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Lessons Learned from the Sea Star Wasting Disease Investigation

Ian Hewson,[1] Mitchell R. Johnson,[2] and Brandon Reyes-Chavez[1]

[1]Department of Microbiology, Cornell University, Ithaca, New York, USA; email: hewson@cornell.edu

[2]Department of Biology, San Diego State University, San Diego, California, USA

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Abstract

Marine invertebrate mass mortality events (MMEs) threaten biodiversity and have the potential to catastrophically alter ecosystem structure. A proximal question around acute MMEs is their etiologies and/or environmental drivers. Establishing a robust cause of mortality is challenging in marine habitats due to the complexity of the interactions among species and the free dispersal of microorganisms from surrounding waters to metazoan microbiomes. The 2013–2014 sea star wasting disease (SSWD) MME in the northeast Pacific Ocean highlights the difficulty in establishing responsible agents. In less than a year of scientific investigation, investigators identified a candidate agent and provided at the time convincing data of pathogenic and transmissible disease. However, later investigation failed to support the initial results, and critical retrospective analyses of experimental procedures and reinterpretation of early findings disbanded any candidate agent. Despite the circuitous path that the investigation and understanding of SSWD have taken, lessons learned from the initial investigation—improving on approaches that led to misinterpretation—have been successfully applied to the 2022 *Diadema antillarum* investigation. In this review, we outline the history of the initial SSWD investigation, examine how early exploration led to spurious interpretations, summarize the lessons learned, provide recommendations for future work in other systems, and examine potential links between the SSWD event and the *Diadema antillarum* MME.

1. INTRODUCTION

Metazoan mass mortality events (MMEs) can shape ecosystem structure by removing important consumers that influence the outcome of interspecific competition for space and resources. These alterations can further lead to phase shifts, where ecosystem momentum may eventually lead to functional extirpation of affected organisms that experience poor recovery over decadal scales. While MMEs and their downstream impacts are frequently observed on land and in freshwater habitats (Richard et al. 2020, Robinson et al. 2019), they are less frequently encountered in marine environments, with the exception of those occurring in commercially or economically relevant species (Delroisse et al. 2020) or when the loss of species and subsequent impacts threaten tourism value (Alvarez-Filip et al. 2019). However, geographically concerted scientific attention to loss of ecologically but perhaps not (immediately) economically important species has led to the description of several marine mass mortalities in the past 50 years.

Sea stars (Asteroidea; Echinodermata) are described as keystone species in coastal ecosystems (Paine 1966), where they play various roles as consumers of benthic invertebrates, influencing benthic community structure to favor taxa that are not directly consumed (e.g., macroalgae). Sea stars are also among the most extensively studied marine invertebrate classes in the disciplines of benthic ecology and developmental biology since some species are easily reared in the laboratory. Echinodermata is described as a boom–bust phylum because of its members' rapid swings in population density (Uthicke et al. 2009), but the mechanisms of their nonconsumptive mortality are remarkably poorly understood. Infectious diseases of aquatic invertebrates overall have garnered significant interest over the past 30 years, and research into their causes has accelerated since the advent and greater accessibility of approaches to understand microbial diversity (e.g., high-throughput nucleic acid sequencing platforms). The confluence of technique development, increased accessibility, and more frequent encounters with marine mass mortalities (Burge et al. 2014) has thus permitted extensive research into their drivers. Here, we focus on the challenges of evaluating disease in ecologically valuable echinoderm communities and best practices for action when a novel outbreak is observed.

2. RED ALERT! THE SEA STAR WASTING DISEASE MASS MORTALITY OF 2013–2014

Sea star wasting disease (SSWD, also called sea star wasting syndrome) describes a suite of grossly abnormal signs that include deflation or loss of turgor, ray loss, lesions (body wall necrosis or ulceration), and abnormal behavior (ray dysplasia) (**Figure 1**). The condition has been reported for over a century (Mead 1898), including in specimens from the Gulf of Maine, Mediterranean Sea, Kattegat, and Gulf of California (reviewed in Hewson et al. 2019). SSWD signs accompanied population declines in the Gulf of Maine and more widely across the North American Eastern Seaboard from 2012 to 2015 (Bucci et al. 2017). Gross abnormalities consistent with SSWD were observed in sea stars in the Channel Islands, California, in 1997 (Eckert et al. 2002) and were further observed on Vancouver Island in 2008 (Bates et al. 2009) but were not associated at the time with population decline or other evidence of a regional MME.

Beginning in the boreal summer of 2013, locally intensive mortality of the ochre star (*Pisaster ochraceus*) was noted along the Pacific coastline, first near Olympic National Park and then more widely across the region in subsequent months, leading to population declines in subsequent years across the entire coast, from Baja California to Alaska (Miner et al. 2018). At the time, there were no basin- or regional-scale physicochemical anomalies (strong El Niño–La Niña events, marine heatwaves, etc.) that may have explained the widespread occurrence of the abnormal condition (Dawson et al. 2024). Pacific observations of abnormalities continued through the fall in

Figure 1

Pisaster ochraceus specimens obtained from Olympic National Park in (*a*) September and (*b*) October 2013. The left image in panel *b* shows a grossly normal specimen, while all others were classified as abnormal. Photos provided by Steven Fradkin.

other asteroid species, including the sunflower star (*Pycnopodia helianthoides*). Veterinarians at the Vancouver and Seattle Aquariums noted significant mortality in sea stars both in natural populations around their facilities and within their sea stars on display. These aquaria lost a substantial fraction (in some cases all) of their sea stars; however, no other invertebrates were lost. Some specimens that were lost had been on display for over 40 years (M. Haulena, personal communication). The loss of asteroids in dramatic fashion generated considerable public interest through the popular press. The condition was quickly named sea star melting by researchers and the popular press based on the gross changes observed in affected specimens. The SSWD moniker was given to the condition because of gross similarities to an earlier event on Vancouver Island (Bates et al. 2009).

Alarmingly, asteroids with lesions fitting descriptions of SSWD were reported within weeks of its initial appearance in Santa Cruz, California, and Sitka, Alaska (1,200 and 1,300 km away from the Salish Sea, respectively) at sites routinely monitored by the Multi-Agency Rocky Intertidal Network (**https://marine.ucsc.edu**), and the condition seemed to progress southward along Big Sur (the Monterey Bay Aquarium reported loss of specimens as well) and northward toward San Francisco Bay in subsequent months. The condition was also noted in Southern California within four months of the Salish Sea report. The rapid apparent spread and arrival in Central California and ultimate spread to Southern California within a few months generated significant concern among veterinarians, ecologists, and aquarium managers who sought to understand and ameliorate the condition. The condition was initially not reported along the coastline from Point Reyes through the Olympic Peninsula but eventually was observed on the Oregon coast (Menge et al. 2016) and northward along the British Columbia and southeast Alaska coasts by May 2014 (Konar et al. 2019).

An obvious question emerged: What caused the condition? Due to the large areal extent of affected asteroids through the second half of 2013 and potential longshore and regional spread, early investigations focused on potentially infective agents (i.e., pathogens) (Hewson et al. 2014).

Researchers rapidly consulted the available literature to discern whether the MME was novel or had been previously encountered. The aforementioned abnormal conditions in the Channel Islands and elsewhere were largely overlooked for a variety of reasons: The literature was not in digital forms or was not available at several institutions (e.g., conference proceedings; Eckert et al. 2002), the literature was isolated to a few observations in disparate species (Staehli et al. 2008) or detailed only aquarium-kept specimens (abnormalities may have been due to husbandry; Christensen 1970), or the literature was very old and the language used to describe the condition had changed in subsequent years (so it was not easily searchable; Mead 1898). Finally, information on asteroid mortalities from the North American Eastern Seaboard was not in peer-reviewed literature at the time (Bucci et al. 2017). The 2013 Pacific MME was considered at the time novel or different because of its geographic extent, even though the gross abnormalities were highly similar to those of the Atlantic event. In particular, the co-occurrence of the condition in public aquaria and adjacent waters from which seawater was drawn for displays had not been previously documented and suggested a transmissible agent.

3. THE RAPID MICROBIAL ECOLOGY INVESTIGATION INTO SEA STAR WASTING DISEASE

Investigators initially solicited the network of scientists for tissue samples to enable comparative analyses of grossly normal and abnormal specimens. Samples from grossly normal specimens of any affected species, along with abnormal specimens, were shipped to complementary microbiology and histopathology laboratories. By mid-2014, microbiological investigators had a total of 314 specimens, with complementary specimens of nearly all samples submitted to histopathology labs.

The initial investigation into microbial associates comprised comparative surveys of viruses, bacteria, and archaea (Hewson et al. 2014, 2018). Because there was no information on microbiome composition by tissue type at the time, initial work focused on ray cross sections, including body wall, pyloric caeca, and gonad tissues (all of which were extracted in a single sample from each sea star). Only a handful of pathogens were known for asteroids (Jangoux 1987), such as the scuticociliate *Orchitophrya stellarum*, which had previously been reported in *P. ochraceus* in the late 1990s (Bouland & Jangoux 1988), but as these were not observed by light microscopy, they were not investigated further. PCR of kinetoplastid endosymbionts of amoebae (Feehan et al. 2013) yielded no successful detections among any normal or abnormal specimen.

At the time, there was very little information on asteroid microbiome (i.e., bacteria and archaea) composition, especially among species that were affected, except for reports of subcuticular bacteria (Holland & Nealson 1978, Kelly & McKenzie 1995, Kelly et al. 1995). Bacterial community fingerprinting [automated ribosomal RNA (rRNA) intergenic spacer analysis; Fisher & Triplett 1999], which was outdated at the time for wider community descriptions but served to rapidly highlight differences between communities based on bacterial species-level length heterogeneity in the 16S–23S rRNA gene spacer region, identified no strong differences between abnormal and grossly normal sea stars with the exception of a single amplicon-length heterogeneous band that was in some of the abnormal sea stars but consistently absent in grossly normal sea stars (Hewson et al. 2018). This band, based on downstream 16S rRNA sequencing, comprised a member of Bacteroidota (most similar to *Marivirga* sp.), a member of Pseudomonadota (most similar to Gammaproteobacteria recovered from other marine invertebrates and within the Chromatiales), a member of Spirochaetota (most similar to *Salinispira* sp.), and a member of Tenericutes (most similar to *Spiroplasma* sp.). Viral metagenomics (Breitbart et al. 2002), which was applied mostly based on DNA ($n = 28$ specimens) but also based on RNA from four *P. helianthoides* specimens, also showed no viral genotype exclusively associated with SSWD. However, one single-stranded DNA

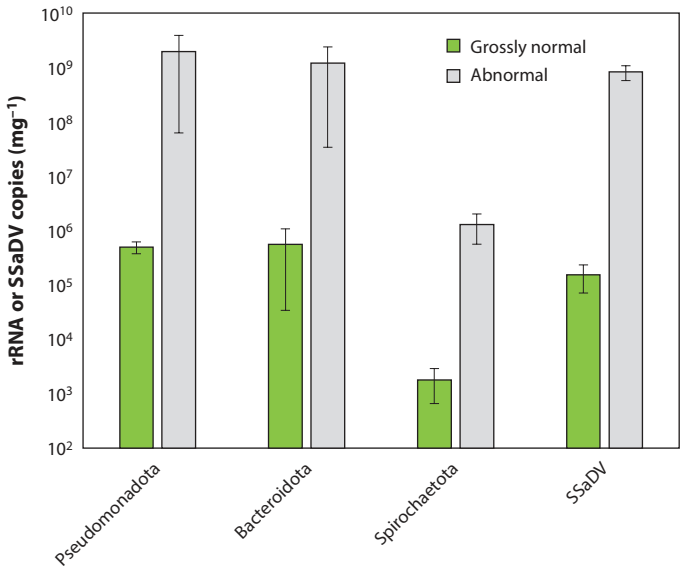

Figure 2

Bacterial rRNA and SSaDV loads in grossly normal and abnormal specimens of *Pycnopodia helianthoides* as observed in the 2013–2014 mass mortality. Error bars show standard error. Abbreviations: rRNA, ribosomal RNA; SSaDV, sea star–associated densovirus.

virus, a densovirus (Parvoviridae, a group that includes viruses infecting terrestrial insects), was more consistently detected in some (but not all) abnormal sea stars and fewer grossly normal sea stars. At the time, echinoderm densoviruses were a relatively recent discovery, having been recovered from Hawaiian sea urchins the year prior using the same approach (Gudenkauf et al. 2014).

Based on the overall pattern of microbial associates, investigators designed quantitative PCR (qPCR) assays around the bacterial taxa and the densovirus identified in surveys and determined their abundances across all samples collected at the time. Four of five candidate agents were enriched in abnormal specimens when compared with grossly normal specimens (**Figure 2**; Tenericutes were not detected by qPCR), but notably, each was also positively identified by qPCR in grossly normal specimens. The three detected bacterial taxa were not further investigated because they comprised taxa that were well-known copiotrophs: Spirochaetes had been observed for decades as subcuticular denizens of echinoderms and so were unlikely to be primary disease agents, and the Bacteroidota and Pseudomonadota were well known to respond to elevated organic matter in seawater and so were considered likely to be stimulated by decaying tissues (i.e., potentially saprobic taxa). The identified densovirus, on the other hand, represented the most promising associate, since at the time viruses were mostly assumed to be pathogenic in nature. The presence of this genotype in grossly normal specimens was considered to be due to preclinical exposure, or infection that occurred but had not yet progressed to observable disease signs, since grossly normal specimens were collected simultaneously with abnormal specimens.

Based on initial identification of a candidate densovirus genotype called the sea star–associated densovirus (SSaDV), researchers rapidly organized challenge experiments using the virus-sized fraction (VSF, functionally defined as tissue homogenates that were filtered to remove material larger than 0.22 μm) to test for pathogenicity (i.e., to satisfy Koch's postulates). Injection of this material generated SSWD signs in two of these experiments, performed in series from late March through April 2014 (Hewson et al. 2014) (but see Section 4.1). qPCR was then used to

detect SSaDV in specimens that became abnormal, providing evidence that agents in the VSF were transmissible and that SSaDV was the prime candidate for agents within that size fraction (Hewson et al. 2014).

Parallel histopathologic investigation, which by nature is slower than sequencing-based surveys due to extended sample decalcification time and requires trained veterinary histopathologists to observe multiple tissue thin sections per specimen, did not observe any microbe associated with lesions, nor any inclusion within tissues that may have indicated viral or other invasive microbial attack (i.e., virion matrices, hypernucleation, or hyperplasia) (Bucci et al. 2017, Oulhen et al. 2022). Instead, histopathologists observed nonspecific inflammation accompanied by diffuse coelomocyte recruitment, edema, cleft formation, and in some specimens necrosis of papulae and other tissues (Oulhen et al. 2022). Parvoviruses, which are among the smallest known viruses at 20–50-nm diameter, do not form virion lattices or undergo cellular changes during infection except for nucleus swelling, which was not observed in sea stars. Transmission electron microscopy of lesion tissues identified very few virus-like particles. The inconsistency between histopathologic results and SSaDV qPCR results was considered at the time to be influenced by observational artifacts, or perhaps to indicate that SSaDV was missed entirely by histopathologic resolution.

Overall, the results suggested that SSWD was a transmissible condition and that SSaDV was the best candidate microorganism for etiology. Based on available information, the results of this investigation were published in the paper "Densovirus Associated with Sea-Star Wasting Disease and Mass Mortality" in the *Proceedings of the National Academy of Sciences* in November 2014 (Hewson et al. 2014). The publication gained wide public media attention and has been cited 357 times in the peer-reviewed literature as of March 2024. Continued investigation into SSWD etiology and its impacts on the genetic structure of host populations as well as its impacts on benthic communities has been supported by more than US$4.8 million awarded from the US National Science Foundation over the decade since that publication. As of March 2024, a total of 57 peer-reviewed papers or reviews have focused on SSWD. Of these, most have described causes or environmental correlates of the disease, impacts of the disease on population structure of hosts, or impacts of the condition on benthic ecosystem structure.

4. SEA STAR WASTING DISEASE ETIOLOGY *QUOD ERAT DEMONSTRANDUM*?

Shortly after the Hewson et al. (2014) publication, wider concerns surfaced about the mismatch between veterinary histopathology, which showed a lack of association with any microbial agent, and the microbiology survey, which concluded that SSaDV caused SSWD. Subsequent challenge experiments identical to those performed in the Salish Sea, using VSF material, did not yield SSWD signs in either *P. helianthoides* or other affected species (Hewson et al. 2018). Furthermore, virome surveys recovered highly similar densoviruses (>90–95% amino acid similarity across the nonstructural gene) in grossly normal asteroids from the northeast Atlantic Ocean (Jackson et al. 2016, 2020), East Asia (Hewson et al. 2018), and New Zealand (Hewson & Sewell 2021). A survey of echinoderms from the Salish Sea in January 2016 revealed the widespread occurrence of SSaDV among many echinoderms in the region (Hewson et al. 2018). As part of Hewson et al.'s (2014) study, museum specimens were screened by qPCR for SSaDV, and several generated positive results. However, further attempts to sequence SSaDV from additional museum specimens, complementing those examined in the 2014 study, failed to generate amplicons homologous to densoviruses (I. Hewson, unpublished data). These inconsistencies and new information led to reconsideration of SSaDV or other viruses as etiological agents for the condition. Sampling and experimental design for results supporting a viral etiology (and SSaDV candidacy) may have led to misinterpretation and informed downstream efforts to understand drivers of other MMEs.

4.1. Were Koch's Postulates Satisfied?

Challenge experiments were performed to test the hypothesis that viruses (and SSaDV specifically) caused SSWD by satisfying Koch's postulates (Koch 1893). These criteria state that a candidate microbe must be identified in most (if not all) cases of disease among populations and not in unaffected populations. Additionally, the microbe must elicit consistent disease signs when challenged against naive hosts and must be identified in specimens that had disease signs after challenge. Isolation of SSaDV in culture was not possible due to the lack of immortal echinoderm cell cultures. In the context of SSWD, at least three VSF challenge experiments on *P. helianthoides* were performed in the Salish Sea in January and February 2014; however, none led to SSWD in treated specimens. Following these experiments, additional challenges were performed on *P. helianthoides* in a closed artificial seawater aquarium system in Ithaca, New York, but all specimens died with signs consistent with SSWD (small lesions) after seven days of incubation. This inconsistency—where a VSF challenge either elicited no response or resulted in SSWD in all specimens—resisted identification of a causal agent. In mid-March 2014, a challenge experiment with five VSF-amended and five heat-treated VSF-amended specimens (i.e., controls) bore fruit: Four of the treated sea stars that were challenged with the VSF developed small lesions and experienced ray autotomy. In all cases, they also experienced ray dysplasia (curling) that appeared to precede the development of lesions. The challenged stars had been used in earlier VSF experiments. Heat-treated controls for this successful experiment did not experience SSWD signs.

This promising result among a backdrop of other negative results led to a second challenge experiment comparing a VSF prepared from specimens in the prior experiment with heat-treated VSF as controls. The copy number in the tissue homogenate was low ($\sim 1.5 \times 10^5$ copies mL^{-1}) and was prepared from specimens that had generated lesions in the first round of the experiment. Due to regulatory difficulties in obtaining fresh sea stars, the experiment enrolled several control individuals from the first challenge (two used in VSF treatment and two used as controls in the new experiment), five specimens that had been in captivity for several months (three used in VSF treatment and two used as controls), an additional specimen that had been used as a control in a previously undocumented feeding trial (one used in VSF treatment), and several freshly collected specimens (four used in VSF treatment and six used as controls). Specimens were sacrificed within 24 h of lesion appearance in the second experiment, and controls were assigned to each treated star (based on size and time of collection) and sacrificed when the complementary treated specimen was harvested. The removal of control specimens from the experiments over time may have contributed to the apparent strong response of *P. helianthoides* to VSF inoculation (8 of 10 VSF specimens developed lesions) compared with controls (which may have developed lesions over time but did not before harvesting), especially since stars were harvested at the same time as their cohort based on age. It is not possible, for example, to say how long control specimens would have remained grossly normal since they were lost progressively as respective treatments were removed.

All specimens, including controls, had some SSaDV signal at experiment initiation. SSaDV loads in tube feet increased in specimens that were treated until the time of lesion appearance (at which point specimens were harvested), while control specimens increased less or decreased between the start of the experiment and the time of collection (which occurred at different times complementary to specimens forming lesions) when calculated as the change between the initial and final time points. However, analysis of all specimens since the time of any experiment (i.e., including those that served as controls in the first experiment and were then reused in the second experiment, as well as additional time points collected over the course of the experiment) revealed that there was no significant difference in SSaDV load over the course of the incubations between treated and untreated specimens (control, $1.06 \pm 3.24 \times 10^7$ $copies^{-1}$ mg $tissue^{-1}$ d^{-1}; VSF,

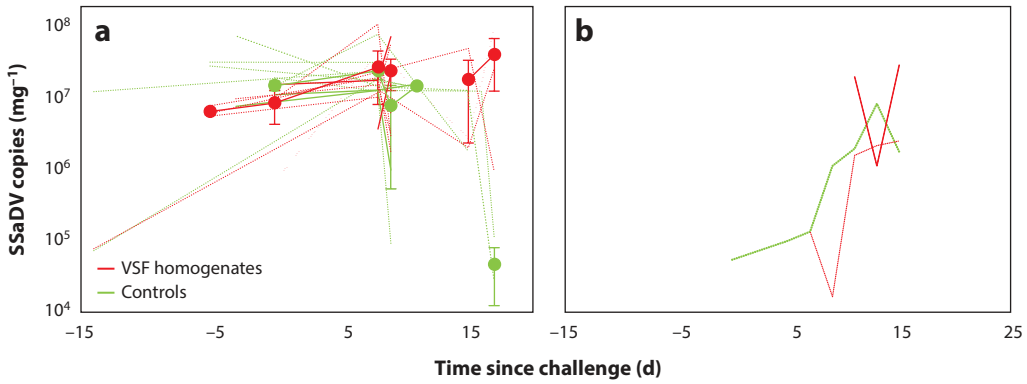

Figure 3

Change in SSaDV loads (normalized by tissue weight) in *Pycnopodia helianthoides* challenged with VSF homogenates (*red*) and controls (*green*) over time in (*a*) Port Townsend, Washington, and (*b*) Ithaca, New York. Most specimens in Port Townsend were sampled before the start of the experiment and included five specimens used in previous experiments. Data from Port Townsend are from the second challenge described by Hewson et al. (2014). Experiments were performed in natural seawater in Port Townsend and artificial seawater in Ithaca. The solid red (VSF) and green (control) lines indicate the mean viral load at time points where more than two asteroids were sampled during the experiment. Missing lines or markers indicate that SSaDV was below the detection threshold (of 104 copies) at that time point. The decrease in control specimens noted by Hewson et al. (2014) was driven largely by decreased viral load at the final time point (17 days after infection). Error bars show standard error. Abbreviations: SSaDV, sea star–associated densovirus; VSF, virus-sized fraction.

$2.62 \pm 1.55 \times 10^7$ mg tissue^{-1} d^{-1}; $n = 10$, Student's *t*-test) (**Figure 3*a***). An additional, previously unreported experiment (in Ithaca, New York) examined variation in coelomic fluid SSaDV load over 15 days in two *P. helianthoides* inoculated with VSF material from an abnormal specimen as well as one control *P. helianthoides*. SSaDV load increased over time in all three specimens observed (**Figure 3*b***), indicating that animal husbandry and time in aquaria were predictors of viral load. In sum, there was little evidence for SSaDV's pathogenicity from the challenge experiments performed in 2014.

Two subsequent experiments with *P. ochraceus* and *Evasterias troschelii* (in 2014 and 2016, respectively), along with a challenge with a *Vibrio* sp. strain isolated from an abnormal *P. ochraceus* specimen, did not yield abnormalities. The inconsistent response of asteroids to VSF challenge was, at the time, believed to be due to the difficulty of performing these experiments and the loss of pathogen virulence, or perhaps the development of resistance due to prior pathogen exposure. However, the lack of additional viral candidates and reanalysis of available data on SSaDV led to reconsideration of the viral or even transmissible agent etiology for this condition. By 2018, the lack of response to viral challenge in repeated experiments, reanalysis, and redesign of qPCR primers and probes and reanalysis of metagenomic data had shown no association between SSaDV and SSWD, leading to the conclusion that SSWD was not associated with the densovirus identified by Hewson et al. (2014) (Hewson et al. 2018). Because of uncertainty about the nature and lability of material prepared in the VSF to which *P. helianthoides* had responded with abnormalities, additional investigations of potential etiological agents were performed.

4.2. Subsequent Investigations in the Northwest Pacific

Subsequent work focused on finding additional candidate agents associated with SSWD by examining the disease progression. Lloyd & Pespeni (2018) examined microbiome variation (assessed through RNA-based approaches) during disease progression in experimental mesocosms and observed an increase in a bacterial genus commonly associated with marine disease in other

metazoans (*Tenacibaculum* sp.) as well as two Pseudomonadota (*Polaribacter* spp. and *Phaeobacter* spp.) through the course of the condition from grossly normal to abnormal. These shifts were concomitant with a decreased abundance of a copiotrophic taxon (*Pseudoalteromonas* spp.) along the same progression. The authors argued that altered normal microbiomes (i.e., dysbiosis) may have permitted other, unidentified pathogens to infect sea stars. It should be noted that the experiment was performed in an aquarium setting with artificial seawater, which is known to cause changes in microbiome structure in other studies (Patin et al. 2018), so it is challenging to parse experimental from disease effects, especially since the vast majority of specimens (29 of 37) became sick over the course of the experiment without external influence (e.g., inoculation of any pathogenic agent). McCracken et al. (2023) reported a similar study in which they used amplicon sequences to examine microbiome variation over time in southeastern Alaska sea stars undergoing SSWD progression. They observed a proliferation of facultative and strictly anaerobic bacterial taxa, notably Clostridia, *Vibrio*, *Moritella*, *Colwellia*, Fusobacteriaceae, and Desulfobulbaceae, which was consistent with observations made during aquarium studies (Aquino et al. 2021). Loudon et al. (2023) and Jackson et al. (2018) examined the core microbiomes of grossly normal sea stars, which included abundant spirochaetes and mollicutes. A comparison of SSWD-affected sea stars from a site in British Columbia and a retrospective analysis of Lloyd & Pespeni's (2018) data suggested that no core microbiome taxa experienced changes in abundance with the condition (Loudon et al. 2023).

The role of protists in SSWD has received considerably less attention than microbiomes, most likely because these are more challenging to differentiate from host marker genes in amplicon library preparation. FioRito et al. (2016) reported the diversity of unicellular protists that produce slime nets (Labyrinthulomycetes) in sea stars, but their association with SSWD was unclear. Examination of multiple RNA viromes (which coextract and sequence bacterial and eukaryotic rRNAs) prepared during the initial 2013 investigation (Hewson et al. 2014) and during observation of virome variation in Santa Cruz, California (Hewson et al. 2020), did not yield any candidate targets, bearing only fungal rRNA gene sequences that were in a pattern inconsistent with animal normal/abnormal state, as well as several ciliate rRNA sequences (see Section 6). Fungal communities have been characterized from grossly normal and abnormal sea stars on Deception Island (Antarctica) but yielded no disease associates (Núñez-Pons et al. 2018). If eukaryotic microorganisms were involved in SSWD abnormalities, they may be loosely associated with animal surfaces (and thus lost during specimen collection), since eukaryotes were not observed in the majority of histopathologic sections of affected tissues, and molecular surveys generally removed all but strongly attached microorganisms through sample collection and preparation.

4.3. Transcriptomic and Genomic Responses of Sea Stars to Wasting

Several studies have compared grossly normal with abnormal specimens by transcriptomics to understand potential drivers. These have mostly observed enhanced expression of genes related to immune system function and programmed cell death (apoptosis) (Fuess et al. 2015, Gudenkauf & Hewson 2015), findings further observed in comparative genomic studies of survivors and their grossly normal cohabitants (Ruiz-Ramos et al. 2020). Fuess et al. (2015) also found changes in expression of nervous system loci, a finding that is echoed in suppressive responses to $MgCl_2$ amendment (Jaffe et al. 2019) and consistent with coelomic fluid Cl^- changes in abnormal stars (Wahltinez et al. 2020, 2023). Population genetic surveys revealed little impact of SSWD on *P. ochraceus* population structure (Schiebelhut et al. 2022a).

4.4. The Organic Matter Enrichment and Surface O_2 Depletion Hypothesis

The absence of any microbe distinctly associated with SSWD in the field and the failed identification of further candidates through challenge experiments suggested that the condition represented

either multiple different pathogenic etiologies that occurred simultaneously (Hewson et al. 2018) or a nonpathogenic etiology. Abiotic factors (e.g., temperature) yielded SSWD in aquarium experiments and were in some cases strongly associated with prevalence in field surveys (Eisenlord et al. 2016, Smith et al. 2023). Further, SSWD was encountered only sporadically from 2015 to 2018. During this period, retrospective analysis of oceanographic data identified significant swings in temperature at affected sites immediately before SSWD onset, along with reduced rainfall in months preceding SSWD (Hewson et al. 2018).

SSWD was noted in *P. ochraceus* housed at the Long Marine Laboratory (Santa Cruz, California) in May and June 2018, which afforded the opportunity to further understand factors that influenced SSWD onset and speed (Aquino et al. 2021). All insults that were tested [abrasion, desiccation, challenge with proteinaceous (against proteinase K–treated) unfiltered tissue homogenates, and slow water turnover time in aquaria] led to faster wasting compared with controls when those specimens were retained in the challenges through the course of the experiments. Microbiome (16S rRNA gene) sequencing revealed that SSWD onset was accompanied by an increase in the relative abundance of obligately and facultatively anaerobic bacteria. Between 2015 and 2018, citizen scientists who repeatedly surveyed sites had established that SSWD occurred primarily in autumn (the same time as the MME in 2013). Combined, these observations led to the hypothesis that SSWD may be related to elevated secondary productivity (and consequently respiration) of microorganisms at the animal–water interface. This hypothesis was reinforced by field-based observations that more rugose (corrugated) species were more affected by SSWD compared with less rugose (smoother) species. Experiments testing this hypothesis through amendment with various organic matter substrates (including naturally collected particulate organic matter) caused faster wasting than controls, suggesting that microorganisms at the animal–water interface may ultimately be behind SSWD (Aquino et al. 2021). It was further hypothesized that elevated primary production, which provides labile dissolved organic carbon (DOC) for bacterial metabolism, may lead to SSWD in a mechanism similar to the dissolved organic matter–disease–algae–microbes (DDAM) hypothesis for coral disease (Haas et al. 2016). Data linking primary production rates, heterotrophic secondary production rates, oxygen depletion on animal surfaces, and SSWD are still lacking, but the absence of microbial associates, the strong seasonal pattern, and experimental response to organic matter enrichment are highly suggestive of an environmental upset or seasonal variability in primary production leading to greater microbial abundance and abnormal tissues. It is interesting to note that experimental transcriptomics suggests that immune and hypoxia response gene repertoires have enhanced transcription in abnormal *P. ochraceus* relative to grossly normal specimens and that transcriptomic responses in these pathways are enhanced before lesions appear (Pespeni & Lloyd 2023).

4.5. Sea Star Wasting Disease Observations Since 2018

Descriptions of SSWD from the late 1800s and throughout the literature (described by various names) suggest that wasting phenomena may be commonplace. Recently, Smith et al. (2022) described an outbreak of SSWD in Northern Ireland, and subsequent work identified temperature as a key effector of the condition (Smith et al. 2023). Lesions consistent with SSWD have also been observed in the Gulf of California (Vergneau-Grosset et al. 2022); the Gulf of Maine (Van Volkom et al. 2021); Port Philip Bay, Australia (Hewson et al. 2019); Deception Island in the subantarctic region (Núñez-Pons et al. 2018); Tauranga, Aotearoa New Zealand (Jones & Sewell 2023); Qingdao, China (Hewson et al. 2019); and recently in McMurdo Sound, Antarctica (Moran et al. 2023). The etiologies of most of these outbreaks, similar to those in the northeastern Pacific Ocean, remain unresolved.

5. LESSONS LEARNED AND FUTURE RECOMMENDATIONS

5.1. If You Cannot See It by Histopathology, It Is Probably Not an Invasive Infectious Agent

Veterinary histopathologic approaches remain the gold standard for defining disease-associated signs. Yet it was not until 2021 that a case definition for any sea star was reported (Work et al. 2021). Examining histopathologic lesion sections of sea stars is time-consuming, since decalcification of ossicles is often needed to examine fine structure of soft tissues (Newton & Dennis 2021). The pace of microbiome research in 2013 was faster than histopathology investigation, the latter of which may have informed the absence of invasive etiological agents. Later case descriptions of SSWD suggested that wasting was a basal-to-surface process that was initiated at ossicles and proceeded to the epidermis (Work et al. 2021). In all case reports, there were few microbial associations (see Section 5.1.1). However, a limitation of histopathology for novel infectious diseases like SSWD was that the extensive decalcification procedure may have caused microbiome components to be lost prior to observation. Additionally, some pathogens (e.g., viruses) may stimulate a systemic immune response and otherwise be invisible or distant from lesion sites. However, it is unlikely that massive systemic infections and a wide array of lesions would be devoid of associated microorganisms visible by histopathology, so this approach remains a necessary complement to the application of microbiological approaches to examine their associates. Future MME investigations should start with light microscopic assessment of coelomic fluid and blood/lymph and examination of lesions via thin section. Histopathologic assessment, which can take time, should occur in parallel with microbiology investigations.

For the 2022 *Diadema antillarum* MME (Hewson et al. 2023), histopathology was engaged in the investigation from the outset, and communication of results between two engaged teams and microbiology investigators was kept to a minimum so as not to bias findings—i.e., the findings were independent. These studies identified ciliates with morphology consistent with *Philaster* spp. both invading epidermal tissues and spines and in coelomic fluid via hematology. It was only after the conclusion of the microbiological investigation, which discovered this ciliate through host transcriptome analysis, that these investigations were communicated between researchers, providing three independent lines of evidence for the association between abnormal urchins and the scuticociliate.

5.2. Dead or Dying Specimens Alone Do Not Aid Much in Identifying Candidate Agents

A key problem with the initial investigation into SSWD, which focused on identifying potential pathogenic microorganisms and viruses, is that it was heavily biased toward grossly abnormal specimens. Of the 314 specimens obtained by soliciting scientists and community members, the overwhelming majority were from abnormal individuals (88%; **Table 1**). Nearly all grossly normal specimens were *P. ochraceus* (69%), and of the wild specimens, most (63%) came from the Salish Sea and Olympic National Park, with fewer from Central (20%) and Southern (14%) California. Two factors influenced this collection: (*a*) Most sites were affected when they were sampled, and thus preclinical infection was often cited as a reason for observing potential pathogenic agents in otherwise healthy tissues, and (*b*) scientific collection permits can take time during marine disease emergencies, and so collection of dead specimens was justified against sacrificing grossly normal specimens. Especially when dealing with species facing extirpation (Montecino-Latorre et al. 2016), there is also a tendency to avoid collecting the survivors, in case they ultimately survive. This created problems downstream in the interpretation of disease associates (e.g., inconsistent

Table 1 Numbers of grossly normal and abnormal specimens by taxon and captivity state

Species	Total	Grossly normal	Grossly abnormal	Captive	Wild
Pisaster ochraceus	110	27	83	19	91
Pycnopodia helianthoides	64	9	55	47	17
Evasterias troschelii	41	3	38	19	22
Pisaster brevispinus	38	0	38	20	18
Pisaster giganteus	25	0	25	6	19
Mediaster aequalis	7	0	7	7	0
Patiria miniata	7	0	7	7	0
Dermasterias imbricata	6	0	6	4	2
Luidia foliolata	6	0	6	6	0
Solaster stimpsoni	6	0	6	2	4
Stylasterias forreri	2	0	2	2	0
Leptasterias hexactis	1	0	1	0	1
Orthasterias koehleri	1	0	1	0	1
Total	314	39	275	139	175

statistical comparisons). Conservative collection of grossly normal specimens in equal number to abnormal specimens is critical to avoid such issues in future MMEs.

In the investigation of the 2022 *D. antillarum* MME, an equal number of grossly normal and abnormal specimens were collected from affected sites. The total number of specimens collected (typically three from each tissue state) provided sufficient statistical *n* for downstream analysis, and along with reference specimens (see Section 5.3) facilitated identification of microbiome constituents that corresponded with abnormal urchins. A wider suite of sublethal specimens (e.g., tube feet in sea stars or spines in urchins) may be collected from both grossly normal and abnormal specimens once disease associates are identified to provide added statistical rigor to associations.

5.3. The Most Valuable Samples for Microbial Investigation May Involve the Destruction of Apparently Healthy Specimens

A key missing component of the early SSWD investigation was the availability of reference (pristine) sea star specimens for comparative analyses. By the time that investigators were aware of the SSWD MME, abnormal specimens had been noted across a very wide geographic range, and state and institutional policies resisted sampling of remaining specimens. Hence, collection of specimens from unaffected sites, well away from any abnormal specimens, was not possible. Because so little was known about the microbial diversity of grossly normal specimens, the lack of reference material made the identification of disease associates more complicated. Yet reference specimens are vital for distinguishing between normal microbiome constituents and potential pathogens. Predicting the emergence of novel MMEs is not possible (there was no prior warning of the SSWD MME, as indeed was the case for *D. antillarum* MMEs in 1983 and 2022), but museum specimens collected before the disease event may be useful for comparisons with contemporary events. Regardless, microbiological investigations should place a priority on collecting reference specimens from sites well away from disease fronts.

Fortunately, in the 2022 *D. antillarum* MME, reference materials from before event onset are available both in museum collections and from frozen specimens at sites that were later affected by the condition (*D. antillarum* has been monitored continuously since the 1983–1984 MME). Furthermore, rapid efforts to sample specimens at sites well away (i.e., on the other side of islands) from the disease front allowed examination of reference specimens to establish normal

constituents. Regulatory agencies and policy provided very rapid (within weeks) permission to sample specimens, recognizing the value of both reference and abnormal specimens. Reference specimens were vital to understand that the condition was uniquely associated with a ciliate most closely related to *Philaster apodigitiformis* and was absent in reference sites, since several grossly normal specimens at affected sites bore *P. apodigitiformis* (i.e., they had been exposed). Without reference material, delineation of *P. apodigitiformis* as a candidate agent would not have been possible. Collection and maintenance of preserved echinoderms (or indeed other invertebrates) that are amenable to microbiome work should be supported to enable downstream candidate pathogen identification should a disease occur.

5.4. "Grossly Abnormal" Is Prone to Inter-Investigator Variation

In the absence of formal veterinary case definitions, assignment of health state to a named disease is not possible. Hence, specimens that are grossly abnormal are just that—grossly abnormal. Even within that categorization, there can be considerable variation among observers based on experience and observation level (**Figure 1**). For example, some specimens reported as having lesions in the field during the early investigation into SSWD were later determined to be normal, with prominent madreporites misreported as lesions. Similarly, the drive for investigator inclusion in wider investigations led to several samples being misidentified as abnormal even though there was little or no SSWD at specific sites to which scientists had access. Hence, photographic evidence at a minimum should be considered vital for each specimen collected in order to reduce inter-observer error. Even so, there is much variation among individuals considering tissue states, which is especially concerning given the lack of case definitions. This is particularly true during challenge or other experiments, where abnormal signs may be confused with stress responses to animal handling or husbandry, especially when gross descriptions of abnormalities encompass a wide range of anomalies (**Figure 4**). In the SSWD challenge experiments, a primary sign was curled or backfolded rays, which is a normal response of sea stars to injury on ray aboral surfaces (Schiebelhut et al. 2022b). Lesions, which grossly occur when outer epidermis is lost and subcutaneous body wall tissues are exposed, may result from a number of insults.

Reducing inter-investigator variation in defining disease states is best ameliorated by veterinary case definitions, which may identify disease-defining signs. However, these can take time to generate (which is often incompatible with rapid MME spread) and may point to microscopic features that are inaccessible in the field. At minimum, clear photographs of specimens intended for laboratory analyses should be collected. Furthermore, one or a few laboratories should coordinate and perform comparative investigations on specimens with clearly identified tissue states. In the 2022 *D. antillarum* investigation, specimen health state was easily recognizable by gross observation: Specimens were observed during the day away from rock crevices, they moved on oral spines (as opposed to tube feet), and their spines initially drooped before they were lost entirely. Investigators initially focused comparative analyses on the clearly identified grossly normal specimens at affected sites (i.e., those that had none of these signs), at reference sites (where no abnormalities were noted and well away from the disease front), and on abnormal specimens that had drooped and unresponsive spines and were found away from rock crevices. Collections for initial comparative investigations were performed by two research groups before a wider suite of specimens from additional investigators were solicited.

5.5. Samples Stored in a Commercial or Home Freezer Are Only So Useful

The accessibility of scientific laboratories does not always correspond to the sites of MMEs. In the 2013–2014 SSWD MME, investigators were flooded with offers to assist in sample collection

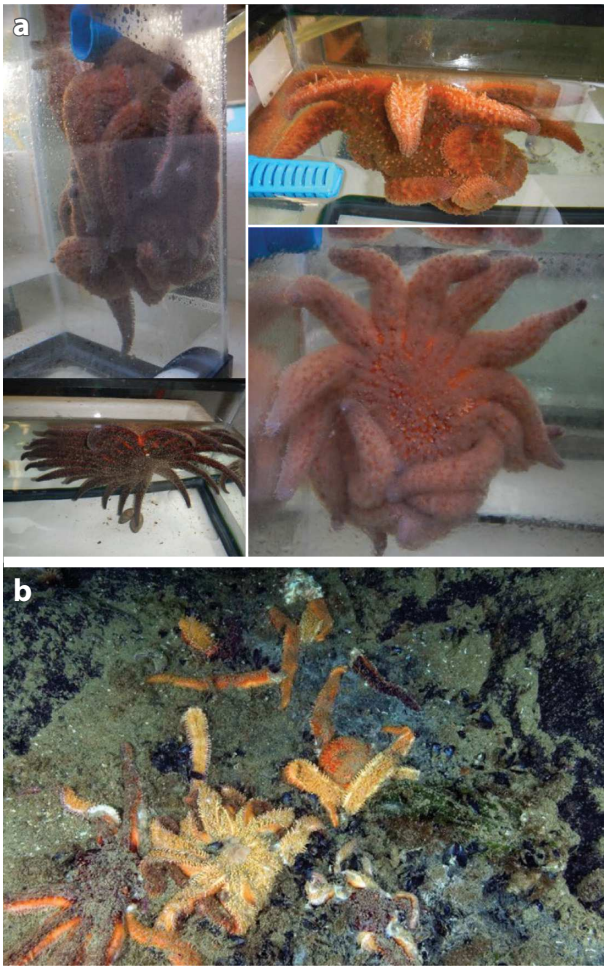

Figure 4

Sea star wasting abnormalities (*a*) during the April 2014 VSF challenge and (*b*) in the field near Gower Point, British Columbia, Canada, in *Pycnopodia helianthoides*. In the VSF challenge, arm curling, which is associated with epidermal injury, was recorded in three of five specimens challenged (the bottom left photo in panel *a* shows a grossly normal control) 12–18 h before lesions were noted and the animals euthanized for downstream microbiology investigation. Abbreviation: VSF, virus-sized fraction. Panel *a* photos provided by Morgan Eisenlord; panel *b* photo provided by Martin Haulena.

from community scientists and scientists without prior experience in molecular microbiological approaches. While information on the extent of SSWD was obtained from such individuals, the specimens collected, preserved, and transported to the investigating laboratories may have caused difficulties in downstream interpretation due to the decay of tissues, proteins, and nucleic acids. There were also difficulties in determining the health states of collected specimens, since they were not provided by individuals experienced in examining disease signs and often were not accompanied by photographs of specimens in the field.

DNA and RNA have variable stabilities under different preservation methods, and RNA can have different decay rates depending on physical structure. For example, messenger RNAs (mRNAs), which are typically short stretches of transcribed RNA, decay on average much faster

than structural RNAs (rRNAs). Viral RNA may be stable within intact virions for longer than host mRNA, rRNA, or transfer RNA (tRNA). RNA has slower decay in commercial or domestic freezers but is best preserved at −80°C or in liquid N_2 (–196°C). Genomic and viral DNA is more stable than RNA at both −20°C and −80°C. Since many specimens in 2013–2014 were kept at −20°C for variable time periods prior to analysis, this precluded examination of RNA in most specimens. DNA and RNA are also less stable in ethanol, although high-quality DNA may be obtained from museum specimens (Chalifour et al. 2022). A common approach to preserving RNA at room temperature is to use Invitrogen RNA*later* (or other similar solutions, such as Zymo Research DNA/RNA Shield). This product is generally inaccessible to community scientists. It should be noted that RNA*later* is not compatible with viral metagenomic approaches, since it physically destroys the viral particles and therefore does not allow for virion purification before sequencing. Transportation of specimens under conditions appropriate to downstream analysis of RNA and DNA is also complicated. Shipping materials on dry ice (−78.5°C) or in liquid N_2 requires specialized training and certification in safe practices. Ethanol is considered a hazardous good for transport but is subject to exemptions for small quantities. Shipping of materials in RNA*later* is not subject to International Air Transport Association standards or other courier or mail hazardous goods regulations. A large number of specimens analyzed for the initial 2013–2014 work were shipped on wet ice (i.e., ice blocks at 0°C), which may have led to the decay of tissues and associated microorganisms during transport.

For the 2022 *D. antillarum* MME, investigators focused on a few sites (St. John and Saba) where samples could be collected and transported in liquid N_2 (Hewson et al. 2023). While transportation of specimens in a dry shipper was expensive, focusing on only two affected sites allowed assessment of microorganisms without the negative effects of decay processes or the use of preservatives. Subsequent specimens of animal tissues and coelomic fluid were fixed in RNA*later* or DNA/RNA Shield for transport; however, these were used only for the verification of specific putative pathogens and were not used to identify transcriptomic responses of the host.

5.6. Challenge Experiments Are Challenging and May Provide Spurious Evidence of Transmission

A key question around the inability to generate additional SSWD responses with VSF material during the 2014–2018 investigation was why the experimental sea stars used for successful challenge in 2014 generated abnormalities in 80% of tested specimens. Here, we outline evidence that the VSF challenge design may have led to spurious results due to differential enrichment of DOC between treatments.

Challenge experiments in the 2013–2014 investigation were designed based on prior work examining the impacts of enhanced viral pressure on bacterioplankton community structure (Hewson & Fuhrman 2006, Hewson et al. 2003, Schwalbach et al. 2004). In these experiments, viruses were enriched from seawater through 0.2-μm filtration, concentration, and amendment to ambient seawater. The controls for these experiments were filtered viral concentrates that had been heat treated (boiled) to inactivate microbes and denature biological molecules (e.g., enzymes). This treatment of viral concentrates accounted for labile, high-molecular-weight dissolved organic material that was co-concentrated with viruses, amendment with which may stimulate bacterioplankton growth and lead to bacterial community artifacts. Hence, asteroid challenge experiments employed filtered tissue homogenates (filtered to remove material larger than 0.22 μm and leaving viruses and other smaller microorganisms, organic molecules, and other inorganic constituents) against heat-treated tissue homogenates. Ideally, experiments to satisfy Koch's postulates for individual pathogens may involve isolation of those candidate agents in culture; however, the lack of immortal cell lines against which viral candidates may be isolated for

any marine invertebrate prevented this approach. Similarly, purification of viruses or other small cellular entities away from coextracted organic and inorganic materials in homogenates could have been performed by, e.g., cesium chloride density gradient centrifugation but were not performed due to a lack of suitable laboratory equipment and demand for rapid investigation. Hence, VSF responses must be taken with caution, since the response may well be due to perturbations from any number of other coextracted compounds, some of which may be heat labile or modified by heating in controls. Experiments where specimens are incubated in water that has previously held abnormal sea stars (with controls of normal sea stars) (Bucci et al. 2017)—including those that are filtered to select microbial size fractions—likewise need to be interpreted with caution since they may be enriched with organic material that emanates from decaying sea stars or with elevated inorganic compounds (e.g., NH_3^+) that may be toxic to echinoderms.

The approach whereby the VSF is compared with a heat-treated fraction may also provide spurious results due to differences in organic nutrient concentrations. Filtration of tissue homogenate may lyse cells and other tissue debris, liberating intracellular contents and resulting in greater protein and DOC concentrations than it does in crude tissue homogenates (**Figure 5**). Paradoxically, heating of VSF material, which may liberate additional intracellular and capsid-bound material smaller than 200 nm, reduces (in a limited survey; **Figure 5**) overall DOC and protein contents, presumably due to condensation or organic molecule breakdown into inorganic constituents. In the successful 2014 experiment, the order of heat treatment for control specimens (pre- and postfiltration) may also have influenced the experiment. Sea star tissues are rich in collagen (O'Neill 1989), which gelatinizes above 100°C and recollagenizes below 50°C (Bozec & Odlyha 2011). In VSFs that are filtered prior to heating, most collagen would be removed. This effect may have affected results since collagen is labile to many marine microorganisms. The gelatinization of collagen and retention of gelatin on 0.22-μm filters may retain large macromolecules and microorganisms, which results in their lower concentration in material that is filtered after heating. Hence, comparisons of VSF and heat-treated VSF may induce different organic matter fields available to bacteria and other microorganisms, and overall responses to VSF of potential agents may be biased toward copiotrophic taxa that thrive on higher DOC concentrations.

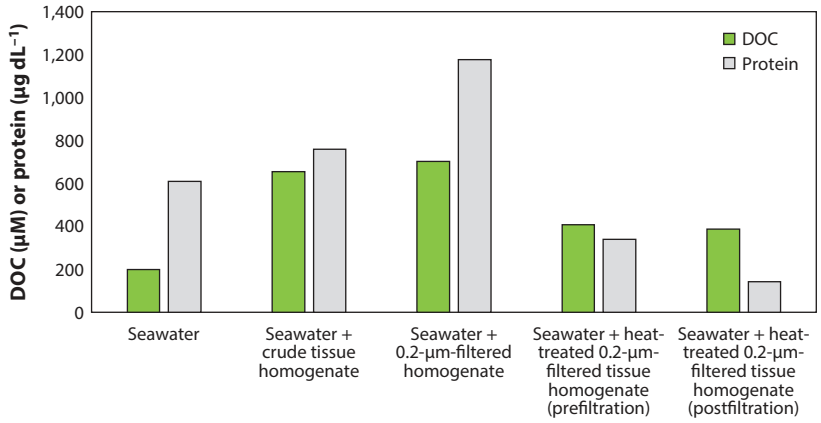

Figure 5

Concentrations of DOC and protein in 50 mL of Gulf of Mexico seawater with crude tissue homogenate, VSF, and heat-treated (pre- and postfiltration) VSF amendment. The VSF was prepared by homogenizing 1 g of *Pycnopodia helianthoides* body wall and epidermis per Hewson et al. (2014), and the amendments were 0.25 mL of prepared material in 50 mL of Gulf of Mexico seawater. Abbreviations: DOC, dissolved organic carbon; VSF, virus-sized fraction.

Future studies of suspected viral agents should focus on isolating viruses away from coextracted compounds through density gradient centrifugation or other purification methods, perhaps in concert with broad-scale antibiotics to inhibit bacterial growth. Furthermore, experiments employing the approach where abnormal specimens are cohoused with normal specimens, or where abnormal specimens are housed in aquaria and water from those aquaria is then used to challenge normal specimens, should be modified to include additional controls with euthanized specimens to account for saprobic or opportunistic taxa. Attention should also be paid to organic matter quantity and ammonia levels in challenge aquaria, since these may provide spurious responses in challenged specimens.

Fortunately, in the context of the 2022 *D. antillarum* MME, the suspect agent was easily cultivated from coelomic fluid collected from an abnormal urchin in the Florida Keys. The ciliate was then purified from enrichment cultures by simple dilution. Nevertheless, *P. apodigitiformis* cultures are not axenic, and there were a number of bacterial taxa, including those forming globulose biofilms. Hence, challenge experiments were performed in naive *D. antillarum* specimens using 5-μm-filtered *P. apodigitiformis* culture as controls. This approach ensured that the effect—which occurred in 60% of specimens—was due to the ciliate and not to bacteria, archaea, other microorganisms (e.g., viruses) or culture media that were amended during challenge.

6. ARE SEA STAR WASTING DISEASE AND THE 2022 *DIADEMA ANTILLARUM* MASS MORTALITY LINKED?

The finding of a ciliate responsible for mass mortality stimulated recent interest in their potential roles in SSWD. Initial examination of specimens to look for potential agents ignored larger eukaryotes since they were not observed by light microscopic examination. Rather, ciliates have been observed as endo- and ectosymbionts of echinoderms for some time (Barel & Kramers 1977 and references therein). rRNA-based molecular approaches are also mostly incompatible with animal microbiome investigations since primers target both host rRNAs (which are abundant) and protistan rRNAs (which are far less abundant than those of the host). Some part of this may be overcome with blocking primers (Del Campo et al. 2019) or CRISPR approaches (Zhong et al. 2021), which had not been developed at the time of the 2013–2014 investigation for sea stars. Histopathologic assessment observed ciliates on the surfaces of epidermal tissues of many specimens, but their pattern of association was inconsistent with the disease state, and in at least two species the proportion with ciliates was higher in grossly normal specimens than in abnormal sea stars (**Table 2**). Histopathology did not observe any invasive ciliate cells (or any other eukaryote; A. Newton, personal communication).

Several ciliates were observed among contig sequences assembled from viromes and directly sequenced from DNA specimens collected during the Bodega Bay organic matter enrichment experiment and during temporal observation of *P. ochraceus* wasting in Santa Cruz (Aquino et al. 2021, Hewson et al. 2020). The most common were philasterine ciliates, including those most closely related to *Homalogastra* spp. and *Paralembus digitiformis* (**Figure 6**). The pathogenic

Table 2 Proportions of histopathology specimens in which ciliates were observed on outer epidermis (A. Newton, personal communication)

Species	Grossly abnormal	Grossly normal	Total observed
Evasterias troschelii	1.0	1.0	11
Leptasterias hexactis	0.7	1.0	12
Pisaster ochraceus	0.6	0.4	61
Pycnopodia helianthoides	0.5	0.6	33

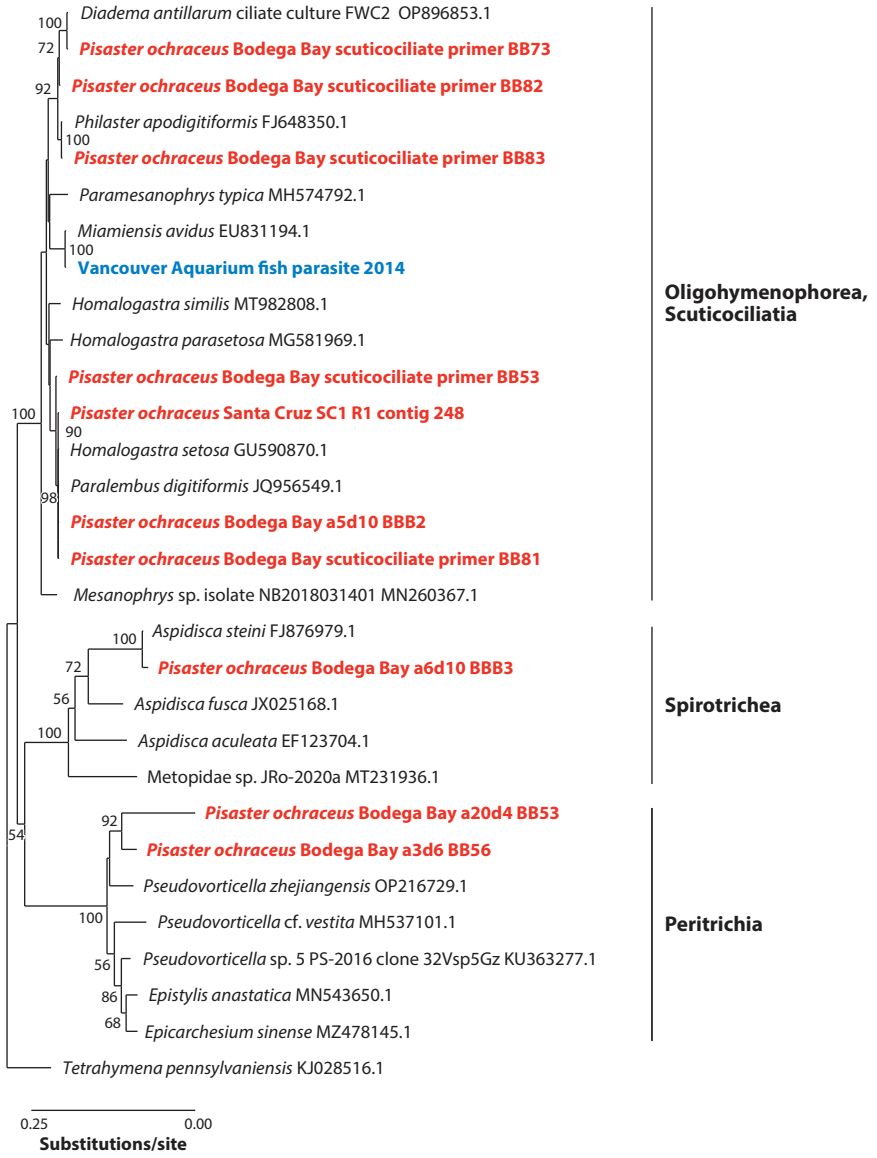

Figure 6

Ciliophora sequences recovered from virus-sized metagenomes and amplified by PCR using primers targeting the *Diadema antillarum* scuticociliatosis–associated *Philaster* clade (Vilanova-Cuevas et al. 2023) from Bodega Bay and Santa Cruz organic matter amendment studies (Aquino et al. 2021) and from an ulcerative lesion on *Anthias* sp. from the Vancouver Aquarium recovered in 2014. The phylogenetic reconstruction was based on a 392-nucleotide alignment (MUSCLE; Edgar 2004) of the 18S rRNA gene and constructed by neighbor joining and UPGMA in MEGA X (Kumar et al. 2018). Abbreviations: MEGA X, Molecular Evolutionary Genetics Analysis X; MUSCLE, multiple sequence comparison by log expectation; rRNA, ribosomal RNA; UPGMA, unweighted pair-group method with arithmetic means.

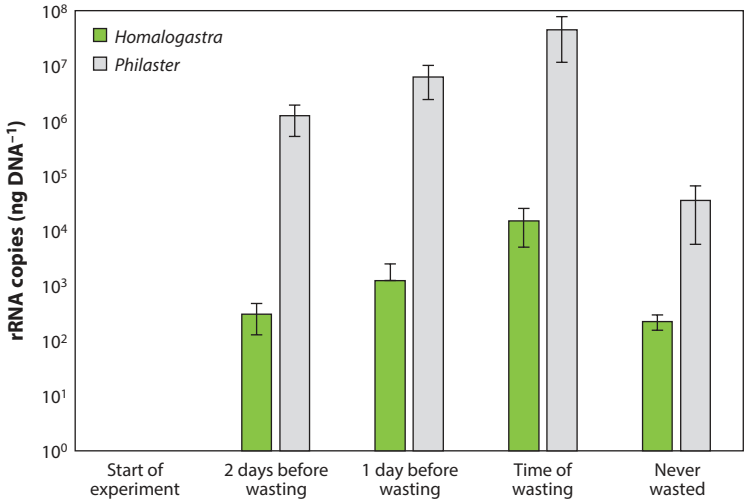

Figure 7

Ciliate rRNA gene abundance in swab samples collected from sea stars during the organic matter challenge on *Pisaster ochraceus* in July 2019 (Aquino et al. 2021). The copy number was determined by TaqMan qPCR using primers designed around the *Homalogastra*-like sequence recovered from viral metagenomes and the *Philaster apodigitiformis* primer/probe set from Hewson et al. (2023). Error bars show standard error. Abbreviations: qPCR, quantitative PCR; rRNA, ribosomal RNA.

scuticociliate *Miamiensis avidus* (then known as *Philasterides dicentrarchi*) was present in the Vancouver Aquarium at the time of SSWD, since we recovered it from a specimen of *Anthias* sp. (a teleost) that became ill at the time. Sequences of *Philaster* spp. were also found among contig spectra. The remaining ciliate sequences were hypotrich and peritrich ciliates.

We further investigated the dynamics of two scuticociliates over time in the Bodega Bay organic matter enrichment experiment (Aquino et al. 2021) by designing qPCR primers and probes around the 18S rRNA gene of the *Homalogastra*-like contig and applied a primer set previously developed based on the 28S rRNA gene of *P. apodigitiformis* (Hewson et al. 2023). Both ciliates were absent in animals at the start of experiment, but both had abundances of 10^2–10^3 and 10^6–10^7 rRNA copies μL^{-1} extract after 24–48 h (**Figure 7**). The abundance of both ciliates generally increased over time and was greatest at the time lesions appeared. Although less abundant, these same ciliates were present on asteroids that never wasted during the course of the study (14 days). These results suggest that ciliates become enriched by experimental conditions or under enrichment with various organic substrates but provide no evidence of any association with SSWD. Interestingly, amendment of organic substrates reduced overall ciliate loads across all affected sea stars, suggesting that they were consuming matter released from dying individuals. Additionally, a survey of 30 echinoderm specimens collected from San Diego, California, in September 2023 and 3 asteroid specimens affected by SSWD from Portaferry, Northern Ireland, did not yield any positive detections using the *P. apodigitiformis* 28S rRNA gene primer/probe set (Hewson et al. 2023).

As bacterivores, ciliates are clearly consumers of microorganisms that are stimulated by the production of organic matter, which is produced by phytoplankton and released through a variety of mechanisms, including decay of metazoans. In the context of SSWD, there is no evidence that ciliates are implicated in abnormalities, but amplification of *Philaster* sp. by qPCR from decaying experimental specimens is intriguing. While the qPCR primer set is known to provide

false positives due to close relatives (and are based on a conserved gene), retrospective analyses of available specimens from the region using primers that selectively amplify the *D. antillarum* scuticociliatosis–associated *Philaster* clade (Vilanova-Cuevas et al. 2023) should be prioritized.

7. CONCLUSIONS

The mysterious nature of SSWD has generated considerable attention from the scientific community and public alike. More than 10 years of concerted microbiological and veterinary investigation have failed to yield distinct causative agents, despite earlier reports. Inadequate experimental design and biased sampling may have contributed to the opaque results. Despite this, lessons learned from the 2013–2014 investigation may be used to guide further efforts to understand marine invertebrate diseases. Understanding the ecology of microorganisms associated with grossly normal specimens would strongly aid in the identification of novel candidate microbes or dysbiotic microbial assemblages. Future investigations should remain agnostic to cause (including whether the condition is transmissible or a response to stressors) until independent lines of investigation concur and should resist pressures to identify specific groups of candidate agents. Instead, investigations should begin with simple and easily deployable microscopic investigations, then follow with parallel histopathology and microbial surveys that include reference specimens and sample across biological realms.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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