities.
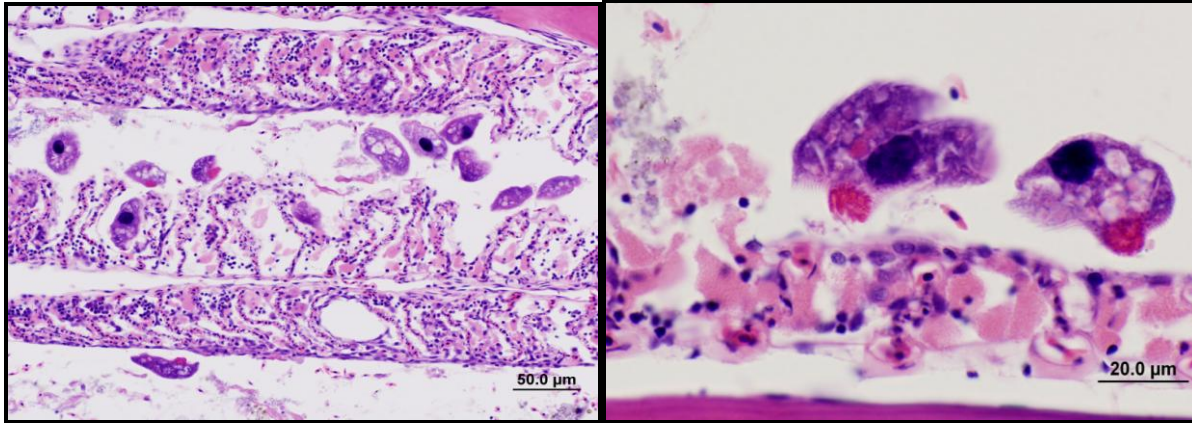
Figure 2. Gill histology of fish BFK09-43 (*Microspathodon chrysurus*) showing heavy infection of *Brooklynella* sp. and associated severe gill pathology. Left image: low magnification showing numerous ciliate parasites (basophilic bodies with dense, almost black staining of the nuclei between two gill filaments) and extensive damage to secondary gill lamellae. Right image: Closeup of three feeding ciliates with associated disruption of secondary lamellae, inflammation and chloride cell hyperplasia (numerous large eosinophilic cells).
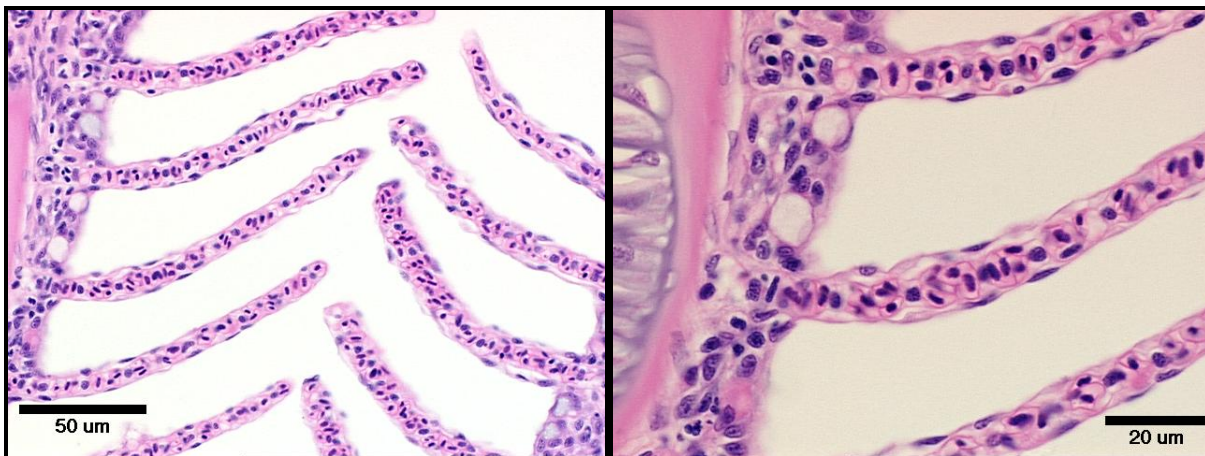

Figure 3. Histology of normal fish gill structure. Left: portions of two adjacent primary gill filaments and their secondary lamellae. Right: Closeup of portions of three secondary gill lamellae showing the osmoregulatory epithelium (at base of 2° lamellae), respiratory surfaces, pilar cells, and erythrocytes.

Significant pathology in the tissues and organs of the alimentary tract, including liver, exocrine pancreas, stomach and intestine was common in many of the fish. These observations are tabulated in T**ables 5-8** of the attached **Appendix**. Histologically, most of the fishes (19/22 = 86%) exhibited liver pathology including some combination of mild to severe hepatocellular ceroidosis (accumulation of a pathological pigment called ceroid within the cytoplasm of hepatocytes, and a marker for sublethal cell damage), elevated macrophage aggregates (aggregations of macrophages, an avidly phagocytic host defensive cell, replete with ceroid pigments), fatty change (accumulation of lipid droplets within hepatocytes), widespread, diffuse single cell necrosis and inflammation. In many fish exhibiting these clinical signs there was evidence of bacterial infection of the tissues associated with focal necrosis. This was true especially in liver and in the alimentary tract (stomach and intestines). In the 12 seargent major (*Abudefduf saxatilis*) obtained by targeted spear fishing, histologic analysis additionally demonstrated diffuse pancreatitis with widespread acinar cell degranulation and necrosis. In some of these fish there was evidence of possible bacterial infection of the exocrine pancreatic tissues. Pancreatitis was observed in 10 of the 12 (83%) seargent majors, whereas two fish (17%) appeared histologically normal (**Table 4**). Two of these fish also exhibited V. *harveyi* infection and one additional fish exhibited a V. *communis* infection (total bacterial infection prevalence = 25%). Only one other species, the French grunt (*Haemulon flavolineatum*: BFK09-14), exhibited similar pancreatic pathology. This fish did not exhibit bacterial infection but showed severe gill pathology, probably associated with *Brooklynella* sp. infection. Other tissues and organs exhibited little or no significant pathology (**Table 4**). Several metazoan parasitic infections were observed (**Table 4**) including digenetic trematode metacercariae in the heart of the dusky squirrelfish (BFK09-15) and the seargent major (BFK09-50), larval nematode infections in the gonad of a yellow damselfish (BFK09-43) and in the mesenteries of a squirrelfish (BFK09-44) and digenetic trematodes in the intestine of a seargent major (BFK09-62). None of these mild infections caused significant pathology and are not considered to play a role in the 2009 Bermuda reef fish die-off.

Ongoing work: Additional work is required to clarify the underlying causes of the pathology observed in the alimentary tract and associated organs (e.g., liver, pancreas). Special stains (e.g., Gram stain) are currently being performed to determine if bacterial infections are associated with any of these pathologies. The pancreatitis observed in a high number of seargent majors additionally requires more histopathology (e.g., special stains) in order to determine the underlying causes. It is interesting to note that very few seargent major exhibited infection by *V. harveyi* and it is possible that some other disease process is active in this species. This additional work will be required for preparation and submission of a manuscript detailing these findings.

16S rRNA sequencing appears in this case to identify most isolates involved in the fish kill event as *Vibrio harveyi*. Several species of *Vibrio*, (e.g. *V. rotiferianus*, *V. campbelii*) however, are very closely related to *V. harveyi,* and multilocus sequencing (i.e. generating sequence data at

more than one genetic location) is necessary to definitively speciate them (Thompson et al., 2005; Thompson et al., 2007). The Gauthier laboratory is currently performing sequencing at *pyr*A, *rpo*A, and *rec*A loci in order to definitively identify isolates as *V. harveyi*, as related species, or as novel isolates. Isolates identified as other than *V. harveyi* will also be sequenced at muliple loci.

The presence or absence of virulence factors within individual *Vibrio* spp. has received much attention in the literature, and numerous groups have classified strains of *Vibrio* by the presence of virulence genes and/or *in vitro* and *in vivo* virulence assays such as blood agar hemolysis or inoculation, respectively.   Little work has been done in *Vibrio harveyi* in this regard, although recent work has begun to explore virulence in this species (Rattanama et al., 2009). We intend to test isolates identified as *V. harveyi* in this study for the presence of published virulence factors, including two hemolysin genes (*vhh* and *hhl*), and calcium response component of Type III secretion system (*vcr*D). This will allow comparisons with published *V. harveyi* isolates, and will likely form the basis for future work exploring virulence factors with homologs in other *Vibrio* spp.

Ultimately, it is our goal to produce a baseline characterization of *V. harveyi* isolates from the 2009 Bermuda fish kill event so that isolates from future events may be compared, and potentially, virulence factors associated with outbreak isolates may be identified.

Discussion and Conclusions

We have tentatively identified two specific infectious disease agents that are likely to have played a causative role in the summer 2009 reef fish die-off in the near-shore waters of Bermuda. These are 1) the Gram$^-$ bacterial finfish and shellfish pathogen, *Vibrio harveyi* and 2) a highly pathogenic gill ciliate tentatively identified as *Brooklynella* sp. Eleven of the 26 fish specimens (42%) evaluated in this study yielded bacterial growth on tissue/organ culture. Ten of these 11 positive fish (91%) were infected with *Vibrio harveyi;* in some cases several tissues and organs within an individual fish were positive for bacterial infection. Seventeen of 22 fishes (77%) analyzed histologically exhibited moderate to severe gill pathology and 11 (50%) of them showed histologic evidence of infection by *Brooklynella* sp. Based on its ubiquity among the fishes examined in this study (as identified using the Biolog Gene III System), *Vibrio harveyi* is of interest for further study in the context of last year's fish kill event. Similarly, based on the high prevalence of the gill ciliate *Brooklynella* sp. and the severe gill pathology accompanying this infection, this protozoan parasite is also of further interest. We hypothesize that both of these infections played a significant role in the 2009 Bermuda reef fish die-off.

The family Vibrionaceae is comprised largely of Gram$^-$ heterotrophic bacteria that are ubiquitous in aquatic environments (Colwell 1984). Members of this group are opportunist pathogens and include some that infect humans (Blake et al., 1980) and others that are primary or secondary pathogens of finfish, shellfish and other aquatic organisms (e.g., Zhang and Austin, 2000;

Alcaide 2003; Ruangpan et al., 1999). *Vibrio harveyi* is a natural inhabitant of seawater and most commonly isolated in tropical marine ecosystems. It is a common pathogen of marine invertebrates and vertebrates, including lobster, shrimp, seabass, seahorses, amberjack and sharks (Oakey et al. 2003), however it is not pathogenic to humans. Luminous vibriosis caused by *V. harveyi* is an important cause of mortality in commercial shrimp farming and has been documented in many other crustaceans, all of which glow in the dark when infected (Diggles 2000). Phenotypic discrimination between *V. harveyi* and other fish pathogens including *V. splendidus* has been reported, but not all of the distinguishing tests are used within the Biolog assay. Alternatively, PCR has been used to identify *V. harveyi* (Oakey et al. 2003), although cross reactivity of primers with *V. splendidus* as well as certain other vibrios was reported.

16S rRNA sequencing of bacterial isolates from the 2009 Bermuda reef fish die-off effectively identifies most of the isolates obtained in this study as *V. harveyi* and largely supports, and in some cases clarifies our phenotypic characterizations using the Biolog system. Several species of *Vibrio*, (e.g. *V. rotiferianus*, *V. campbelii*) however, are very closely related to *V. harveyi,* and multilocus sequencing (i.e. generating sequence data at more than one genetic location) is necessary to definitively speciate them (Thompson et al., 2005; Thompson et al., 2007). Some of this work is being continued in the Gauthier laboratory, because we have identified several closely related species in our samples.

Brooklynella hostilis is a ciliate protozoan parasite that infects the gills and, to some minor degree, the skin of many different marine reef fishes. It is a common problem in marine aquarium fish culture and can result in severe tissue damage including destruction of the respiratory and osmoregulatory gill epithelia, hemorrhage, intense inflammation, gill hyperplasia and lamellar fusion, all of which in combination can lead rapidly to hydromineral imbalance, osmoregulatory compromise and death (Lom and Nigrelli, 1970). The prevalence of this ciliate infection was not fully appreciated in our initial examination of the fish using fresh squash preparations of gill tissues because the parasites apparently degrade rapidly once the host fish has died and is placed on ice. The histologic analysis however provided evidence of severe gill pathology in the majority of the fish and evidence of the ciliate infection in 50% of them. Therefore this infection is hypothesized to have played an important role in the reef fish die-off last summer. It is likely that *Brooklynella* sp. is endemic to Bermuda reef fishes and probably always present on a proportion of individuals in very low numbers. Lom and Nigrelli (1970) suggest that the parasite may become pathogenic when the host-parasite balance is altered by changing environmental conditions. Environmental stressors (e.g., reduced water quality, elevated temperature, etc) may elicit the integrated stress response in fishes (Bonga 1997). This could lower their resistance to infection and favor sudden explosive replication and a more aggressive histophagous feeding response in the ciliate, resulting in extensive damage to the gills. This is frequently seen in salt water aquaria and can result in rapid mortality of the fish (Lom and Nigrelli, 1970). It is also very likely that the severe gill damage associated with *Brooklynella hostilis* infections provides a portal of entry for secondary infections, including

those by opportunistic vibrios such as *V. harveyi*. Although gill tissues were not cultured for bacteria in this study, histopathology indicates that compromised gills of fishes infected with *Brooklynella* sp. were in many instances colonized by unidentified microbial organisms, including bacteria.

At the time of the fish kill, speculation about the underlying causes of the die-off included possible infection, harmful algal blooms, or stress associated with unusually high water temperatures and attending lowered oxygen carrying capacity of the water, increased nutrient levels or other contaminants due to runoff, or perhaps the unusually large quantities of coral spawn seen on the reefs that summer. It remains tempting and appropriate to speculate that environmental factors played a critical role in this coral reef fish die-off. Temperature seems especially worthy of consideration in this context. Extended periods of elevated temperature and attendant reduced oxygen carrying capacity of the water may have negative effects on physiology, immune function, growth and reproductive output of fishes modulated through the integrated stress response (Bonga, 1997). Elevated temperature may additionally augment proliferative capacity, infectivity and virulence/pathogenicity of heterotrophic microbial organisms. It is very likely that temperature, elevated by only several degrees C for extended time periods, is stressful to tropical reef fishes. A very common histologic observation in these fish was extensive bacterial colonization of the damaged gill tissues. A possible scenario is that elevated summer temperatures stimulated proliferation of normally low-intensity gill ciliate infections in the reef fishes. Resulting damage to the gills provided a portal of entry for opportunist bacterial pathogens, in this case *V. hrveyi (*and others), with fish succumbing to combined respiratory compromise and secondary bacterial septicemias. Alternatively, different reef fish species may be succumbing to different infectious agents to which they are particularly susceptible (*Brooklynella* sp. vs *V. harveyi*) in association with a suite of common but undetermined underlying environmental stressors. Finally, the observation of consistent acute pancreatitis in the seargent major may be evidence of another unrelated disease process in this species modulated by environmental stress. These scenarios are hypothetical and it is not known to what extent if any, environmental perturbations influenced the course and outcome of *V. harveyi* and *Brooklynella* sp. infections in Bermuda reef fishes. The interactions between fish populations, pathogens and the environment are complex and the impacts of infectious disease on population dynamics and stock structure are only recently being investigated (Gauthier et al., 2008). Much additional work needs to be done to determine if temperature or other environmental stressors played a role in the 2009 Bermuda reef fish die-off.

During the period when fish were dying and washing on shore, there was concern about potential human health impacts from contact with, or consumption of, tainted reef fishes and even unaffected pelagic species. Our results suggest that concerns for human health are largely unfounded, given that neither *V. harve*yi nor *Brooklynella* sp. has been reported to infect humans. Both are known to infect and cause disease only in marine organisms and have no demonstrated zoonotic potential. It is recommended however that marine species exhibiting

external signs of disease or that are dead or dying should not be handled by people and should also not be consumed.

Recommendations for Future Research: The summer of 2009 in Bermuda was one characterized by atypically warm and still waters. This in part has led to speculation that anthropogenic impacts, in particular climate change, may be the proximal underlying cause of this, and prior Bermuda reef fish health events. Thus it is very likely that reef fish die-offs and the associated public and fisheries concerns arising thereof, will recur in Bermuda. It is critical that research on Bermuda reef fish health issues be continued. With the projected future rise in sea surface temperatures, it is very important that we obtain baseline information on Bermuda reef fish health now. Only in this way can ever-changing environmental stressors be related to specific wild finfish health issues, and more generally to overall coral reef health and ecology. The immediate cause(s) of the 2009 reef fish die-off in Bermuda are likely tied to two infectious agents, the pathogenic ciliate parasite, *Brooklynella* sp., and the heterotrophic marine bacterium, *Vibrio harveyi*. As both are ubiquitous in tropical marine reef systems and rarely cause disease outbreaks in the wild, we hypothesize that currently unknown underlying environmental variables are modulating disease expression in Bermuda reef fishes. We recommend that the Bermudian government consider implementing the following, all of which we would be happy to participate in.

1. Initiate a limited, cost-effective fish health monitoring program on a few selected coral reef communities around Bermuda. This might focus on the summer months only or may be active during all seasons. Such a program might be as simple as enlisting the recreational diving and fishing communities through Island dive and bait shops in order to document visual changes in fish health and to report observations to some central well-advertised telephone hotline. We currently have a similar setup for a striped bass disease investigation that is working very well. It is likely that reliance on sporadic land- or water surface-based observations of deaf and dying fishes will significantly underestimate and delay reporting of any fish disease or mortality events.

2. Initiation of routine fish tissue collections and physicochemical environmental parameter measurements on select Bermuda reefs several times a year. These efforts might be cost-effective if incorporated into existing environmental monitoring programs currently being undertaken through BIOS or other Bermudian government entities. Many of these sorts of data may already be routinely collected.

3. Fund a Bermudian citizen to pursue advanced training (Masters or Ph D degree) in aquatic animal health issues so that this specific scientific expertise can be brought to Bermuda directly and reliance on outside experts is minimized in future.

4. Support future investigations should another die-off occur. We plan to continue our initial investigation and to pursue additional work on the pathology, infectivity, molecular

identification of virulence genes in *V. harveyi* and the role of environment in modulating disease expression (e.g., laboratory disease challenge studies). We strongly believe that *V. harveyi* will be a viable model to investigate the ecology of infectious disease in tropical marine environments and plan to continue some level of effort in the absence of dedicated funding. Along this line, we realize that the Bermudian government does not command the resources to fund these extensive multi-disciplinary studies. We therefore propose seeking additional funding from other sources. One that immediately comes to mind is the British Overseas Territories Environmental Program (OTEP). We would like to pursue a joint application that includes scientists within the Bermuda government (e. g., Dr. Fred W. Ming, Bermuda of Environmental Protection), at BIOS (Dr. Tim Noyes), VIMS (Drs Vogelbein and Kator) and Old Dominion University (Dr. David Gauthier), and perhaps other individuals with expertise in the required disciplines.

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