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DISEASES OF AQUATIC ORGANISMS
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OPINION PIECE

Technical pitfalls that bias comparative microbial community analyses of aquatic disease

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ABSTRACT: The accessibility of high-throughput DNA sequencing technologies has attracted the application of comparative microbial analyses to study diseases. These studies present a window into host microbiome diversity and composition that can be used to address ecological theory in the context of host biology and behavior. Recently, comparative microbiome studies have been used to study non-vertebrate aquatic diseases to elucidate microorganisms potentially involved in disease processes or in disease prevention. These investigations suffer from many well-described biases, especially prior to sequence analyses, that could lead to misleading conclusions. Microbiome-focused studies of aquatic metazoan diseases provide valuable documentation of microbial ecology, although, they are only a starting point for establishing disease etiology, which demands quantitative validation through targeted approaches. The microbiome approach to understanding disease is most useful after laboratory diagnostics guided by pathology have failed to identify a causative agent. This opinion piece presents several technical pitfalls which may affect wider interpretation of microbe–host interactions through comparative microbial community analyses and provides recommendations, based on studies in non-aquatic systems, for incorporation into future aquatic disease research.

KEY WORDS: Microbiome · Virus · Eukaryotes · Community · Disease

1. INTRODUCTION

Improvements in sequencing technologies and costs over the last decade have allowed elucidation of the complex interaction between microbial communities and their hosts, known as the host-associated microbiome (Lederberg & McCray 2001). An attractive extension of fundamental microbiome ecology is its relationship to disease (Koren & Rosenberg 2006, Egan & Gardiner 2016). Numerous studies have identified links between microbiome composition (MC) and vertebrate conditions, e.g. obesity (Turnbaugh et al. 2006), susceptibility to infection by commensal microorganisms (Chang et al. 2008) and metabolic and cardiovascular diseases (Ordovas & Mooser 2006, Wen et al. 2008). While most studies focus on human microbiomes, there is increasing interest in their use in studying wildlife diseases (Galan et al. 2016). In aquatic habitats, metabarcoding and metagenomic approaches are gaining popularity in the study of aquacultured taxa (reviewed in Alavandi & Poornima 2012, Gómez-Chiarri et al. 2015, Munang'andu 2016, Munang'andu et al. 2017).

While studies of the association between MC and aquatic diseases have taken place since the late 1990s (Cooney et al. 2002, Pantos et al. 2003), renewed interest has spawned from greater accessibility of high-throughput sequencing (HTS)-based metagenomic and metabarcoding approaches and bioinformatics/analytical tools (Table 1). These studies extend from earlier fundamental microbial ecological investigations asking: Who is present and why?

Microbial community analyses in the context of disease are, however, subject to many biases, including those imposed by sampling strategy (e.g. Tedjo

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Table 1. Summary of nucleic acid template (NAT) extraction, quantification and standardization in studies of aquatic invertebrate diseases comparing asymptomatic to disease-affected microbial communities in/on tissues. NAT extraction protocols — 1: Agencourt DNAdvance Bead Extraction Kit; 2: bead-beating, freeze–thaw and phenol–chloroform extraction; 3: CTAB extraction and isopropanol extraction; 4: GE Illustra TriplePrep Kit; 5: Macherey-Nagel NucleoSpin Soil Kit; 6: MoBio PowerPlant DNA Extraction Kit; 7: MoBio PowerSoil DNA Kit; 8: MoBio Soil DNA Isolation Kit; 9: MP Biomedicals FastDNA Spin Kit; 10: organic extraction protocol; 11: Proteinase K, guanadinium isothiocyanate and isopropanol extraction; 12: QBiogene FastDNA Kit; 13: QIAmp DNA Stool Mini Kit; 14: Qiagen AllPrep DNA/RNA Mini Kit; 15: Qiagen DNeasy Blood & Tissue Kit; 16: Qiagen DNeasy Plant Kit; 17: Qiagen Mini Kit; 18: Qiagen RNeasy Plus Kit; 19: Roche High Pure Template PCR Preparation Kit; 20: sodium dodecyl sulfate, potassium acetate and isopropanol extraction; 21: Stratec RTP Bacteria Mini Kit; 22: sucrose, sodium dodecyl sulfate, lysozyme, Proteinase K and phenol–chloroform extraction; 23: TRIzol Reagent Kit; 24: Zymo ZR Soil Microbe DNA MicroPrep Kit; 25: Zymo Quick gDNA Kit; 26: Zymo ZR Tissue and Insect Kit; 27: Zymo ZR Viral DNA Kit. NATQ: nucleic acid template quantity (ng if mass or µl if volume); B: bacteria; C: cyanobacteria; V: viruses; F: fungi; MB: metabarcoding of 16S rRNA; CL: clone libraries of 16S rRNA; LH-PCR: length heterogeneity PCR; LASL: linker-amplified shotgun libraries; ϕ29: ϕ29 DNA polymerase; VM: viral metagenome; BM: bacterial metagenome; TRFLp: terminal restriction fragment length polymorphism; F: fluorescence based; UV: spectrophotometer based; M: mass of NAT; V: volume of NAT; NR: not reported

Phylum/host	Disease	Target domain	Approach used	NAT extraction protocol	NAT quantification	Template standardization	NATQ	Reference
Arthropoda								
Litopeneaus vannamei	Early mortality/ acute pancreatic necrosis disease	B	MB	24	F	M	200	Cornejo-Granados et al. (2017)
Homarus americanus	Epizootic shell disease	B	LH-PCR	9	NR	NR	NR	Meres (2016)
H. americanus	Epizootic shell disease	B	LH-PCR	NR	NR	NR	NR	Meres et al. (2012)
H. americanus	Shell disease	B	MB	7	NR	V	0.5–2	Feinman et al. (2017)
Litopenaeus vannamei	Unnamed disease event	B	MB	13	NR	M	50	Xiong et al. (2015)
L. vannamei	Unnamed disease event	B	MB	13	NR	M	50	Xiong et al. (2017)
Synisoma nadejda	Acidification impacts	B	MB	25	NR	M	~10	Aires et al. (2018)
Cnidaria								
Gorgonia ventalina	Aspergillosis	B	LASL/ϕ29-VM	18	NR	V	1	Hewson et al. (2012)
Montastrea annularis and *Diploria strigosa*	Black band disease	C	TRFLP	2	NR	V	5–30	Frias-Lopez et al. (2004)
Montastraea, Orbicella and *Pseudodiploria*	Black band disease	B	MB	7	NR	NR	NR	Meyer et al. (2016a)
Montastraea cavernosa	Black band disease	B	MB/BM	7	NR	NR	NR	Meyer et al. (2017)
Favites sp. and *Favia* sp.	Black band disease	B	CL	7	NR	V	1–2	Barneah et al. (2007)
Coral	Bleaching	V	LASL-VM	3	NR	NR	NR	Marhaver et al. (2008)
Acropora millepora	Bleaching	B	ϕ29-VM	NR	NR	NR	NR	Littman et al. (2011)
Porites lobata	Bleaching	B	MB	14	UV	M	5–35	Hadaidi et al. (2018)
Porites lutea	Bleaching	B	MB	19	UV	M	50	Pootakham et al. (2018)
Acropora millepora	Bleaching	B	CL	22	UV	NR	NR	Bourne et al. (2008)
Montastraea, Orbicella and *Pseudodiploria*	Dark spot syndrome	B	MB	7	NR	NR	NR	Meyer et al. (2016b)
Stephanocoenia intersepta	Dark spot syndrome	B/F	DGGE	15	NR	M	20	Sweet et al. (2013)
Pacifigorgia cairnsi	Necrotic patch disease	B	MB	7	UV/F	V	1	Quintanilla et al. (2018)
Eunicea flexuosa and *Pseudoplexaura porosa*	Physical injury	B	MB	NR	NR	NR	NR	Shirur et al. (2016)
Porites astreoides	Unnamed disease event	B	MB	7	NR	M	~15	Meyer et al. (2014)
Paramuricea clavata	Unnamed disease event	B	MB	19	F	M	10	Vezzulli et al. (2013)
Porites compressa	Various stress impacts	B	ϕ29-BM	17	NR	M	1	Vega Thurber et al. (2009)
Gorgonia ventalina and *Orbicella faveolata*	Warming impacts	B	MB	7	F	M	14	Tracy et al. (2015)
Acropora darmicornis	White band disease	B	MB	1	NR	NR	NR	Gignoux-Wolfsohn et al. (2017)
A. darmicornis	White band disease	B	MB	1	NR	NR	NR	Gignoux-Wolfsohn & Vollmer (2015)
Acropora hyacinthus	White band disease	B	MB	6	NR	NR	NR	Pollock et al. (2017)
Montastrea faveolata	White band disease	B	CL/PhyloChip	11	NR	M	50	Sunagawa et al. (2009)
Pavona duerdeni and *Porites lutea*	White band disease	B	Microarray	16	UV/F	NR	0.15–0.5	Roder et al. (2014)
Porites lutea	White patch syndrome	B	MB	5	NR	M	10	Séré et al. (2013)
Montastraea annularis	White plague disease	V	ϕ29-VM	10	NR	NR	NR	Soffer et al. (2014)
M. annularis	White plague disease	B	DGGE	12	NR	V	1.5	Pantos et al. (2003)
Acropora palmata	White pox disease	B	MB	3	NR	NR	NR	Lesser & Jarett (2014)
Echinopora lamellosa	White syndrome	B	MB	15	NR	M	2	Smith et al. 2015)
Orbicella faveolata	Yellow band disease	B	CL/PhyloChip	6	NR	M	20	Closek et al. 2014)
Montastrea faveolata	Yellow band disease	B	DGGE	15	NR	NR	NR	Cróquer et al. (2013)

(Table 1 continued on next page)

Table 1 (continued)

Phylum/host	Disease	Target domain	Approach used	NAT extraction protocol	NAT quantification	Template standardization	NATQ	Reference
Echinodermata								
Tripneustes gratilla	Bald sea urchin disease	B	MB	21	F/qPCR	NR	NR	Brink et al. (2019)
Odontaster validus	Unnamed disease event	B/F	MB	3	NR	V	1.5	Núñez-Pons et al. (2018)
Acanthaster planci	Unnamed disease event	B	MB	26	UV	NR	NR	Hój et al. (2018)
Pisaster ochraceus	Wasting	B	MB	23	UV	V	2	Lloyd & Pespeni (2018)
Asteroids	Wasting	V	ϕ29-VM	27	NR	V	1	Hewson et al. (2014)
Mollusca								
Pinctada fucata martensii	Akoya oyster disease	B	LASL-BM	NR	NR	NR	NR	Matsuyama et al. (2017)
Saccostrea glomerata	*Marteilia sydneyi* infection	B	CL	2	NR	M	1–50	Green & Barnes (2010)
Crassostrea gigas	Unnamed disease event	B	MB	15	NR	NR	NR	King et al. (2019)
C. gigas	*Vibrio* infection	B	MB	4	UV	M	20	Lokmer & Wegner (2015)
Porifera								
Ircinia fasciculata	Unnamed die-off	B	MB	15	UV/F	NR	NR	Blanquer et al. (2016)
Geodia barretti	Unnamed disease event	B	MB	8	NR	V	1	Luter et al. (2017)
Aplysina aerophoba	Unnamed disease event	B	DGGE	21	NR	NR	NR	Webster et al. (2008)
Rhodophyta								
Delisea pulchra	Bleaching	B	MB	NR	NR	V	1	Zozaya-Valdes et al. (2015)

et al. 2015), nucleic acid extraction and processing (Kennedy et al. 2014), PCR biases (see below), sequencing chemistry (Clooney et al. 2016) and bioinformatic analyses of sequence libraries (Allali et al. 2017). This opinion piece focuses on biases introduced by amplification-based protocols, such as PCR (Mullis et al. 1986) and ϕ29 polymerase-based multiple displacement amplification (Dean et al. 2001). To overcome the extraordinarily small amount of genetic material in individual cells relative to input DNA requirements for HTS sequencing, researchers employ PCR (Mullis et al. 1986) to amplify extracted mixed community DNA (i.e. template). Historically, PCR-amplified material was cloned into competent *E. coli* prior to plasmid-based sequencing (Olsen et al. 1986, Pace et al. 1986). PCR-independent approaches (e.g. λ phage libraries and bacterial artificial chromosomes) (Schmidt et al. 1991, Stein et al. 1996) were applied to study MC for 2 main reasons: (1) to link conserved molecular housekeeping genes to metabolic functional genes and (2) to avoid biases associated with PCR amplification. PCR biases are introduced by primer design (Baker et al. 2003, Wang & Qian 2009, Youssef et al. 2009, Nossa et al. 2010, Gantner et al. 2011, Kumar et al. 2011, Soergel et al. 2012, Klindworth et al. 2013, Hugerth & Andersson 2017), skewed template-to-amplicon amplification ratios (Polz & Cavanaugh 1998), nucleic acid extraction variability (Polz et al. 1999), relative amplicon length (Suzuki et al. 1998) and secondary structure characteristics (Suzuki & Giovannoni 1996), preferential amplification based on G+C:A+T composition of targets and between-species rRNA copy number (Suzuki et al. 1998, Větrovský & Baldrian 2013) and primer choice biases (e.g. reviewed by Klindworth et al. 2013). Many HTS-based approaches, especially those used in the study of aquatic diseases, employ some type of amplification to reach input thresholds for sequencing.

Relative abundance of microbial taxa in samples of disease-affected tissues over time in comparison to asymptomatic tissues over time is often used to infer — somewhat vaguely — their potential roles in disease processes as either beneficial (relative abundance declining with disease) or potentially pathogenic (relative abundance increasing with disease) taxa (Koren & Rosenberg 2006, Egan & Gardiner 2016). The biological and ecological characteristics of these taxa are further extrapolated from genomic elements (detected in metagenomes or metatranscriptomes), or by linking housekeeping genes to predicted genome elements (Cha-Aim et al. 2012, Langille et al. 2013). While these comparisons generate testable hypotheses regarding the association between microorganisms and disease, they are rarely tested epidemiologically or experimentally. Moreover, few attempts are ever made to link the suspect infectious agent to tissue pathology at the microscopic level (Work & Meteyer 2014). There are underappreciated artifacts and biases introduced during sampling, DNA extraction and HTS library preparation which could affect interpretation of MC surveys. Furthermore, geographical and ecological factors may drive MC (e.g. Schöttner et al. 2012), which may confound differences relating to the disease itself.

2. BACTERIA FOLLOW DISSOLVED ORGANIC CARBON

Quantitative comparison of amplification-generated microbial community profiles — where overall microbial abundance is unknown — potentially leads to problems in downstream interpretation of disease or healthy association. Observations of greater representation of viral (Hewson et al. 2014, Soffer et al. 2014), bacterial (Lloyd & Pespeni 2018) or fungal (Sweet et al. 2013) operational taxonomic units in disease-affected tissues relative to asymptomatic tissues may, in part, be driven by variations in overall microbial abundance.

There have been few studies examining bacterial abundance associated with aquatic metazoans and fewer still comparing the abundance of microorganisms between asymptomatic and disease-associated states (Tables 1 & 2). This is likely due to the time effectiveness of processing multiple samples or complications in counting DNA-containing particles against a backdrop of autofluorescence when using epifluorescence microscopy (Garren & Azam 2010, Leruste et al. 2012) or flow cytometry (Bettarel et al. 2016). Flow cytometry may be advantageous in estimating the abundance of microorganisms associated with aquatic hosts because of heterogeneity in cell distribution across membrane filters used in epifluorescence microscopy (Muthukrishnan et al. 2016). The abundance of bacteria and viruses in coral mucus and tissues is generally enriched relative to surrounding seawater by 3- to 10-fold (Table 2). Differences in overall microbial abundance are expected between disease-affected and asymptomatic tissues and their immediate overlying waters. Because heterotrophic

Table 2. Microbial abundances reported in association with marine invertebrates. WSS: white spot syndrome; WS: white syndrome; VLP: virus-like particle; –: no disease investigated

Host organism	Disease	Sample location	Result	Abundance	Reference
Acropora digitifera	–	Various	Bacterial abundance significantly lower on white coral skeleton than on natural coral rubbles	$1.08 \pm 0.01 \times 10^3$ cells ml^{-1} in skeleton; $1.82 \pm 0.01 \times 10^6$ cells ml^{-1} on coral rubble	Islam et al. (2016)
Echinopora lamellosa	WSS	Lesions prior to complete tissue loss	Bacterial abundance significantly higher in degrading tissues but no difference with ampicillin treatment	$2.9–3.0 \times 10^7$ cells cm^{-2} in asymptomatic and ampicillin-treated WSS-affected corals; 3.5×10^7 cells cm^{-2} in WSS-affected corals (no significant difference)	Smith et al. (2015)
Alveopora, Favia, Fungia, Heteroxenia, Lobophyllia, Platygyra, Turbinaria	–	Mucus	Viral abundance	$4–11.5 \times 10^7$ VLP ml^{-1}	Leruste et al. (2012)
Fungia rupanda, Acropora formosa	–	Mucus	Viral/ bacterial abundance	$2.3–5.4 \times 10^7$ VLP ml^{-1} and $4.5–9.2 \times 10^6$ cells ml^{-1}	Nguyen-Kim et al. (2015)
Pocillopora damicornis	–	Coral surfaces	Bacterial abundance	1.3×10^5 and 35.9×10^5 cells cm^{-2} when enriched with peptone	Garren & Azam (2012)
Porites lobata	–	Mucus	Bacterial abundance	$5.3–17.9 \times 10^5$ cells ml^{-1}	Garren & Azam (2010)
Seria histrix, Echinopora lamellosa, Echinopora sp.	WS	Not stated	Bacterial and *Vibrio* abundance in some cases elevated, in cases decreased in WS-affected tissues	~1×10^6 to 1.2×10^8 cells cm^{-3} in asymptomatic tissues; 1×10^6 to 6.4×10^8 cells cm^{-3} in WS-affected tissues	Luna et al. (2010)
Oculina patagonica	–	Mucus and tissue	Bacterial abundance	6.2×10^7 cells cm^{-2} in mucus and 8.3×10^8 cells cm^{-2} in tissue	Koren & Rosenberg (2006)

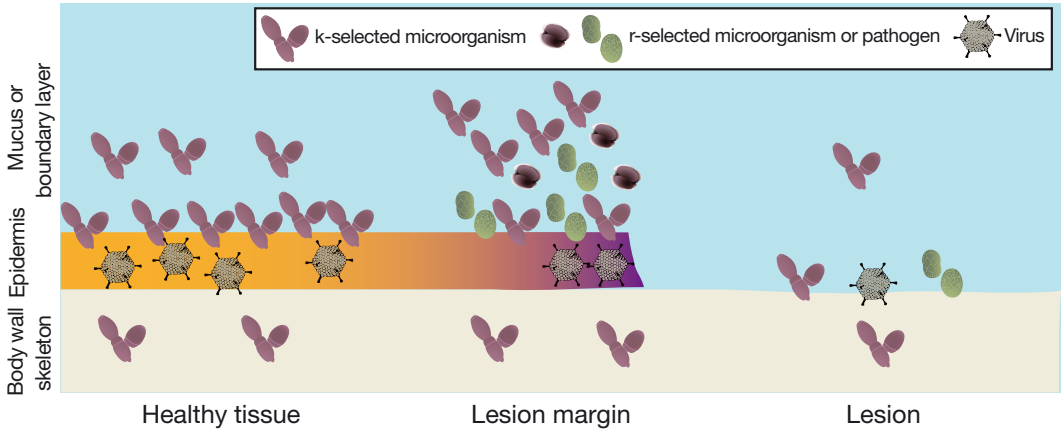

Fig. 1. Conceptualization of bacterial abundance on or near apparently healthy and lesion surfaces of hard skeleton-bearing metazoa (e.g. cnidarian). Healthy tissues include the basal and surface body wall with epidermis which may exude organic matter (mucus), which in turn supports bacterial proliferation. At lesion margins, decomposing tissues support bacterial growth (saprophytes and opportunists, in addition to pathogens). In the center of lesions, epidermal tissues may be lost, and the underlying skeleton may support fewer bacteria since organic matter is in lower quantity. Conversely, the bare skeleton may support the growth of opportunist, surface-associated microorganisms

bacterioplankton consume organic carbon (Ducklow 1983), they likely thrive on surfaces on which there is high dissolved organic carbon (DOC) availability (Fig. 1). These may include both unaffected tissues of metazoa which exchange DOC with overlying waters through excretion or exudation of mucus (Ducklow & Mitchell 1979) and disease-affected tissues which undergo degradation to DOC. Cooney et al. (2002) noted that DNA extracts were more consistently amplifiable using universal 16S rRNA primers from black band disease-affected than asymptomatic coral tissues, suggesting a greater abundance of bacteria in disease-affected tissues. Microscope counts of bacteria by Luna et al. (2010) demonstrated significantly higher bacterial abundance in white syndrome-affected corals compared to asymptomatic tissues in 15 comparisons but also observed 2 species in which bacterial abundance was lower in lesions compared to healthy tissues (the location of samples around or within lesions was not reported). Likewise, abundance of bacteria was higher in white spot-affected *Echinopora lamellosa* tissues compared to controls, but disease-affected corals that were treated with the antibiotic ampicillin were no different from healthy tissues (Smith et al. 2015). It is clear that bacteria on aquatic animal surfaces have a very large capacity to respond to nutrient inputs. For example, Garren & Azam (2012) noted a 10-fold change in bacterial abundance in peptone-enriched coral mucus relative to controls, a finding which corresponds with observations of coral mucus as an important source of bacterial nutrition in coral reef ecosystems (Wild et al. 2004a,b, 2005, Huettel et al. 2006). On the other hand, in areas of low metazoan cell abundance such as proteinaceous or mineralized matrices which are characteristic of lesions in many invertebrate taxa (e.g. tissue loss in corals; Work & Aeby 2006), the abundance of bacteria may be lower than that in surrounding tissues because of minimal DOC on which they depend. For instance, Islam et al. (2016) reported that overall bacterial abundances were 3 orders of magnitude lower in clean coral skeletons than in natural rubbles.

A wide range of template DNA quantities are used in analyses of aquatic invertebrate disease (Table 1). Variation in nucleic acid extraction protocol also has significant impacts on observed bacterial composition (Kennedy et al. 2014, Sinha et al. 2017). Among 50 studies comparing disease-affected and asymptomatic tissues of aquatic invertebrates, 27 different nucleic protocols were used (in 5 studies, the nucleic acid extraction protocol was not reported) (Table 1). These observed variations in MC structure urge caution in interstudy comparisons. Extraction kits utilizing mechanical lysis (e.g. bead beating) are recommended since they lead to higher DNA yield (e.g. Henderson et al. 2013), higher bacterial diversity (e.g. Maukonen et al. 2012) and better extraction from bacteria with more chemically resistant cell walls (e.g. gram-positive and spore-forming bacteria) (e.g. Salonen et al. 2010, reviewed in Pollock et al. 2018).

Assuming an average of 25 ng per reaction (Table 1) and average bacterial DNA quantity per cell of 2 fg (Vrede et al. 2002), an estimate of total bacterial load in DNA templates is ~1 × 10^7 cells. In

contrast, studies standardizing by volume of extracted DNA typically use 1 to 2 µl of extracted DNA. Most commercially available DNA extraction kits demand <200 mg of tissue and result in 30 to 100 µl of DNA extract. Based on average abundances of bacteria in coral tissues or mucus (~10^5 cells ml^{-1}) and assuming that microbial DNA comprises only ~10% of total DNA extraction quantity, PCR templates therefore effectively target ~2000 microbial cells. Considering that extraction efficiencies of DNA are rarely close to 100%, it is likely that the total number of individual microbial particles captured in HTS surveys is considerably less than reported. Hence, microbial DNA assayed by PCR may be only a tiny fraction of overall microbial community DNA. While capturing abundant constituents of microbial communities, comparisons with lower effective sampling depth (before PCR) may result in skewed proportions of rarer constituents, which in turn may lead to spurious identification of candidate disease-associated taxa.

We recently compared the total extracted DNA quantity of 34 body wall biopsy punch samples (5 mm diameter) collected from asteroids during a temporal study of sea star wasting. After 14 d in an aquarium system, individual asteroids began to show signs of wasting, and by 19 d, all individuals (n = 12) had died. Initially, we sampled grossly normal tissues, but after 14 and 19 d, we collected tissue from the center of lesions. The overall quantity of DNA extracted from each sample decreased from 0 to 19 d (Fig. 2). Notably, 5 of 12 total samples collected at 19 d had DNA quantities below the detection threshold (determined by PicoGreen fluorescence). Studies which standardize DNA template based on extracted volume, particularly for samples in which DNA concentration is below detection thresholds, will suffer from inherent biases associated with variable template amounts discussed in Section 3. This may be exacerbated by inaccurate DNA quantification. Quantification of DNA with UV spectroscopy, for example, which is widely used in studies of microbial communities associated with diseases, has well-known inaccuracies in tissue settings as a result of co-extracted organic molecules (Holden et al. 2009). For this reason, it is recommended that binding dye fluorescence-based approaches over UV spectroscopy-based approaches be used to quantify template DNA. It is worth noting that the current version of the NanoDrop spectrophotometer (One/OneC; part number ND-ONE-W; Thermo Fisher Scientific) includes a new 1 sample contaminant identification protocol (Acclaro) which may provide utility to microbiome research.

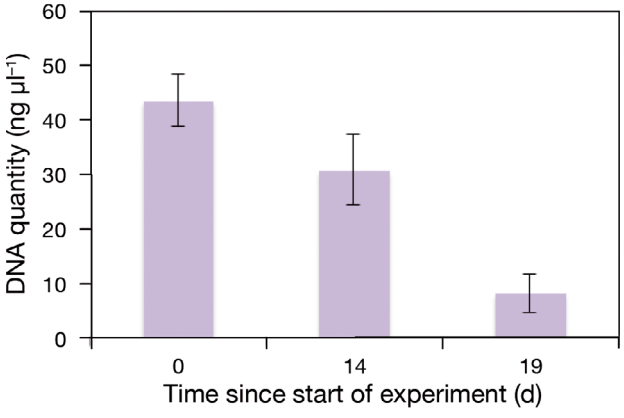

Fig. 2. Quantity of DNA extracted from tissue samples (5 mm biopsy punch of body wall tissues) of *Pisaster ochraceus* (Asteroidea, Echinodermata) during an experiment in August 2018. After 14 d individuals began to waste, and by 19 d animals experienced widespread lesions. Punches were taken from clinically normal tissues before the appearance of lesions or from lesion margins after the appearance of sea star wasting at 14 and 19 d. Error bars = SE. Decrease in DNA quantity was only significant ($p < 0.05$, Student's *t*-test) between 0 and 19 d

3. ASSUMPTIONS OF EQUAL SAMPLING

Sampling with equal depth is a key assumption in any β diversity analyses comparing the similarity of biological communities quantitatively (i.e. through analysis of relative abundance patterns). On average, more unique taxa will be present in samples sequenced in greater depth than in samples with shallow depths (Hughes & Bohannan 2004). Because of this, when comparing different sizes, it is advised to use similarity indices that place greater importance on richness (i.e. presence/absence; qualitative) than on entirely quantitative metrics (Hughes & Bohannan 2004, Cardoso et al. 2009). Quantitative interpretation of microbial community patterns based on PCR is achieved by normalizing input (template) nucleic acid quantities between samples (Borneman & Triplett 1997, Hewson & Fuhrman 2004, Brown et al. 2005). Reducing PCR biases in contemporary HTS-based approaches is achieved by normalizing overall template DNA quantity in PCR by either volume or mass of DNA, normalizing PCR amplicon quantity between samples during library preparation, manipulating PCR cycling conditions and combining replicate PCR products. Equal sampling of PCR amplicons is achieved by rarifying library sizes to the same number of sequences (Aird et al. 2010, Kennedy et al. 2014), where resampled rarefaction analyses show complete coverage. There can be concerns with false

positives in rarified library comparisons (McMurdie & Holmes 2014). Additionally, rarifying DNA sequence libraries does not account for potentially unequal microbial abundances between samples (i.e. sampling that occurs at the PCR stage as template material) and may be best examined by qualitative over quantitative metrics. Kennedy et al. (2014) evaluated the effects of template mass variation on taxonomic profiles of bacterial communities and deduced that DNA template quantity had significant impacts on profile variability across a variety of samples. This study also emphasized optimization of template concentration to minimize variability in studies comparing microbial communities. Seashols-Williams et al. (2018) found that varying template amounts did not affect the relative abundances of major bacterial genera in libraries but caused significant variation in the relative abundance of rarer genera.

Biases in the amplification of templates prior to HTS are not limited to PCR-based approaches. Rolling circle amplification (also referred to as strand displacement amplification and multiple displacement amplification [MDA]; Dean et al. 2001) uses ϕ29 DNA polymerase to generate large quantities of amplified material from extremely small (<1 ng) template quantities. Pinard et al. (2006) found that MDA generated biases that were 119 to 165 times greater than unamplified controls, while Yilmaz et al. (2010) showed pronounced unidirectional skewing in 16S rRNA libraries when templates were MDA amplified. For example, while systematic biases in overrepresentation of single-stranded DNA (ssDNA) genomes in virome libraries prepared from standardized template quantity do not impact β diversity analyses (Yilmaz et al. 2010, Parras-Moltó et al. 2018), the overrepresentation of ssDNA genomes (especially circularized templates, like plasmids) in low-template libraries (Kim et al. 2008) is cause for concern when comparing samples of variable template amounts. Other genomic approaches demanding HTS sample preparation (e.g. linker-amplified shotgun libraries; Breitbart et al. 2002) rely on at least 1 PCR step and experience the same biases as noted above.

Standardizing template quantities (~equal sample sizes) is rarely performed in studies of aquatic animal diseases. In a survey of 50 studies comparing MC in aquatic disease-affected and asymptomatic tissues, 21 did not specify PCR template amounts, and a further 12 studies standardized PCR templates by volume of extracted material or lysate (Table 1). While standardization of template DNA mass is appropriate when the majority of template DNA originates from targets, this approach poses problems when the proportion of microbial target nucleic acids to non-target nucleic acids (i.e. from host) is unknown. This is further exacerbated by the potentially large contribution of host DNA relative to microorganisms in studies of host-associated microorganisms (i.e. microbiomes).

One approach to overcoming the problem of sampling variation in MC studies proposed by Seashols-Williams et al. (2018) employs quantitative PCR (qPCR) using universal 16S rRNA PCR primers to standardize bacterial genome equivalents prior to HTS library preparation. It is, however, worthwhile noting that apparently universal primer sets rarely encompass all known bacterial 16S rRNAs. For example, Wang & Qian (2009) found that in 30 primer sets used in community analyses, most had >90% coverage of known 16S rRNAs in the Ribosomal Database Project. However, 30% had <90% coverage (Wang & Qian 2009). Hence, even when using qPCR to standardize genome equivalents, care must be taken to select primer sets targeting the phylogeny of bacteria expected as constituents of the microbiome. Because viruses lack universally conserved genes, this approach is not viable in studies of viral composition. However, quantifying total viral load in tissues by microscopy (Leruste et al. 2012), especially after purification of isolated viruses (Marhaver et al. 2008, Thurber et al. 2009, Soffer et al. 2014), and standardizing quantities between metaviromes offers an alternate approach. While these approaches may lead to truly quantitative comparisons between disease-affected and asymptomatic MC, they are also subject to contamination issues noted below.

4. REAGENT CONTAMINANTS ARE EVERYWHERE

Another critical source of error in comparative microbial community analyses is the presence of contaminating DNA in molecular biology reagents or laboratories. Our knowledge of such contaminants comes from research areas where low abundance of target genes is expected. Zehr et al. (2003) reported the widespread occurrence of diverse nitrogenase (*nifH*) genes in PCR reagents, several of which were also recovered from a broad range of low-abundance studies, including arthropod microbiomes. Contamination has been noted in 16S rRNA surveys of low-biomass environments (Tanner et al. 1998, Grahn et al. 2003, Salter et al. 2014, Glassing et al. 2016, Lauder et al. 2016, Kim et al. 2017, Karstens et al. 2018, Velásquez-Mejía et al. 2018, Stinson et al.

2019, Weyrich et al. 2019) including ancient, human-built environmental, geomicrobial or forensic DNA (Willerslev et al. 2004, Fierer et al. 2008, Witt et al. 2009, Dunn et al. 2013, Weyrich et al. 2017). Many of these contaminants originate from ultrapure water (McFeters et al. 1993, Nogami et al. 1998, McAlister et al. 2002) or PCR reagents (Shen et al. 2006). Alarmingly, many of the contaminants share similarity with normal inhabitants of the environments targeted in sampling strategies (Salter et al. 2014, Glassing et al. 2016, Sheik et al. 2018). Because of the relatively high cost of sequencing (even with the advent of HTS), negative or positive controls, including blank samples, are rarely included in HTS microbial surveys. Yet, these are critical for understanding the diversity of potential contaminants specific to reagents and laboratory settings and are highly recommended for all community-level studies. For example, Naccache et al. (2013) identified a parvovirus–circovirus hybrid associated with seronegative hepatitis (A-E). This discovery led to further epidemiological investigation until it was resolved that the virus did not originate from template material but rather was present in spin columns used in DNA extraction. Optimal strategies for accounting for reagent or laboratory contaminants are not yet fully resolved; however, subtracting sequences matching negative control contaminants (Sheik et al. 2018), cleaning PCR reagents prior to use (reviewed in Eisenhofer et al. 2019) or identifying contaminant sequences in sequencing libraries upon reporting of results (Glassing et al. 2016) are recommended. Considering variability in template quantities used to amplify communities, it is possible that disease-affected tissue microbiomes could contain disproportionate numbers of contaminant sequences. When comparing disease-associated microbial communities to those from asymptomatic tissues, the inclusion of negative controls and accounting for detected sequences in those controls is a significant consideration. The use of positive controls (e.g. mock communities) is also recommended to identify systematically overrepresented operational taxonomic units in microbiome analyses and to assess extraction biases (Eisenhofer et al. 2019).

5. NEGATIVE CONTROLS MAY NOT ALWAYS BE NEGATIVE

The presence of non-microbial DNA in microbial community surveys may also artifactually increase amplification of contaminants. Carrier DNA (i.e. non-template DNA) is frequently used in DNA extraction protocols and in PCR-based approaches when targeting rare template DNA, in particular when trying to avoid overexpression artifacts in expression vectors (Ellison et al. 2006). The addition of carrier DNA improves the efficiency of template amplification (Handt et al. 1994, Köhler et al. 1997). This effect is thought to be a result of lower adsorption of low-quantity templates to plasticware in the presence of higher overall DNA quantities or reduction in encounters between templates and degrading enzyme activities (Handt et al. 1994), especially when using degraded DNA. In studies of host-associated microbiomes, the presence of non-template DNA may act as a carrier in PCR amplification of low-level targets (Handt et al. 1994, Köhler et al. 1997) but at the same time cause efficient amplification of contaminating DNA. This effect would not be observed in deionized water negative controls at the PCR stage. Hence, the use of carrier DNA (e.g. non-microbial purified DNA) is recommended in 16S rRNA library preparation. Unfortunately, there is no way to account for the carrier DNA effect in metagenomic surveys, since all nucleic acids are amplified during this approach.

6. MISLEADING RESULTS IN COMPARATIVE AQUATIC DISEASE MICROBIAL SURVEYS

To illustrate the difficulties of unequal sample sizes and contaminants, I present here an example of a promising candidate pathogen which turned out to be a red herring. Viral metagenomics was used to explore viruses associated with lesions of a sea fan wasting disease, which affected the gorgonian octocoral *Gorgonia ventalina* in Puerto Rico from 2012 to 2013. Gorgonians comprise a skeleton of proteinaceous material (gorgonin) over which lie 3 cell layers. Samples from asymptomatic tissues and from the center of lesions were homogenized separately and viral metagenomes prepared following standard protocols (Thurber et al. 2009) where MDA was applied to equal volumes of extracted viral DNA. Quantitative comparative analyses of metagenome read recruitment revealed a number of contiguous sequences (contigs) that were present in lesions and absent in apparently healthy tissue, and a number of these were annotated viral based on translated nucleotide–protein BLAST (BLASTx) comparison to the non-redundant database at NCBI (Fig. 3). Based on read coverage, a promising, highly represented CRESS DNA virus-like disease-associated contig was chosen

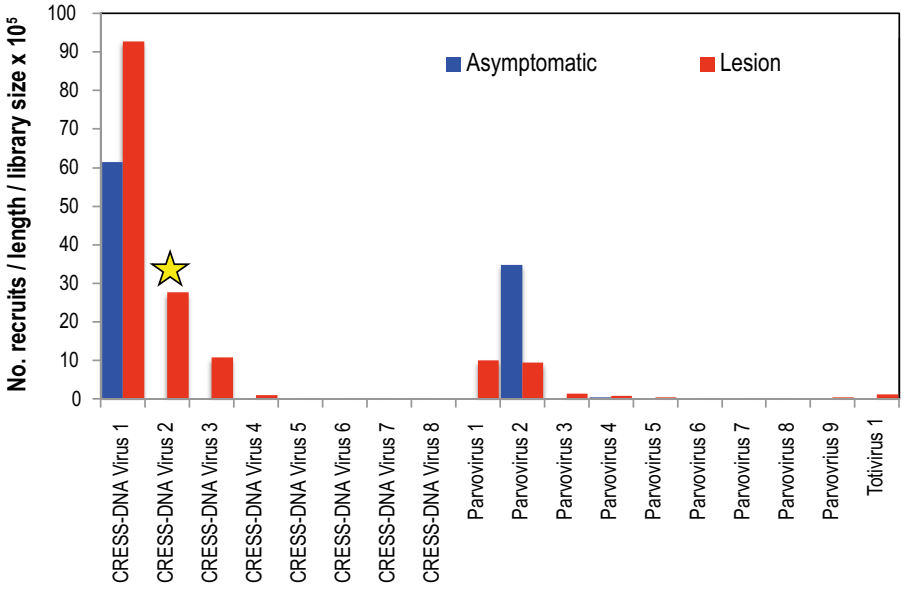

Fig. 3. Contig length and library size normalized read recruitment against 18 viral genome sequence bins. Binning was performed by pairwise BLASTn analysis with a cut-off of e < 10^{-20}. Annotation of contigs was performed based on comparison by BLASTx against the non-redundant database at NCBI. CRESS DNA Virus 2 was selected for downstream quantitative analyses since it had the greatest recruitment of any viral sequence bin that was absent in asymptomatic tissues

for a wider survey comparing lesions (n = 20) and apparently healthy tissues (n = 20) by quantitative PCR. However, the candidate disease-associated contig could not be detected in any sample, including those used as templates for metavirome construction. The origin of the disease-associated contig sequence was never determined; however, it may represent a reagent contaminant or very rare genotype that became well represented in libraries after amplification with MDA. I have observed this phenomenon in several other studies of aquatic diseases, which emphasizes the importance of post-genomic confirmation of putative pathogen detection by complementary approaches.

7. CAN WE REALLY INFER PATHOGENICITY BASED ON OBSERVED OR PREDICTED GENES?

Most HTS studies to date annotate microbial communities at lowest to genus level based on percent similarity across sequence cover or by phylogenetic clustering patterns (Huson et al. 2007). However, the identification of pathogens based solely on phylogeny is extremely problematic since closely related bacteria — even at the genus level — share disparate traits (Perna et al. 2001, Denef et al. 2010, Soto et al. 2014). For example, identification of known pathogenic and known beneficial bacterial genera is weak and may lead to spurious interpretations (Lamb et al. 2017). Another mechanism by which the ability of bacteria to generate pathology is inferred is through genomic analyses or by connecting predicted metabolic genes with housekeeping genes in metabarcoding surveys (Langille et al. 2013, reviewed in aquaculture settings by Ortiz-Estrada et al. 2019). The gene ontology most consistently used to ascribe pathogenicity is virulence factors (VFs), which most commonly allow bacteria to attach to surfaces, evade or suppress host immune systems, enter/exit cells, or obtain nutrients from hosts. While many VFs are specific to pathogens, homologs of VFs occur widely in non-pathogenic bacteria (Holden et al. 2004, Pallen & Wren 2007, Niu et al. 2013) and environmental bacteria (Persson et al. 2009), where they play a role in nutrient acquisition, are present on genomic islands (possibly indicating horizontal gene transfer) or may represent loose annotations to known clinical strains of poorly resolved function. Among 1901 *Vibrio* spp. genomes available in the Integrated Microbial Genomes database as of March 2019 (Joint Genome Institute), all but 13 bear genes annotated as VFs within clusters of orthologous groups. Such VFs include many that may have variable expression depending on environmental conditions, and not all VFs generate pathology exclusively (Kimes et al. 2012). An analysis by Niu et al. (2013) identified a greater number of orthologous VFs in apathogenic genomes of bacteria than in pathogens. Hence,

assigning the trait of pathogenicity based solely on taxonomic identification or the presence of VFs, especially those based on loose similarity to sequenced representatives, is extremely problematic.

8. MATCHING SEQUENCING-BASED APPROACHES WITH PATHOLOGY

It is clear that biases in comparative community analyses to highlight disease-associated microorganisms prohibit direct implication of pathogenicity. However, these approaches may provide important targets for further comparisons and classical pathology investigations. For example, the identification of the bacterial pathogen *Xenohaliotis californiensis* (a *Rickettsia*-like organism; Friedman et al. 2000), which causes withering syndrome in *Haliotis cracherodii*, was only possible after inclusion of molecular approaches (Antonio et al. 2000, Friedman et al. 2000). Histopathology and field observations alone suggested starvation or high water temperatures (Tissot 1988), or association with a coccidian parasite (Friedman et al. 1993), and it was not until these were complemented by ecological and molecular approaches that the etiology was established (Friedman et al. 2000, reviewed in Burge et al. 2016). In a similar way, agents identified in comparative disease/asymptomatic surveys require classical pathological approaches to facilitate understanding of pathology but are to date rarely matched in marine disease investigations.

9. RECOMMENDATIONS

Koch's postulates represent the gold standard for surveys of disease-causing microorganisms yet remain difficult to demonstrate for many marine microorganisms that cannot be cultured or manipulated experimentally, demanding the use of alternate approaches in uncultivated settings (Fredricks & Relman 1996, Byrd & Segre 2016). For example, disease in Atlantic salmon *Salmo salar* caused by *Tenacibaculum dicentrarchi* was diagnosed only when sequence-based approaches identified the bacterium, which then facilitated challenge experiments (Klakegg et al. 2019). HTS surveys of housekeeping genes and metagenomes provide valuable insight into the identity of microbial consortia associated with metazoans and their lesions. However, amplification-based surveys with unknown template input may not accurately or quantitatively represent microbial communities and cannot differentiate organisms that may be causing lesions from organisms that are contaminants. Microbial community surveys implicating disease-associated taxa must be followed by quantitative validation, and inference of disease should not rest only on predicted or observed genomic features. Furthermore, they should be used as a supplement to the standard suite of tools used to investigate animal diseases and localize the presence of the suspect agent to the lesion at the cellular level (Work & Meteyer 2014, Work 2015) (Fig. 4). Several strategies exist for reducing bias in microbial community surveys as they relate to disease investigation: normalizing microbial DNA templates through microscopic counts or quantitative PCR; reducing the use of quantitative statistics for comparing communities and focusing instead on qualitative statistics; using statistics to address the compositionality of microbiome data (i.e. Gniess; Morton et al. 2017); standardizing reporting of sample location (lesion center, lesion margin, asymptomatic tissues), nucleic acid extraction protocol and DNA quantification protocol; performing negative controls parallel with each HTS run and reporting those sequences in concert with

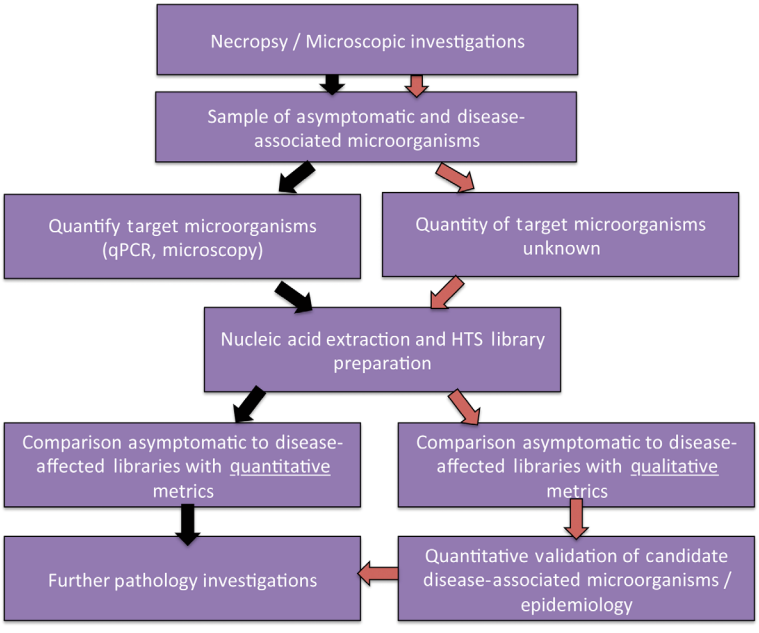

Fig. 4. Conceptualized workflow for using amplified material to elucidate potential pathogens as targets for pathology investigations. Red arrows denote predominately qualitative strategies; black arrows denote potential quantitative strategies

survey results; and performing negative controls with non-target template (carrier nucleic acids) in amplification-based surveys. Finally, comparative surveys should avoid implication of etiology or causation based on taxonomic affiliation or inferred (or observed) genome elements alone. A union of classical microbiological and modern molecular approaches (Burge et al. 2016) along with attempts to localize the agent to host cells, host pathology and host cell pathogenesis may be more appropriate in distinguishing the roles of microbiome constituents in aquatic invertebrate diseases.

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