Vol. 109: 257–261, 2014
doi: 10.3354/dao02736

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 3

NOTE

Aspergillosis in the common sea fan *Gorgonia ventalina*: isolation of waterborne hyphae and spores

Victoria J. Troeger[1], Paul W. Sammarco[1,2,*], John H. Caruso[1]

[1]Department of Ecology and Evolutionary Biology, Tulane University, New Orleans, Louisiana 70118, USA
[2]Louisiana Universities Marine Consortium (LUMCON), 8124 Highway 56, Chauvin, Louisiana 70344, USA

ABSTRACT: The octocoral disease aspergillosis is caused by the terrestrial fungus *Aspergillus sydowii.* The possibility of secondary (horizontal) transmission of aspergillosis among common sea fans *Gorgonia ventalina* would require waterborne transmission of hyphae and/or spores. A laboratory filtration experiment confirmed that fungal hyphae and spores were shed into the water by infected fans. This suggests that secondary infection might be possible in this species. It remains to be determined whether healthy fans actually develop aspergillosis after contact with hyphae-laden water.

KEY WORDS: Aspergillosis · Sea fan · *Gorgonia ventalina* · Gorgonian · Secondary transmission · Hyphae · Spores · *Aspergillus sydowii* · Octocoral · Disease

INTRODUCTION

Within a few years of its description, the causative agent of aspergillosis in gorgonians (Fig. 1) was identified as *Aspergillus sydowii*, a common terrestrial fungus (Smith et al. 1996, Geiser et al. 1998). *A. sydowii* is commonly found associated with the Caribbean sea fan *Gorgonia ventalina* (Cnidaria, Gorgonacea). However, the mechanism by which this fungus enters and spreads in the marine environment is not fully understood. *A. sydowii* cannot reproduce in seawater (Smith et al. 1996); yet despite this, it has spread throughout the known range of this common sea fan (Smith 1998, Porter et al. 2001, Weil et al. 2002, Kim & Harvell 2004, Smith & Weil 2004, Weil & Rogers 2011). It has been hypothesized that the marine environment is experiencing a constant input of the fungus through river discharge, associated sedimentation, and windborne dust (Shinn et al. 2000, Jolles et al. 2002, Garrison et al. 2006).

An analysis of spatial distribution of this disease in a sea fan population in the Florida Keys (USA) has suggested that the disease may be spread through secondary transmission and infection, i.e. from sea fan to another sea fan (Jolles et al. 2002). It is not yet known whether the hyphae are shed into the water. Infected fans experience tissue necrosis, and infected tissue contains high concentrations of hyphae (Smith et al. 1996). These hyphae, if shed into the water, could contribute to the spread of the disease, but hyphal export has not been documented. In addition, *A. sydowii* apparently does not produce reproductive spores in seawater (Smith et al. 1996). If spores were released, they could likewise potentially contact neighboring sea fans and cause infection. Laboratory experiments have demonstrated that contact between injured sea fans and hyphae-infused gauze,

*Corresponding author: psammarco@lumcon.edu

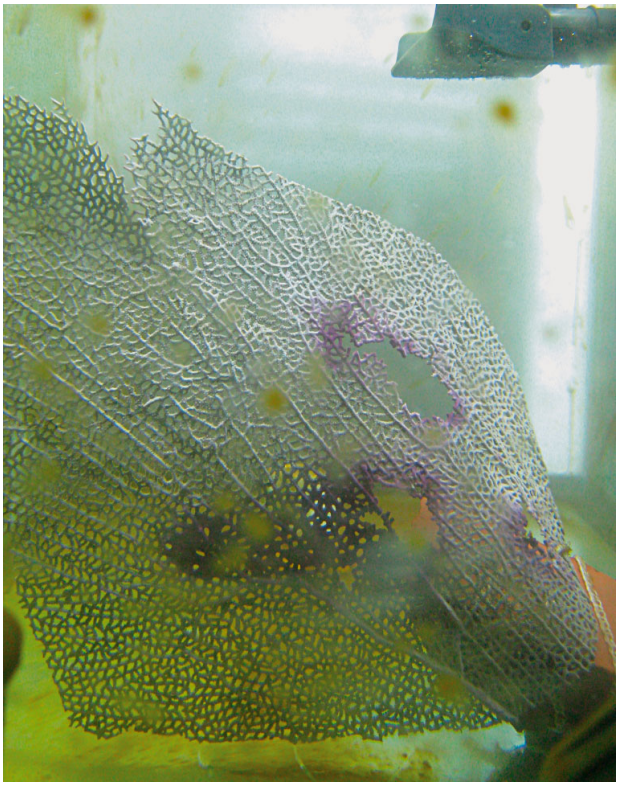

Fig. 1. Sea fan *Gorgonia ventalina* infected with aspergillosis. Obvious indications of disease are lesions and purpling of tissue. Infected fans were used as the source of hyphae and spores for the experiment

as well as direct tissue contact, leads to the development of aspergillosis (Smith & Weil 2004). This successful infection did not require the presence of reproductive spores (Smith et al. 1996).

Here we attempted to determine experimentally whether infected fans shed (1) live hyphae and/or (2) spores into seawater.

MATERIALS AND METHODS

Colonies of *Gorgonia ventalina* were collected, of which some were healthy and others showed obvious signs of aspergillosis ('purpling' of tissue, galling, development of lesions; Fig. 1). These were obtained from the Florida Keys Reef Tract with the assistance of the Tropical Research Center of Mote Marine Laboratory, Summerland Key, Florida. Upon arrival at Tulane University, infected and healthy sea fans were kept individually isolated from each other and placed into several 113 l tanks in an aquarium room.

The original design of this experiment was a 1-way replicated ANOVA design, using 8 to 9 sea fans. Three tanks were used. One held a diseased sea fan with obvious signs of aspergillosis. In addition, 1 tank held a healthy fan receiving seawater from the diseased fan. The last tank was a control, holding a fan immersed in re-circulating seawater, with no introduction of water from diseased fans. In the laboratory, sea fan holdfasts were fastened to terracotta bricks with twine to anchor them to the bottom of the tanks. The experiment was to be replicated in time in 3 trials.

The aquarium setup used for this experiment followed that of Dunn et al. (2012). In the aquaria, *G. ventalina* were provided with medium to strong water flow. Water flow was created using a combination of power filters (Marine Emperor 280 BIO-Wheel powerfilter) and powerheads (Marineland Maxi-Jet 400 Pro Powerhead) attached by suction cups. Powerheads ran continuously, creating constant flow and a water cycling rate of 25 times h^{-1} $aquarium^{-1}$. A combination of heaters (Eheim 150 W) and fans was used to reduce excessive heat from the lights and maintain water temperature. Since these sea fans are autotrophic and require photosynthetically active radiation, actinic lighting was provided, using 2 broad-spectrum (broad wavelength) high-intensity 10 000 K bulbs (Ushio®) derived from a 400 W metal halide ballast (PFO®). Timers were used to create a 12:12 h light:dark photoperiod. Heaters (Eheim 1050 W) were used to maintain temperatures at 25°C. Tanks were covered with glass to reduce evaporation and introduction of airborne contaminants. An ultraviolet (UV) sterilizer (Green Killing Machine® UV Sterilizer w. Powerhead) controlled potential contamination from bacteria, algae, and other biotic material and was used for 24 h prior to introducing sea fans into the tanks.

Ammonia, nitrate, nitrite, pH (8.2–8.4), temperature, and salinity (34–35 ppt) were measured and recorded regularly throughout the experiment. Ammonia, nitrate, and nitrite levels were measured using Salifert® test kits. Artificial saltwater (Instant Ocean®) was prepared in 189 l batches with water from a reverse-osmosis deionization unit, and salinity was maintained between 34 and 35 ppt, as measured by a handheld refractometer.

Tanks were prepared for the introduction of specimens without the aid of live rock or sand in order to prevent potential fungal contamination. Instead, frozen food-grade shrimp were added to the tank to promote the growth of *Nitrobacter* and *Nitrosomonas.* Once ammonia, nitrites, and nitrates reached negligible levels, the tanks were considered safe for the introduction of corals.

For the experiments, we used diseased sea fans that showed obvious signs of aspergillosis, including purpling (polyps had necrotized and the purple gorgonin could be seen), galling, and development of lesions (Fig. 1). Once collected, specimens were shipped overnight from Florida to the laboratory in Louisiana. Each fan was placed in an individual bubble-wrap bag filled with oxygenated sea water, and was then sealed in a Ziplock® bag to prevent cross-contamination during the shipping process (E. Bartels pers. comm.).

The first experimental tank contained a sea fan displaying obvious signs of aspergillosis (i.e. purpling of tissues, galls, and tissue necrosis). The tank was fitted with a 2 mm Millepore® filter (Fig. 2). Water pumped from the bottom of the diseased sea fan tank was passed through the filter and re-circulated back into the source tank. Initially, this filter was replaced every 3 d. During the second trial, however, the frequency of filter change was increased, and the filter was replaced daily. After removal, the filter was stained with hematoxylin and subsequently acridine orange, using the fluorescent method described by Pickett et al. (1960). The stained filter was then checked for the appearance of hyphae, under an epifluorescent microscope at 100× or 200× magnification. This staining method causes fungi to fluoresce brightly against a dark background. Fungi may be identified to genus using this technique. *Aspergillus* fluoresces green when stained with acridine orange. The green is recognizable against a black background, since the sister stain, hematoxylin, stains all other living tissue in the sample black (Pickett et al. 1960). For comparative purposes, a piece of the healthy portion of the diseased source colony was stained. No hyphae were found on the healthy portions of the sea fan colonies.

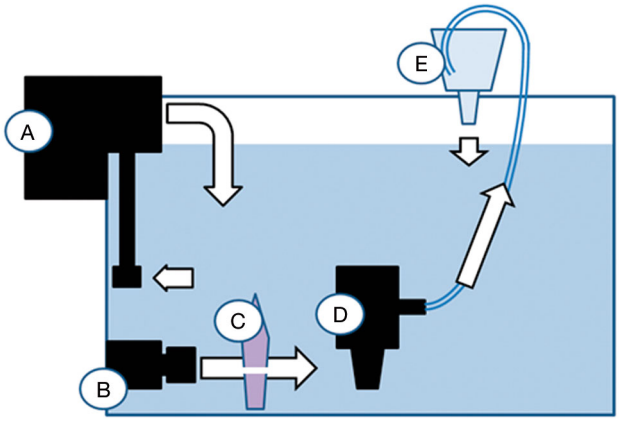

Fig. 2. Schematic of experimental aquarium holding infected sea fans *Gorgonia ventalina*: (A) biofilter containing nitrifying bacterial populations; (B) powerhead pumping water across the surface of the sea fan; (C) sea fan infected by aspergillosis; (D) modified powerhead for pumping water above tank; (E) funnel containing a 2 µm pore size Millipore® filter receiving pumped water, filtering it, and allowing it to return to the tank

The second experimental tank was constructed to determine whether healthy fans would develop signs of aspergillosis when in contact with water containing hyphae. A healthy fan was placed in a tank that had 50 % of its volume replaced each day with water from a tank containing a diseased fan. Daily visual observations of the fan's condition were made, and photographic records were kept. A qualitative scale was used to describe the course of disease over 2 wk: 1 = healthy; 2 = initial signs of infection occurring; 3 = clear signs of infection; and 4 = mortality. In the case of lesions developing, areas of the lesions were recorded over time. MATLAB was used to analyze the photographic records to determine the amount of fan that succumbed to disease through time.

Corals were fed once a day if their polyps were extended, at which time filters were turned off for 30 min. Polyps were recorded daily as either 'not extended,' 'moderately extended,' or 'fully extended.' In general, sea fans feed constantly, and concomitantly have their polyps extended. Polyp retraction was used as measure of stress, as this species has the ability to fully retract its polyps (see Sammarco et al. 1987).

Trials lasted for a maximum of 2 wk. Tanks were cleaned before introducing new fans by emptying, scouring, and heavily rinsing them in fresh water and removing algae and other fouling organisms. Cleaning chemicals were not used. Heaters, pumps, and terracotta bricks were cleaned in a similar manner. The biowheel and power filter cartridges were rinsed repeatedly in clean salt water to preserve their beneficial *Nitrobacter* and *Nitrosomonas* bacterial colonies. Tanks were refilled with freshly made, clean salt water. The experimental tanks were sterilized for 12 to 24 h with UV light prior to the introduction of new sea fans.

Due to challenging technical problems, the final experimental design was different from the original. Some of the sea fans experienced stress during shipping and died soon after arriving at the laboratory. Signs of stress appeared within 24 h of their arrival. This reduced experimental replication; only 2 replications could be completed. For this reason, this study should not be considered quantitative in nature. It is only descriptive of general observations made during the investigation and should be considered a preliminary study. Nonetheless, the qualita-

tive information produced by the experiment was informative.

RESULTS

For all replicate samples of the first trial, *Aspergillus* hyphae (denoted by green fluorescence) were found on the filters (Fig. 3A). This occurred on all other sample dates. During the second trial, when sampling frequency increased, the same occurred. Hyphae were observed in all cases; all samples fluoresced a vivid green. Since the stains were vital ones, all hyphae were confirmed alive when sampled. In 1 case, a small item fluoresced red. According to Pickett et al. (1960), this could have been either *Coccidiodes* or *Rhinosporidum* (Fig. 3B). In 4 out of 9 cases, material that appeared to be fungal spores and conidia were also observed in the filter samples (Fig. 4).

DISCUSSION

This study suggests that waterborne transport of fungal material from infected sea fans may be possible. Heretofore, this has not been documented (Smith et al. 1996). It should be noted that there are potential limitations to interpretation of these results. For example, in nature, bacteria and fungi are found on, and likely contribute to, normal healthy physiological processes in corals (Gil-Agudelo et al. 2006). *Gorgonia ventalina* also possesses a variety of species of *Aspergillus,* which may be found on both diseased and healthy colonies (Toledo-Hernandez et al. 2008). In addition, not all strains of *A. sydowii* are pathogenic to sea fans (Smith & Weil 2004). Moreover, the staining method used here to detect fungi will only identify such to genus (Pickett et al. 1960). Despite these potential limitations, however, it may be assumed that *A. sydowii* was at least among those species whose hyphae were collected. This was due to the obvious expression of aspergillosis in the experimental specimens. In fact, Toledo-Hernandez et al. (2008) showed that *A. sydowii* can be present on the surface of sea fans exhibiting no signs of aspergillosis.

The confirmation of the presence of hyphae and spores in seawater derived from diseased fans opens the door to the possibility of secondary infection occurring in healthy sea fans, derived from infected colonies (see Smith & Weil 2004). It is likely that either the hyphae or spores could initiate aspergillosis in a sea fan susceptible to infection in nature. It is possible that spores could be produced asexually in *Aspergillus*, but this needs to be confirmed. In addition, what is equally important is that these repro-

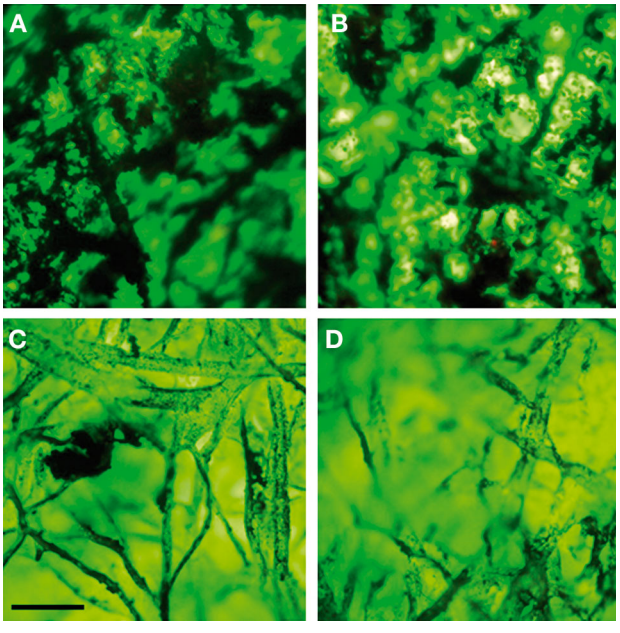

Fig. 3. Epifluorescent micrographs of material filtered from experimental water. (A,B) Examples of material isolated via filtration during the first trial after (A) 3 d and (B) 6 d. The green glowing materials are fungal hyphae. (C,D) Material isolated after 1 d of filtration during the second trial. Note clearer examples of fungal hyphae. *Aspergillus* is denoted by green fluorescence. Scale bar in (C) = 10 µm (applies to all panels)

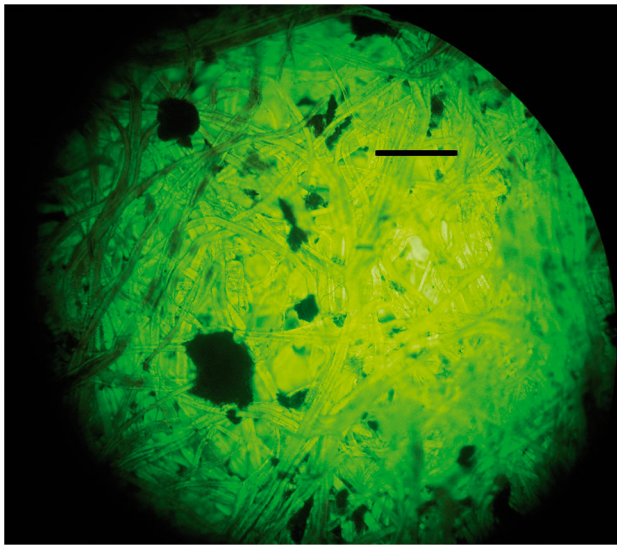

Fig. 4. Epifluorescent micrograph showing evidence of spores and spore-bearing structures derived from sea fans *Gorgonia ventalina* infected with aspergillosis and filtered from seawater in which they were immersed. Scale bar = 40 µm

ductive propagules are waterborne. The next question to be resolved is whether healthy colonies are indeed subject to infection by either these hyphae or spores once released and transmitted from the diseased colonies. Only further research will answer these questions.

Acknowledgements. We thank B. Rosenheim for comments on an early draft of the manuscript; G. LaFleur (Nicholls State University) for lending aquarium equipment; J. Dunn for advice and supplying aquarium equipment; G. Glotzbecker for assistance in setting up the aquarium system; M. Blum for lending laboratory space for conducting the experiments; B. Hall for lending use of his epifluorescent microscope; F. Chen and L. Ling for assistance in the use of that instrument; J. Leslie and D. Battistela for administrative support; E. Bartels and Mote Marine Laboratory for collecting and shipping the sea fans; members of various online coral reef fora for advice on aquarium techniques; D. Henry for general support; and A. Foy, A. Krantz, and M. Knowlton for assistance with transport. This study was supported by the Tulane University Center for Engaged Learning and Teaching (CELT) and Newcomb-Tulane College.

LITERATURE CITED

Dunn JG, Sammarco PW, LaFleur G Jr (2012) Effects of phosphate on growth and skeletal density in the scleractinian coral *Acropora muricata*: a controlled experimental approach. J Exp Mar Biol Ecol 411:34–44

Garrison VH, Foreman WT, Genualdi S, Griffin DW and others (2006) Saharan dust — a carrier of persistent organic pollutants, metals and microbes to the Caribbean? Rev Biol Trop 54(Suppl 3):9–21

Geiser DM, Taylor JW, Ritchie KB, Smith GW (1998) Cause of sea fan death in the West Indies. Nature 394:137–138

Gil-Agudelo DL, Myers C, Smith GW, Kim K (2006) Changes in the microbial communities associated with *Gorgonia ventalina* during aspergillosis infection. Dis Aquat Org 69:89–94

Jolles AE, Sullivan P, Alker AP, Harvell CD (2002) Disease transmission of aspergillosis in sea fans: inferring process from spatial pattern. Ecology 83:2373–2378

Kim K, Harvell CD (2004) The rise and fall of a six-year coral-fungal epizootic. Am Nat 164(Suppl 5):S52–S63

Pickett JP, Bishop CM, Chick EW, Baker RD (1960) A simple fluorescent stain for fungi. Am J Clin Pathol 34:197–202

Porter JW, Dustan P, Jaap WC, Patterson KL and others (2001) Patterns of spread of coral disease in the Florida Keys. Hydrobiologia 460:1–24

Sammarco PW, La Barre SC, Coll JC (1987) Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef. III. The relationship between toxicity and morphology. Oecologia 74:93–101

Shinn EA, Smith GW, Prospero JM, Betzer P, Hayes ML, Garrison V, Barber RT (2000) African dust and the demise of Caribbean coral reefs. Geophys Res Lett 27: 3029–3032

Smith GW (1998) The environment decade Q&A. Sci Am Frontiers Archive, Oct. 1998. Recorded Interview. Available at www.pbs.org/safarchive/3_ask/archive/qna/3291_gwsmith.html (accessed March 2013)

Smith GW, Weil E (2004) Aspergillosis of gorgonians. In: Rosenberg E, Loya Y (eds) Coral health and disease. Springer, New York, NY, p 279–288

Smith GW, Ives LD, Nagelkerken IA, Ritchie KB (1996) Caribbean sea-fan mortalities. Nature 383:487

Toledo-Hernandez C, Zuluaga-Montero A, Bones-Gonzales A, Rodriguez JA, Sabat AM, Bayman P (2008) Fungi in healthy and diseased fans (*Gorgonia ventalina*): Is *Aspergillus sydowii* always the pathogen? Coral Reefs 27:707–714

Weil E, Rogers CS (2011) Coral-reef diseases in the Atlantic-Caribbean. In: Dubinsky Z, Stambler N (eds) Coral reefs: an ecosystem in transition. Springer, New York, NY, p 465–491

Weil E, Urreiztieta I, Darzon-Ferreira J (2002) Geographic variability in the incidence of coral and octocoral diseases in the wider Caribbean. Proc 9th Int Coral Reef Symp, Bali, Indonesia, 2:1231–1238

Editorial responsibility: Garriet Smith, Aiken, South Carolina, USA

Submitted: May 15, 2013; Accepted: February 20, 2014
Proofs received from author(s): May 19, 2014