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Crown-of-thorns sea star, *Acanthaster* cf. *solaris*, have tissue-characteristic microbiomes with potential roles in health and reproduction

Lone Høj,[a#,b] Natalie Levy,[a,b,c*] Brett K. Baillie,[a] Peta L. Clode,[d,e,f] Raphael C. Strohmaier,[a] Nachshon Siboni,[a,**] Nicole S. Webster,[a,b,g] Sven Uthicke,[a,b] David G. Bourne[a,b,c]

[a]Australian Institute of Marine Science, Townsville, Queensland, Australia.
[b]AIMS@JCU, Division of Research & Innovation, James Cook University, Townsville, Queensland, Australia
[c]College of Science and Engineering, James Cook University, Townsville, Queensland, Australia.
[d]Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Perth, Western Australia, Australia
[e]School of Biological Sciences, The University of Western Australia, Perth, Western Australia, Australia
[f]The Oceans Institute, The University of Western Australia, Perth, Western Australia, Australia
[g]Australian Centre for Ecogenomics, University of Queensland, Brisbane, Queensland, Australia

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[#]Address correspondence to Lone Høj, l.hoj@aims.gov.au.
[*]Present address: Natalie Levy, School of Zoology, Tel Aviv University, Tel Aviv, Israel.

[**]Present address: Nachshon Siboni, Climate Change Cluster, University of Technology Sydney, Sydney, New South Wales, Australia.

ABSTRACT Outbreaks of coral-eating crown-of-thorns sea stars (CoTS; *Acanthaster* spp. complex) cause substantial coral loss, hence there is considerable interest in developing prevention and control strategies. We characterised the microbiome of captive CoTS and assessed whether dysbiosis was evident in sea stars during a disease event. Most tissue types had a distinct microbiome. The exception was female gonads, which were highly variable amongst individuals. Male gonads were dominated (>97% of reads) by a single *Mollicutes*-related OTU. Detailed phylogenetic and microscopy analysis demonstrated the presence of a novel *Spiroplasma*-related bacterium in the spermatogenic layer. Body wall samples had high relative abundance (43-64% of reads) of spirochetes, likely corresponding to subcuticular symbionts reported from many echinoderms. Tube feet were characterised by *Hyphomonadaceae* (24-55% of reads). Pyloric caeca microbiomes had high alpha diversity, comprising many taxa commonly found in gastro-intestinal systems. The order *Oceanospirillales* (genera *Endozoicomonas* and *Kistimonas*) was detected in all tissues. A microbiome shift occurred in diseased individuals, although differences between tissue types were retained. The relative abundance of spirochetes was significantly reduced in diseased individuals. *Kistimonas* was present in all diseased individuals and significantly associated with diseased tube feet, but its role in disease causation is unknown. While *Arcobacter* was significantly associated with diseased tissues and *Vibrionaceae* increased in diversity, no single OTUs were detected in all diseased individuals suggesting opportunistic proliferation of these taxa in this case. This study shows that CoTS have tissue-characteristic bacterial communities and identifies taxa that could play a role in reproduction and host health.

IMPORTANCE

Coral-eating crown of thorns sea stars (CoTS; *Acanthaster* spp. complex) are native to the Indo-Pacific, but during periodic population outbreaks they can reach extreme densities (>1000 starfish per hectare) and function like a pest species. On the Great Barrier Reef, Australia, CoTS have long been considered one of the major contributors to coral loss. There has been significant investment in a targeted control program using lethal injection, and there is interest in developing additional and complementary technologies that can increase culling efficiencies. The biology of CoTS has been studied extensively but little is known about their associated microbiome. This cultivation-independent analysis of the CoTS microbiome provides a baseline for future analyses targeting the functional role of symbionts, the identification of pathogens, or the development of reproduction manipulators.

INTRODUCTION

Crown-of thorns sea stars (CoTS; *Acanthaster* spp., excluding *Acanthaster brevispinus*) are corallivorous carnivores that display long-term boom-bust population cycles with densities reaching plague proportions. CoTS were previously thought to belong to a single species, *Acanthaster planci*. It is now recognised that there are at least four species in the Indo-Pacific, and the name *Acanthaster solaris* is proposed for the Pacific species that is native to the Great Barrier Reef (GBR) in Australia (1). Here, this species will be referred to as *Acanthaster* cf. *solaris* or crown-of-thorns starfish (CoTS). Four population outbreaks of CoTS have been documented on the GBR since the 1960's (2, 3) and it was estimated that CoTS contributed to approximately 42% of the decline in coral cover on the GBR in the period from 1985 to 2012 (4). As a consequence, local management options for CoTS have received considerable attention (5-7).

Marine invertebrates have associated microbiomes that play major roles in their biology, including settlement induction, development, metamorphosis, reproduction, digestion, and nutrition (8). Despite their critical importance to host health, studies of echinoderm microbiology are scarce, and most have been triggered by disease outbreaks in the wild (9, 10) or in aquaculture facilities (11, 12). Recently however, molecular surveys of bacteria associated with healthy sea urchins (13-15), holothurians (16), and the coelomic fluid of the sea star species *Patiria pectinifera* and *Asterias amurensis* (17) were reported. Many echinoderms, including many sea stars, have subcuticular bacteria (SCBs) localised in the lumen between epidermal cells and the outer cuticle (18-22). The presence of SCBs appears to be related to host classification, in most cases at family level (20). Although SCBs have not previously been investigated for the family *Acanthasteridae*, they have been detected in other members of the order *Valvatida* (20, 22). While the functional role of SCBs is not clear, it has been hypothesised that they can provide nutrition and antimicrobial protection (22).

To date, all studies of bacteria in CoTS have been cultivation-based (23-28), biasing our understanding of their microbiome and precluding assessment of total microbial diversity in this ecologically important sea star. Sutton and Trott (1987) found that seasonal factors had no effect on microbial composition in apparently healthy individuals and suggested that the most dominant bacterial type could be a specific symbiont. *Vibrio*, *Photobacterium*, and *Pseudoalteromonas* species have been isolated from healthy CoTS (25-28). Several potential pathogens have also been isolated from CoTS displaying disease symptoms (lesions, tissue degeneration, loss of turgor and collapsed spines) including *Vibrio* spp., *Pseudomonas*, and *Moraxella* (24, 26, 27). *Vibrio* has been a focus of CoTS microbiology research to date, but without a culture-independent assessment of the total microbial community it is difficult to ascertain their relative importance to host health state.

There is increasing appreciation that many diseases in humans, and most likely also in marine systems, are linked to microbial imbalance (dysbiosis) or polymicrobial infections (29). This challenges the traditional approach of attempting to isolate single pathogenic agents by standard methods in order to understand and describe marine diseases, and emphasises the need to investigate the total microbiome in healthy as well as diseased individuals. The aim of the current study was to provide a microbial baseline for different *A.* cf. *solaris* tissues and determine how these change during the onset of disease. Healthy and diseased individuals were sampled from COTS held in outdoor tanks and the microbiomes associated with body wall, tube feet, pyloric caeca and gonads (Fig. 1) were analysed by amplicon sequencing of 16S rRNA genes, histology and electron microscopy. The taxonomic position of one dominant phylotype was analysed in more detail by cloning and Sanger sequencing of the corresponding 16S rRNA genes.

RESULTS

The microbiome of healthy *A.* cf. *solaris* tissues. Healthy CoTS displayed significant tissue-differences in their microbiome based on weighted Unifrac distances (PERMANOVA: Pseudo F 10.38, p = 0.0001; ANOSIM: R 0.7854, p = 0.0001) and individual OTUs (PERMANOVA: Pseudo F 5.30, p=0.0001; ANOSIM: R 0.7037, p=0.0001). More specifically, the male gonad microbiome differed from all other tissues based on individual OTUs (PERMANOVA and ANOSIM: $p<0.05$).

The male gonad microbiome was dominated by a single OTU, classified by QIIME to the order *Anaeroplasmatales* (Anaeroplasmataceae_OTU1; 96.0-99.6% of reads) (Fig. 2, Fig. S1). This dominance of a single OTU resulted in a tight cluster in PCoA plots for male gonad tissue samples (Fig. 3, Fig. S2), a high Dominance value (Fig. S3), and low values for evenness (Shannon), species richness (observed species), Fisher's alpha, and overall

123 phylogenetic distance (PD whole tree) (Fig. S3). The same OTU was detected in all healthy
124 tissue samples, albeit at lower relative abundances (pyloric caeca: 2.7-7.7%; female gonads:
125 0.2-79.8%; tube feet: ≤ 0.1%; body wall: <0.1% -3.4%) (Fig. S1, Fig. S4). This single OTU
126 was significantly associated with male gonads and explained 9.0% of the dissimilarity
127 between healthy tissues overall (Table S1). In particular, it explained large proportions of the
128 dissimilarity between male gonads and tube feet (22.1%) or body wall (19.1%), but also
129 between male gonads and female gonads (11.8%) or pyloric caeca (8.3%) (Table S1). The
130 only other order detected in male gonads at an average relative abundance >1% was
131 *Oceanospirillales* (0.0-3.5%) (Fig. 2).
132 The phylogenetic position of the dominant OTU in male gonads was analysed in greater
133 detail. Nine 16S rRNA gene clones derived from male gonads were Sanger sequenced and
134 found to have 99.7-100% sequence identity across the analysed 1495 bases. A representative
135 clone had 99.6% identity, including two single base deletions present in all clones, to a 16S
136 rRNA gene sequence recovered from a scaffold previously generated for male gonads from a
137 CoTS collected near Okinawa, Japan (5). The closest sequence matches in the nr/nt database
138 were two uncultured *Mollicutes* clones from the chiton *Leptochiton boucheti* (HE663394;
139 85% sequence identity) (30), and from the jellyfish *Cotylorhiza tuberculata* (LT599040; 83%
140 sequence identity) (31). The closest matches in the 16S ribosomal RNA database were
141 *Spiroplasma platyhelix* (GU993266; 80% sequence identity) (32) and *Spiroplasma ixodetis*
142 (GU585671; 81% sequence identity) (33). These results were supported by the generated
143 phylogenetic tree (Fig. 4). The sequences derived from CoTS male gonads (GBR and
144 Okinawa) clustered closely together, with the chiton-derived sequence as the closest relative.
145 The cluster formed a deep branch with the *Spiroplasma*-derived lineages, which include the
146 *Spiroplasma* clades (Citri-Chrysopicola-Mirum, Apis, and Ixodetis) and the Mycoides-
147 Entomoplasmataceae clade (34) (Fig. 4). Transmission electron microscopy of male gonads

148 detected cells compatible with both helical and pleiomorphic or intermediate forms of
149 *Spiroplasma* in the spermatogenic layer (Fig. 5A), linking the dominant retrieved bacterial
150 sequences to the characteristic morphologies of this taxon (35).

151 Female gonads displayed large variation in their microbiome with the relative abundance
152 of *Oceanospirillales* and *Anaeroplasmatales*-related sequences in particular different
153 amongst individuals (Fig. 2). One sample was dominated by order *Oceanospirillales* (85.5%)
154 (Fig. 2), of which nearly all reads (>99.9%) were classified as belonging to
155 *Endozoicomonaceae* (genus *Endozoicomonas*, family *Hahellaceae*) (Fig. 6). Another sample
156 had high relative abundance of the *Anaeroplasmatales*-related sequences (79.9%) (Fig. 2,
157 Fig. S1), driving this sample towards the male gonad samples in PCoA plots (Fig. 3, Fig. S2).
158 One OTU related to *Caulobacterales* was significantly associated with female gonads despite
159 explaining <2% of the dissimilarity between female gonads and other individual tissues
160 (Table S1).

161 Body wall samples from healthy individuals had a high relative abundance (45.1-65.8%)
162 of unassigned reads; largely belonging to two OTUs (Unassigned_OTU1: 38.6-61.7%;
163 Unassigned_OTU2: 1.5-12.5%) (Fig. S1). BLAST searches for representative sequences
164 showed that these two OTUs are related to spirochetes previously detected in marine
165 invertebrates (Table S2). Hence, they were grouped and labelled 'Marine spirochetes,
166 BLAST id' to discriminate them from other unassigned OTUs in Fig. 2. Unassigned_OTU1
167 was significantly associated with body wall samples (Table S1). It explained relatively large
168 proportions of the dissimilarity between body wall and female and male gonads (8.5%, and
169 14.4% of the dissimilarity, respectively) (Table S1). Both of the marine spirochete-related
170 OTUs were detected in all healthy and diseased somatic tissue samples, except that
171 Unassigned_OTU2 was absent from one diseased tube feet sample. Hence, our results
172 suggest that marine spirochetes are part of a core COTS microbiome (Table S3).

173 The order *Oceanospirillales* accounted for 17.9-51.3% of reads from body wall samples
174 (Fig. 2, Fig. 6), and of those 44.0-99.7% were *Endozoicomonas* (Fig. 6). Three
175 *Endozoicomonas*-related OTUs (Endozoicomonaceae_OTUs 1, 2 and 3) together explained
176 relatively large proportions of the dissimilarity between body wall and other individual
177 tissues, however no individual *Endozoicomonas*-related OTU was significantly associated to
178 the body wall (Table S1). Endozoicomonaceae_OTU1 was detected in all healthy and
179 diseased somatic tissues, and Endozoicomonaceae_OTU2 and 3 were detected in all healthy
180 and diseased body wall and pyloric caeca samples, hence they are likely members of a core
181 COTS microbiome (Table S3). Furthermore, three additional *Endozoicomonas*-related OTUs
182 were present in all healthy and all diseased body wall samples at low relative abundances
183 (Table S3; Endozoicomonaceaea_OTU5, 6 and 7; up to 0.2% each). Only three other taxa
184 were detected in healthy body wall samples at an average relative abundance >1% in at least
185 one individual, namely *Flavobacteriales* (0.1-9.2%), *Anaeroplasmatales* (<0.1-3.9%), and the
186 betaproteobacterial order EC94 (<0.1-1.0%) (Fig. 2).
187 Tube feet samples from healthy individuals had high relative abundance of the order
188 *Rhodobacterales* (24.2-55.3%) (Fig. 3), with nearly all (99.9%) classified to family level as
189 *Hyphomonadaceae*. The *Hyphomonadaceae*-related OTU was significantly associated with
190 tube feet and explained 13.5%, 6.3%, 8.1% and 15.1% of the dissimilarity between tube feet
191 and body wall, pyloric caeca, female gonads, and male gonads, respectively (Table S1). This
192 OTU was present in all healthy and all diseased tube feet samples (Fig S1, Table S3). A large
193 proportion (up to 52.2%) of reads from healthy tube feet were unassigned with the majority
194 (86.5-94.1%) belonging to Unassigned_OTU1, tentatively identified as a marine spirochete
195 as described above. Interestingly, a spirochete-shaped cell was evident in the coelomic
196 epithelium of the tube foot wall (Fig. 5B). Two additional Unassigned OTUs
197 (Unassigned_OTUs 4 and 5) were present in all healthy and diseased tube feet samples

198 (Table S3) and significantly associated with tube feet, despite having low relative abundance
199 (up to 0.7% each) and explaining <2% of the overall dissimilarity between tissue groups
200 (Table S1). BLAST searches for representative sequences indicated that Unassigned_OTU4
201 was related to *Hyphomonadaceae*, while Unassigned_OTU5 had very low sequence identity
202 (<90%) with sequences in public databases with the closest cultured relatives belonging to
203 the phylum *Firmicutes* (Table S2). Another three unassigned OTUs (Unassigned_OTUs 7, 8
204 and 9) were detected in all tube feet samples irrespective of health status (Table S3), albeit at
205 low relative abundances (up to 0.4%). Only three additional orders were present in healthy
206 tube feet at an average relative abundance >1% in at least one individual: *Flavobacteriales*
207 (0.1-21.3%), *Oceanospirillales* (2.0-6.6%) and *Anaeroplasmatales* (<0.1-1.0%) (Fig. 2). An
208 OTU related to *Flavobacterium* explained between 2.5% and 6.7% of the dissimilarity
209 between tube feet and other tissues, however the association was not significant due to large
210 variability between individuals (Fig. S1). The proportion of *Oceanospirillales* reads identified
211 as belonging to the *Endozoicomonas* was low in all healthy tube feet samples (1.7-9.3%)
212 (Fig. 6). Of the six *Oceanospirillales*-related OTUs that were detected in all healthy and
213 diseased tube feet, only two were classified as *Endozoicomonas* (Table S3).
214 Pyloric caeca of healthy individuals had microbiomes with relatively high alpha
215 diversity (Fig S3). This was reflected in a high number of orders with average read
216 abundance above 1% (Fig. 2), and the highest proportion (3.3-4.7%) of reads assigned to
217 orders with relative abundance < 1% each ('Other' in Fig. 2). Unassigned reads constituted
218 up to 41.8%, with 25.0-82.8% of these belonging to the OTUs tentatively identified by
219 BLAST as spirochetes (Unassigned_OTU1 and Unassigned_OTU2) (Fig. 2). A third
220 unassigned OTU (Unassigned_OTU3) also present in all healthy and diseased pyloric caeca
221 (Table S3), was tentatively identified by BLAST as an epsilonproteobacterium (Table S2)
222 and significantly associated with pyloric caeca (Table S1). The relative read abundance of

223 *Oceanospirillales* and proportion of *Endozoicomonas* was in the range 7.0-36.7% and 23.2-
224 99.0%, respectively (Fig. 2, Fig. 6), with individuals following the same trend as for the
225 corresponding body wall samples (Fig. 6). All three *Oceanospirillales*-related OTUs that
226 were detected in all healthy and diseased pyloric caeca belonged to *Endozoicomonas* (Table
227 S3). Another *Endozoicomonas*-related OTU (Endozoicmonaceae_OTU4) was significantly
228 associated with pyloric caeca but explained < 2% of the dissimilarity with other tissues
229 (Table S1). Other orders with relative abundances above 1% in pyloric caeca were
230 *Anaeroplasmatales* (6.0-10.3%), *Flavobacteriales* (1.3-9.4%), *Lactobacillales* (1.8-7.6%),
231 *Actinomycetales* (2.9-5.6%), *Rhizobiales* (1.3-3.9%), *Bacillales* (1.1-3.1%), *Burkholderiales*
232 (0.7-3.0%), *Clostridiales* (1.7-2.6%), *Enterobacterales* (0.5-2.2%), *Pseudomonadales* (0.6-
233 1.6%), *Neisseriales* (<0.1-1.6%), *Vibrionales* (0.3-1.5%), *Caulobacterales* (1.0-1.5%),
234 *Bacteroidales* (0.7-1.3%) and *Xanthomonadales* (0.5-1.2%). (Fig. 2). Individual OTUs
235 related to *Anaeroplasmatales*, *Bacillales*, *Caulobacterales* and *Vibrionales* were detected in
236 all pyloric caeca samples (Table S3), and OTUs related to *Actinomycetales*, *Bacillales*,
237 *Lactobacillales*, *Rhizobiales*, *Burkholderiales*, *Enterobacterales*, and *Vibrionales* were
238 significantly associated with pyloric caeca despite each explaining <2% of the overall
239 dissimilarity between healthy tissue samples (Table S1).

240 **Comparative analyses of healthy and diseased tissues.** Histological analysis revealed
241 tissue disintegration in diseased individuals. Transverse sections of body wall showed
242 reduced tissue integrity, with papulae frequently replaced by voids (Fig. 7). The structural
243 integrity of tube feet was largely retained in diseased individuals, however in some cases the
244 integument was loosening and the non-adhesive epidermis was disrupted. The structural
245 integrity of pyloric caeca was clearly affected. The extent of damage ranged from near-intact
246 areas with few changes, via loosening of the *tunica serosa* and the underlying nervous layer
247 and muscle fibres, to more severe disintegration.

Microbiome 16S rRNA gene profiling of healthy and diseased somatic tissues showed that both 'Tissue' and 'Health Status' explained significant parts of the variation based on phylogenetic distance (Two-way PERMANOVA; p=0.0001 and p=0.0002) and individual OTUs (Two-way PERMANOVA; p=0.0001 and p=0.0126). There was no significant interaction between the two explanatory variables (Two-way PERMANOVA; p>0.05). There was a significant increase in Dominance for diseased relative to healthy pyloric caeca and whilst not significant, there was a general trend of a decrease in all other diversity measures for this tissue type (Fig. S3). In contrast, the opposite trends were seen for diseased relative to healthy body walls (Fig. S3). For tube feet, there were minimal changes in diversity measures between healthy and diseased individuals (Fig. S3).

Increased relative abundance of *Oceanospirillales*- and *Endozoicimonas*-related OTUs together explained more than 12.5% of the dissimilarity between healthy and diseased individuals (Table S4). In particular, there was a clear increase in the relative abundance of *Oceanospirillales* in diseased tube feet (Fig. 2, Fig. 6), mostly due to two OTUs (Oceanospirillales_OTU1 and Oceanospirillales_OTU3) closely related to the type strain of *Kistimonas asteriae* (Fig S1, Table S2). While these OTUs were present in all healthy and all diseased tube feet (Fig S1, Table S3), Oceanospirillales_OTU1 was significantly associated with diseased individuals and explained 3.9% and 12.8% of the dissimilarity between healthy and diseased tissues overall and between healthy and diseased tube feet, respectively (Table S4). Oceanospirillales_OTU3 was also significantly associated with diseased tube feet and explained a further 2.9% of the dissimilarity of healthy and diseased tube feet (Table S4).

An OTU related to the genus *Arcobacterium* (class *Epsilonproteobacteria*, order *Campylobacterales*) was significantly associated with diseased individuals (Table S4) but explained < 2% of the overall dissimilarity between healthy and diseased individuals (Table S4). This OTU was exclusively detected in diseased CoTS, however it was not present in all

273 diseased individuals (Fig. S1). Due to the well-recognised role of *Vibrio* spp. as primary and
274 opportunistic pathogens in marine systems, OTUs classified as *Vibrionaceae* were analysed
275 separately (Fig. S5). While the true diversity of this family is underestimated by the low
276 resolution of the amplified 16S rRNA gene fragment, we did observe statistically significant
277 trends in some diversity indices. The species richness (observed species) and phylogenetic
278 distance (PD wholetree) of *Vibrionaceae*-related OTUs were significantly higher in diseased
279 as compared to healthy individuals. More specifically, species richness and fisher-alpha
280 diversity of *Vibrionaceae* were significantly higher in diseased compared to healthy pyloric
281 caeca (Van der Waerden's *post hoc* test, $p < 0.05$).
282 Three OTUs were significantly associated with healthy tissues overall.
283 Unassigned_OTU1, tentatively identified as a marine spirochete, was significantly associated
284 with healthy individuals, and specifically with healthy body wall and healthy pyloric caeca
285 (Table S4). A *Flavobacterium*-related OTU explained 2.9% of the overall dissimilarity
286 (Table S4) between healthy and diseased tissues, although its presence varied between
287 individuals (Fig. S1). Unassigned_OTU6, which was tentatively identified by BLAST
288 searches as belonging to the phylum *Bacteroidetes* (Table S2), was significantly associated
289 with healthy tissues despite explaining <2% of the overall dissimilarity (Table S4). Several
290 additional OTUs were found to be significantly associated with healthy pyloric caeca:
291 Unassigned_OTU2 and OTUs related to *Streptococcus*, *Rhizobium*, and *Enterobacteriaceae*
292 (Table S4).
293
294 **DISCUSSION**
295 **Microbiomes of healthy *A.* cf. *solaris* tissues.** Microbiome analysis of the ecologically
296 important crown-of-thorns sea star revealed tissue-specific microbial consortia that were
297 largely conserved amongst individuals, with the exception of a variable microbial community

298 in female gonads. Male gonads were primarily colonised by bacteria that likely represent a
299 novel species, if not a new genus or family, within the *Spiroplasma*-derived lineages (34, 36).
300 Closely related sequences have been recovered from male gonads of CoTS from both the
301 GBR and Okinawa, Japan, suggesting the possibility of a host-specific association. The
302 sequence evidence was further supported by the presence of bacterial morphologies
303 consistent with exponentially growing and pleomorphic or intermediate forms of *Spiroplasma*
304 (35) in the spermatogenic layer of male gonads.

305 Mollicutes have been detected in several marine and freshwater invertebrates including
306 bryozoans (37), ascidians (38, 39), chitons (30), shrimp (40-42), crayfish (43), and jellyfish
307 (31, 44). Recently, mollicutes were found to be the dominant bacteria in the coelomic fluid of
308 a low number of the analysed individuals of *A. amurensis* and *P. pectinifera (17)*. The role of
309 mollicutes in marine invertebrates is not yet well understood; but *Spiroplasma penaei* and
310 *Spiroplasma eriocheiris* have been implicated in disease of aquaculture produced prawns (41,
311 45) and crabs (46, 47), respectively. A recently proposed new candidate *Spiroplasma* genus
312 and species, *Candidatus* 'Medusoplasma mediterranei' gen. nov, sp. nov., (31) was described
313 as an intracellular commensal of the jellyfish *Cotylorhiza tuberculata* with a predicted
314 anaerobic metabolism. Interestingly, *Spiroplasma* infection of male gonads in the crayfish
315 *Pacifastacus leniusculus* appeared to reduce sperm production (43). The occurrence and role
316 of mollicutes in a wide range of insects is better documented, where they have been found to
317 occur both intracellularly and extracellularly, and in some cases are implicated in male
318 killings during late embryogenesis and protection of their host against parasites (48). The role
319 of the *Spiroplasma*-related bacterium in CoTS gonads is unknown but worthy of further
320 exploration especially in relation to potential biological control.

321 The observed variation between female gonad samples may be related to differences in
322 the developmental stage of the gonads, which has been shown to strongly influence the

microbiome of other invertebrates such as the sea anemone *Nematostella vectensis* (49). Ovarian transmission has been demonstrated for many symbiotic bacteria including spiroplasmas (50) and oceanospirillales (51) and the detection of high relative abundances of these known symbiotic taxa suggests this possibility for CoTS.

Healthy somatic tissue samples, and in particular body wall tube feet samples, returned a high relative abundance of two OTUs identified via BLAST searches as belonging to the phylum *Spirochaeta*. Spiral-shaped microorganisms are commonly observed by electron microscopy in the subcuticular region of many echinoderms, and are referred to as Type 2 SCB (19, 21). Type 2 SCB have been previously detected in body wall and tube feet of sea stars and while they are usually spirals, they can vary in morphology from straight rods through spirals with long wave-lengths to tightly kinked spirals with short wave lengths (19). In the present study, a likely spirochete cell was detected by TEM in the coelomic epithelium of the tube foot wall. Spirochetes were not reported in previous molecular analyses of echinoderm subcuticular bacteria (18, 22), but it is important to note that Lawrence and co-workers used Proteobacteria-specific primers that would miss the phylum *Spirochaeta*. Spirochaetes are dominant members of the core microbiome of several octocorals including the red coral *Corallium rubrum* (52) and the soft coral *Lobophytum pauciflorum* (53). They are suggested to play a role in host nutrition and possibly microbial community structuring via production of antimicrobials (52, 53). A low representation of Alphaproteobacteria in the *A.* cf. *solaris* body wall contrasts with previous studies of echinoderm subcuticular bacteria, which have suggested that Alphaproteobcateria are relatively abundant and may play important functional roles in sea stars (22), brittle stars (18), and holothurians (22). *Oceanospirillales* were detected in all healthy and diseased somatic tissue samples and in all female gonad samples. The genus *Endozoicomonas* spp. are commonly found in a wide range of marine invertebrates including corals (scleractinian and octocorals), sea anemones, sponges, tunicates, jellyfish,

348 bivalves, snails, tubeworms, as well as fish (54), although they have not previously been
349 reported from echinoderms. Recovered *Endozoicomonas* sequences had high sequence
350 identity (up to 100%) to sequences retrieved from other marine invertebrates (Table S2).
351 Microscopy-based studies have shown *Endozoicomonas* to occur as aggregations in host
352 tissues (54). However, recent whole-genome sequencing of several *Endozoicomonas* strains
353 showed relatively large genomes and the absence of genome reduction, suggesting the
354 existence of a free-living stage (54, 55). In the present study, we were not able to confirm the
355 presence of bacterial aggregates in CoTS body wall and fluorescence *in situ* hybridisation
356 would be required to spatially localise these cells and confirm their identity. *Endozoicomonas*
357 have been suggested to have important functional roles in their host related to nutrient
358 acquisition and provision, structuring of the host microbiome, maintaining health or causing
359 disease (54). Other *Oceanospirillales*-related OTUs showed high sequence identity (up to
360 100%) to sequences previously recovered from corals and sponges, and *Kistimonas* isolated
361 from a wide range of marine invertebrates (Table S2). Interestingly, the genus *Kistimonas*
362 and the species *Kistimonas asteriae* were initially described from isolates retrieved from body
363 wall of *Asterias amurensis* (56), suggesting that *Kistimonas* may be commonly associated
364 with sea stars.

365 The order *Flavobacteriales* (phylum *Bacteroidetes*) was detected primarily in body
366 wall, tube feet and pyloric caeca of two out of the three healthy individuals, with low
367 abundance in the third. The best BLAST match for the representative sequence had low
368 sequence identity (88%) with the *Flavobacteriaceae* genera *Actibacter* and *Namhaeicola*
369 (Table S2). *Flavobacteraceae* genera have previously been isolated from echinoderms,
370 including *Aquimarina* from body wall of *Asterias amurensis* (56), and *Bizionia* and *Olleya*
371 from coelomic fluid of the sea urchin *Strongylocentrotus pallidus* (57). This suggests that

372 *Bacteroidetes*, and more specifically *Flavobacteriaceae*, are common in echinoderms
373 although there may be high variability between individuals and in the genera present.
374 The tube feet microbiome was dominated by a *Hypomonadaceae*-related OTU, which
375 was present in all tube feet samples irrespective of health status and detected at very low
376 abundance in other tissues. The family *Hyphomonadaceae* (class *Alphaproteobacteria*, order
377 *Rhodobacterales*) includes strict aerobic stalked and non-stalked (one genus only) species
378 that divide by binary fission or budding and are capable of living in low nutrient
379 environments (36). The presence of stalked bacteria in tube feet could not be confirmed by
380 histology or TEM. Related sequences were previously detected in body wall of the temperate
381 sea star *Patiriella* sp. (Table S2) (22). Tube feet are part of the water vascular system and
382 trace amounts of fluid could have been trapped inside the lumen of sampled feet. The fluid of
383 the water vascular system is similar to sea water but includes coelomocytes, which mediate
384 cellular immunity in sea stars (58, 59), a little protein, and an elevated potassium ion content
385 (60). It is unknown to what extent fluid in the water vascular system includes bacteria from
386 the surrounding seawater, and future studies should investigate this possibility.
387 Pyloric caeca had the most diverse microbiome of all *A.*cf. *solaris* tissues, likely
388 reflecting the presence of bacteria capable of enzymatic degradation of a variety of feed
389 items, as well as microenvironments with varying conditions. A high number of taxa
390 commonly associated with gastrointestinal tracts of animals were detected including
391 *Actinomycetales*, *Bacillales*, *Bacteroidales*, *Burkholderiales*, *Clostridiales*, *Enterobacterales*,
392 *Flavobacteriales*, *Lactobacillales*, *Neisseriales*, *Pseudomonadales*, *Rhizobiales*, *Vibrionales*,
393 and *Xanthomonadales* (61-64).
394 **Microbiome shifts in diseased individuals.** A microbial dysbiosis (29) was detected in
395 conjunction with declining host health, involving significant shifts in microbial diversity in
396 body wall and pyloric caeca and significant changes in the relative abundance of some OTUs

in all tissues. The most abundant marine spirochete (Unassigned_OTU1) and two OTUs related to *Bacteroidetes* were significantly associated with healthy individuals, emphasising that these groups are characteristic members of healthy *A.* cf. *solaris* microbiomes. In contrast, one OTU related to *Oceanospirillales* (Oceanospirillales_OTU1) and one OTU related to *Arcobacter* (order *Campylobacterales*) were significantly associated with diseased individuals.

Body wall samples from diseased individuals had decreased dominance (increased evenness) and a significant loss of marine spirochetes. This loss could be a direct result of habitat disintegration, however even minor necrosis can attract bacteria capable of colonising and exploiting available nutrients for rapid proliferation, thereby outcompeting symbionts normally present in healthy individuals (65).

Two OTUs (Oceanospirillales_OTU1 and Oceanospirillales_OTU3) related to the genus *Kistimonas* (family *Hahellaceae*) were significantly associated with diseased individuals, and in particular with diseased tube feet. Related bacteria have been identified as pathogens including *Hahella chejuensis*, which was identified as the etiological agent of red egg disease in tilapia hatcheries (66), and *Endozoicomonas elysicola*, which is responsible for epitheliocystis in cobia hatcheries (67). *Kistimonas* has so far been reported as living in close association with invertebrate hosts (56, 68, 69) and their mode of transmission is largely unknown. In this study, we cannot exclude the possibility that the detected *Kistimonas* were present in trace amounts of fluid from the water vascular system trapped in the sampled tube feet. Without more detailed information on the localisation and physiology of *Kistimonas*-related bacteria, it is difficult to speculate on their possible role in CoTS health and disease.

Arcobacter was found only in diseased CoTS but did not occur in all diseased individuals, suggesting that the proliferation of *Arcobacter* may be opportunistic. The order *Campylobacterales* (*Epsilonproteobacteria*), and specifically the genus *Arcobacter*, was

previously found to dominate the gut microbiome of captive raised sea urchins *Lytechinus variegatus* (14, 15). It has also been detected in diseased coral (70, 71) and necrotic and diseased sponges (65, 72). While *Arcobacter* is linked to gastrointestinal disease and bacteraemia in humans and additionally causes disease in rainbow trout (*Oncorhynchus mykiss*), their pathogenicity and virulence mechanisms are still poorly characterised (73). Importantly, not all species and strains are pathogenic with some *Arcobacter* being opportunistic pathogens or commensals (74).

No single *Vibrio*-related OTU was associated with diseased tissues in this study, but the diversity of *Vibrionaceae* increased in pyloric caeca of diseased individuals suggesting opportunistic proliferation of *Vibrio* spp. Although there was no evidence that *Vibrio* spp. caused the disease event described in the present study, it is possible that members of this genus can cause disease symptoms in CoTS under other circumstances.

We note that the dominant taxa *Mollicutes* and *Endozoicomonas* in diseased CoTS include many intracellular bacteria or microorganisms known to occur as dense aggregates in host tissues. It is possible that bacterial cells with an intimate association with host cells or protected by a tightly enveloping membrane (67, 75) can be protected against host immune responses (54) or simply be detectable for a longer period of time after the onset of tissue degradation.

Diseased CoTS individuals are rarely encountered in the wild (76), hence the sampled disease event in captive CoTS represented an opportunity to investigate possible dysbiosis in CoTS tissues. The comparison to ‘healthy’ CoTS was done using individuals from a separate, healthy batch of COTS that were acclimatised in the same aquarium system to minimise any bias introduced by transportation and captivity. While it is expected that a severe disease event would be the strongest driver of the observed differences between ‘healthy’ and ‘diseased’ tissues, it cannot be excluded that some differences were introduced by using

447 CoTS from a different reef collected six weeks later. Several bacterial taxa were present in all
448 analysed individuals in this study, some of which were tissue characteristic and others were
449 present in multiple tissues, and therefore part of the core microbiome of CoTS. The spatial
450 and temporal stability of bacterial communities in wild CoTS should be targeted in future
451 studies, including analysis of the different species in the *Acanthaster* species complex.
452 This study revealed the presence of tissue-specific microbial communities inhabiting
453 gonads, body wall, tube feet, and pyloric caeca of *A.* cf *solaris* and demonstrated that
454 dysbiosis occurs in conjunction with declining host health. The functional role that symbionts
455 play in maintaining or disturbing CoTS health and controlling CoTS reproduction should now
456 be investigated to ascertain whether these microorganisms represent an "Achilles' heel" that
457 could be exploited in future CoTS control efforts.
458

459 **MATERIALS AND METHODS**

460 **Collection and sampling of sea stars.** Sea stars were collected from the northern
461 section of the Great Barrier Reef between Cairns and Port Douglas, Queensland, Australia, by
462 the Crown-of-Thorns Starfish Control Program Project (Table S5). After collection by scuba
463 divers, *A.* cf. *solaris* were transferred immediately to purpose-built 1000 L holding tanks with
464 trays separating individuals and continuous flow of seawater via a spray tower as previously
465 described (77). Trays were transferred to a transporter tank (1000 L) with static seawater and
466 constant aeration and transported by car for 5 hours to the Australian Institute of Marine
467 Science, Townsville, Australia (77). Upon arrival, CoTS were transferred to outdoor tanks
468 (1000 L) with flow-through unfiltered seawater and aeration.
469 CoTS collected in late March 2014, developed symptoms of disease upon transfer to
470 outdoor tanks, including drooping spines and inability to adhere to the tank wall (Fig. 7).
471 Three diseased individuals were sampled for microbiome analysis within a week (D2, D6,

472 D7) and a further two diseased individuals were sampled in the two following weeks (D8,
473 D9). No lesions were visible at the time of sampling nor did any develop in sea stars
474 remaining in the tank. Four individuals (D2, D6, D7, D8) were at an advanced stage of
475 disease progression and possessed little coelomic fluid at the time of sampling, while D9 had
476 more coelomic fluid and appeared to be at an earlier stage of disease progression. CoTS
477 collected in May 2014 were used to obtain baseline information on microbiomes present in
478 tissues of apparently healthy CoTS (individuals H1, H2, H3). These individuals were
479 acclimated in the outdoor tanks for 4-6 days before sampling to confirm their health status
480 after transportation and minimise any tank effects relative to the previous batch. From both
481 healthy and diseased animals, body wall, tube feet and pyloric caeca (digestive gland)
482 samples were obtained (Fig. 1). The selection of tissues was based on the following
483 considerations: 1) Body wall: many echinoderms harbour subcuticular symbionts and lesions
484 and lesions are a commonly reported disease symptom; 2) Pyloric caeca: many invertebrate
485 diseases are initiated in the digestive system before going systemic; 3) Tube feet: in close
486 contact with coelomocytes, relatively easy to sample and produce good quality DNA. In
487 addition, gonads were included in the study due to their role in animal reproduction. Outside
488 of the spawning season, the gonads of *Acanthaster* cf. *solaris* are completely regressed.
489 Hence gonad tissue samples were obtained from apparently healthy animals collected in
490 November 2013 (MG1, MG2) and November 2014 (MG3, MG4, FG1, FG2, FG3). All
491 tissues were dissected using sterile scalpels and stored according to their respective
492 downstream analysis.

493 **DNA extraction, PCR amplification, NGS sequencing.** Samples for DNA extraction
494 were preserved in ethanol (AJA214, Ajax Finechem, now ThermoFisher Scientific, USA)
495 with the exception of gonads, which were preserved in RNA later (ThermoFisher Scientific).
496 Samples in ethanol were left at 4°C for 16 h, then ethanol was exchanged and the sample

497 transferred to -20°C for storage. Samples in RNA later were left at 4°C for 16 h before being
498 transferred to -20°C for storage. DNA was extracted using the ZR Tissue & Insect DNA
499 MiniPrep kit (Zymo Research, USA), as per the Manufacturer's recommendation. The
500 quantity and quality of extracted DNA was assessed by agarose gel electrophoreses and by
501 spectrophotometry using the Nanodrop 2000 (ThermoScientific).

502 Bacterial 16S rRNA genes were amplified and sequenced at the Australian Centre for
503 Ecogenomics (University of Queensland, Australia). Amplification was performed using the
504 primer set 803F (TTAGANACCCNNGTAGTC) and 1392wR (ACGGGCGGTGWGTRC).
505 The primers amplify the V5-V8 region of *Bacteria* and *Archaea* and were selected based on
506 their high coverage. DNA libraries were prepared with the Illumina TruSeq DNA library
507 preparation protocol, followed by Illumina MiSeq 2 x300 bp sequencing.

508 **Bioinformatic/Statistical analysis of amplicon sequences.** Due to the length of the
509 amplified fragments, only reverse reads were used for subsequent analysis. Sequences were
510 trimmed using PRINSEQ lite version 0.20.4 (PReprocessing and INformation of SEQuence
511 data) (78) and Mothur version 1.34.0 (79). Trimmed sequences were exactly 250 bp long
512 with no ambiguities, a maximum of 8 homopolymers, and all windows (window size 4) had
513 an average quality score of at least 15. Trimmed sequences were analysed using the QIIME
514 pipeline (version 1.9.0) (80) with the Greengenes database (81) version 13_8 (97% similarity)
515 as reference.

516 Chimeric sequences were identified using USEARCH v. 6.1. (82) and filtered from the
517 dataset (approximately 1% of reads were removed). Open-reference OTU picking was
518 performed in four steps using UCLUST (82), with a prefilter cutoff of 60%. Singletons and
519 OTUs whose representative sequence could not be aligned with PyNAST were removed.
520 OTUs that were present in the negative extraction control at a relative abundance of more
521 than 0.05% were removed from all samples. Taxonomy was assigned to OTUs by UCLUST.

In addition, BLAST searches were performed for the representative sequence of selected OTUs (see below).

Before diversity analyses, sequences were evenly subsampled to 7824 reads per sample (the lowest read number, Table S6) to remove the effect of sampling effort. The subsampled dataset was also used for Similarity percentage (SIMPER) analysis (83) and to identify OTUs that were significantly associated with a group (see below). The OTU table was filtered to retain only selected taxonomic groups using the QIIME script filter_taxa_from_otu_table.py, and to retain only OTUs detected in all samples in a defined group using the QIIME script compute_core_microbiome.py, as required. Venn diagrams were generated using the R package VennDiagram v. 1.6.19 (84).

Calculated alpha diversity metrics included Dominance, Shannon index, observed species, and PD wholetree. Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and homogeneity of variances was tested using Levene's test, using PAST version 3.04 (85). Variances were generally not homogenous and the number of samples in each group differed, hence differences between means were analysed by the non-parametric Van der Waerden's normal scores test followed by van der Waerden's *post hoc* test (86) with p-values adjusted for multiple comparisons (87), using the R package PMCMR v. 4.1 (88).

Weighted and unweighted Unifrac distance matrixes were generated by QIIME. Principal Coordinates Analysis (PCoA), ANOSIM, two-way PERMANOVA (9999 permutations), and SIMPER analysis were performed using PAST version 3.04 (85). SIMPER analysis was performed on square-root transformed data with the Bray-Curtis similarity measure. The association of each OTU to a particular group of samples was analysed using the function signassoc in the R package indicspecies (89). The p-value was corrected for multiple testing using the Sidak method.

OTUs were selected for further analysis if they explained more than 2% of the dissimilarity between groups (SIMPER) and/or fulfilled the following criteria: 1) were identified by the function signassoc to be significantly associated with a group ($p<0.05$); and 2) had an arithmetic average difference in relative abundance between groups of >0.05% (90). Representative sequences for selected OTUs were used to search public sequence databases (nr/nt, 16S ribosomal RNA sequences (*Bacteria* and *Archaea*)) for closely related matches using BLASTn. The significance level was set at 0.05 in all cases.

Phylogenetic analysis of 16S rRNA gene sequences. Near full length bacterial 16S rRNA gene sequences corresponding to the dominant OTU in male gonads (Anaeroplasmataceae_OTU1) were obtained from male gonads by cloning and Sanger sequencing. Briefly, bacterial 16S rRNA gene sequences were amplified from DNA extracted as described above using the primers 27F/1492R (91). The amplification product was purified using the QIAquick PCR Purification Kit (Qiagen, Germany), and cloned using the TOPO TA Cloning Kit with Competent One Shot TOP10 cells (Invitrogen, USA). Plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen) and Sanger sequenced (Macrogen, Korea) using M13 primers (M13F/M13R-pUC).

The CoTS (submitted as *A. planci*) genome sequencing project (5) used male gonads as their starting material. Screening of early scaffolds (not filtered for bacterial sequences) identified one scaffold generated from a specimen collected near Okinawa, Japan, that included the representative sequence of Anaeroplasmataceae_OTU1. WebMGA (92) was used to extract the full length 16S rRNA gene sequence from this scaffold (oki_scaffold215_size448669).

Related sequences in public databases (nr/nt,16S ribosomal RNA (Bacteria and Archaea)) were identified by Nucleotide BLAST. Identified sequences and 16S rRNA gene sequences from related type strains were downloaded and used to create a maximum

likelihood-based phylogenetic tree (93). CLC Genomics Workbench v. 9.5.3 (Qiagen) was used for sequence alignment, trimming (about 1400 bp), model testing, and tree construction using the neighbor joining algorithm for the starting tree, the GTR substitution model (94), and 1,000 bootstrap replicates. The resulting tree was exported and edited for clarity using Dendroscope (95) and Adobe Illustrator.

Histology and transmission electron microscopy. Samples for histology were fixed in Bouin's fixative for 16 h at 4°C followed by 3 rinses in 3x phosphate buffer saline (1 x PBS: 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and storage in 70% ethanol at 4°C until processing. Body wall samples were decalcified in 10% formic acid. All samples were embedded in paraffin and sections (5 µm) were stained either by hematoxylin and eosin. Mounted slides were inspected by an AxioImager.M2 compound microscope (Carl Zeiss Pty. Ltd., Oberkochen, Germany) and micrographs captured by an Axiocam 503 (Carl Zeiss) microscope camera. The microscope software Zen Blue 2.3 Pro (Carl Zeiss) was used for automated tiling and stitching of images.

Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde +2% paraformaldehyde in 100 mM cacodylate for about 16 hours at 4°C, followed by 2 rinses in 3x PBS, 1 rinse in 1x PBS and storage in 1x PBS at 4°C until processing at the Centre for Microscopy, Characterisation and Analysis at the University of Western Australia. Samples were post-fixed in 1% OsO_4 in PBS and dehydrated in a graded series of ethanol and acetone using a microwave (Biowave, PELCO), before being infiltrated and embedded in Procure-Araldite resin. Sections from healthy male gonads and tube feet were subsequently cut at a thickness of 100 nm on a diamond knife, before being stained with 1% aqueous uranyl acetate and Sato's modified lead citrate for 5 min each. All sections were imaged at 120 kV in a TEM (JEOL 2100) fitted with a digital camera (Orius, Gatan).

596 **Accession numbers.** The raw amplicon data were submitted to NCBI under BioProject
597 SSRP128607, SRA accession SRP128607, and BioSample accession numbers SRX3542029-
598 SRX3542037. Sequences of 16S rRNA gene clones were submitted to NCBI's GenBank with
599 accession numbers MG776016-MG776024.
600
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619
620 **FIGURE LEGENDS**

621 **FIG 1** Schematic drawing of *Acanthaster* cf. *solaris* showing the location of sampled somatic
622 tissues (body wall, tube feet, pyloric caeca) and gonads.
623
624 **FIG 2** Taxonomic composition of amplicon sequences from healthy *Acanthaster* cf. *solaris*
625 tissue samples. Labels reflect the phylum (abbreviated), class, and order. OTUs that could not
626 be assigned to a taxonomic group by the QIIME pipeline are categorized as 'Unassigned',
627 with the exception of two OTUs (Unassigned_OTU1 and OTU2) categorized as 'marine
628 spirochetes' based on their best BLAST matches as discussed in the text. Orders with relative
629 abundance > 1% in at least one sample are shown, with remaining taxa included in the
630 category 'Other'. Abbreviations: A: *Actinobacteria*; B: *Bacteroidetes*; C: *Cyanobacteria*; F:
631 *Firmicutes*: G: *Gemmatimonadetes*; P: *Proteobacteria*; T: *Tenericutes*; MG: male gonads;
632 FG: female gonads; HBW: healthy body wall; HTF: healthy tube feet; HPC: healthy pyloric
633 caeca. The associated number identifies the sampled individual as described in Table S5.
634
635 **FIG 3** Principal coordinates analysis (PCoA) plot based on Bray-Curtis similarities of
636 Hellinger (square-root) transformed OTU abundance data evenly subsampled to 7824 reads.
637 Abbreviations: HBW: healthy body wall; HTF: healthy tube feet; HPC: healthy pyloric caeca;
638 FG: female gonads; MG: male gonads. The number in the sample label identifies the sampled
639 individual as described in Table S5.
640
641 **FIG 4** Maximum Likelihood tree showing the phylogenetic position within the *Mollicutes* of
642 the dominant bacterium in *Acanthaster* cf. *solaris* male gonads. The sequence MG_clone14
643 was cloned from male gonads of *A. cf. solaris* collected from the Great Barrier Reef. The
644 sequence MG_ oki_scaffold215 was extracted from an existing scaffold produced from male

gonads of *A.* cf. *solaris* collected near Okinawa (5). Bootstrap values are based on 1000 bootstrap replications. The scale bar represents the number of substitutions per site.

FIG 5 Transmission electron micrographs from healthy *Acanthaster* cf. *solaris* tissues. A) The spermatogenic layer of a male gonad showing bacterial morphologies (arrowheads) similar to *Spiroplasma* in exponential growth and its pleiomorphic or intermediate forms. Scale bar corresponds to 1 µm. B) A spirochete-shaped bacterium (arrowhead) detected in the coelomic epithelium of tube feet. Scale bar corresponds to 500 nm.

FIG 6 Proportion of reads classified as *Oceanospirillales* and *Endozoicomonaceae* by QIIME for healthy and diseased *Acanthaster* cf. *solaris* tissue samples. Abbreviations: H: healthy; D: diseased; BW: body wall; TF: tube feet; PC: pyloric caeca. The number in the sample label identifies the sampled individual as described in Table S5.

FIG 7 Photos and micrographs showing representative healthy and diseased *Acanthaster* cf. *solaris*. The micrographs were produced by automated tiling and stitching as indicated. a and b: Arms of healthy (a) and diseased (b) individuals. c and d: Hematoxylin and eosin stained sections of body wall from healthy (c) and diseased (d) individuals (5x5 tiles, 10x objective; scale bar corresponds to 500 µm). e and f: Hematoxylin and eosin stained sections of tube feet from healthy (e) and diseased (f) individuals (5x5 tiles, 20x objective; scale bar corresponds to 200 µm). g and h: Hematoxylin and eosin stained sections of pyloric caeca from healthy (g) and diseased (h) individuals (4x4 tiles, 20x objective; scale bar corresponds to 200 µm).

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