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The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance

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Microbes are well-recognized members of the coral holobiont. However, little is known about the short-term dynamics of mucus-associated microbial communities under natural conditions and after disturbances, and how these dynamics relate to the host's health. Here we examined the natural variability of prokaryotic communities (based on 16S ribosomal RNA gene amplicon sequencing) associating with the surface mucus layer (SML) of *Porites astreoides,* a species exhibiting cyclical mucus aging and shedding. Shifts in the prokaryotic community composition during mucus aging led to the prevalence of opportunistic and potentially pathogenic bacteria (*Verrucomicrobiaceae* and *Vibrionaceae*) in aged mucus and to a twofold increase in prokaryotic abundance. After the release of aged mucus sheets, the community reverted to its original state, dominated by *Endozoicimonaceae* and *Oxalobacteraceae*. Furthermore, we followed the fate of the coral holobiont upon depletion of its natural mucus microbiome through antibiotics treatment. After re-introduction to the reef, healthy-looking microbe-depleted corals started exhibiting clear signs of bleaching and necrosis. Recovery versus mortality of the *P. astreoides* holobiont was related to the degree of change in abundance distribution of the mucus microbiome. We conclude that the natural prokaryotic community inhabiting the coral SML contributes to coral health and that cyclical mucus shedding has a key role in coral microbiome dynamics.

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Introduction

Corals live in a well-described mutualism with photoautotrophic endosymbiotic dinoflagellates of the genus *Symbiodinium*, frequently referred to as zooxanthellae (reviewed by Muscatine, 1990). More recently, the concept of the coral holobiont (Rohwer *et al.*, 2002) has been proposed to describe the association of the coral host with a diverse microbial community including representatives of fungi, endolithic algae, bacteria and archaea, of which certain associations are species-specific (Ritchie and Smith, 1997; Rohwer *et al.*, 2002; Koren and Rosenberg, 2006; Carlos *et al.*, 2013). The sum of these microorganisms and their combined genetic material forms the coral microbiome, whose core composition is determined by the host but whose presence allows for metabolic adaptations to local environmental conditions by selection of beneficial genes (Kelly *et al.*, 2014; Ainsworth *et al.*, 2015).

The putative functions of the coral microbiome comprise, among others, the protection against pathogens (Rohwer *et al.*, 2002; Shnit-Orland and Kushmaro, 2009) and the supply and cycling of nutrients (Lesser *et al.*, 2004; Wegley *et al.*, 2007). Gates and Ainsworth (2011) propose that all taxonomic components should be considered as important because of their potential to stimulate the holobiont's functioning. However, the extent of the microbiome's contribution to the health of the coral holobiont and to coral reef resilience remains largely unknown. Whereas the association between the coral and its eukaryotic symbionts of the genus *Symbiodinium* is fairly well characterized (Rowan, 2004; Frade *et al.*, 2008), relatively little is known about the spatial and temporal variation of the coral's prokaryotic microbiome. Recent studies have shown shifts in the microbiome from

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healthy to diseased corals and under altered environmental conditions (Bourne *et al.*, 2007; Mao-Jones *et al.*, 2010), supporting the idea that both the disturbance of the native microbiota and the direct infection by specific pathogens threaten the well-being of corals.

The coral host provides several distinct habitats for its microbial inhabitants—the tissue (Lesser *et al.*, 2004), the gastrovascular cavity (Herndl and Velimirov, 1985), the skeleton (Sharshar *et al.*, 1997) and the surface mucus layer (SML) (Rohwer *et al.*, 2002; Kooperman *et al.*, 2007), each harboring a distinct microbial community (Sweet *et al.*, 2011a). The SML is particularly important for the biology of corals not only as a habitat for a distinctive suite of coral-associated microbes but also due to its nutritional, protective and cleansing roles (Brown and Bythell, 2005). Consisting of polymeric glycoproteins and lipids (Bythell and Wild, 2011), coral mucus provides a nutritious medium on which a diverse assemblage of microbes thrives, many of which are highly host specific (Rohwer *et al.*, 2002). Although coral mucus is in constant contact with the adjacent seawater, their prevalent microbial communities exhibit almost no overlap (Rohwer *et al.*, 2001, 2002; Frias-Lopez *et al.*, 2002). Furthermore, it has been hypothesized that the microbial community in the coral SML operates as a defense barrier and therefore protects the coral against invasive microbes either because of the production of antimicrobial substances or simply because of the occupation of this interface niche (Rohwer *et al.*, 2002; Reshef *et al.*, 2006; Rosenberg *et al.*, 2007; Shnit-Orland and Kushmaro, 2009).

The SML is a very dynamic system whereby its molecular organization and composition vary between coral species and over time (Brown and Bythell, 2005). In addition, the SML experiences sloughing, either continuously or periodically, from the coral surface into the reef environment (Bythell and Wild, 2011). This cycle of structural changes creates habitat dynamics to which microbial communities are likely responding (Nelson *et al.*, 2013).

Corals of the genus *Porites* sp. provide an ideal model system to study natural short-term dynamics of the mucus microbiota because of a clearly recognizable and well-described aging process that precedes periodical sloughing of the entire mucus layer and its reformation (Coffroth, 1991). The SML initially appears as a transparent surface film whose visual appearance slowly changes over the course of a few days into a conspicuous aged mucus sheet. After the release of this aged sheet of mucus into the water column, new fluidic mucus is produced at the surface of the coral leading to a new cycle, suggested to follow a lunar periodicity (Coffroth, 1991). Our rationale is that the temporal transformation and periodical release of mucus from the surface of poritid corals may trigger or relate to the establishment of a microbial succession analogous to the one shown for bacterioplankton after phytoplankton blooms (Teeling *et al.*, 2012).

To better understand the role of the mucus microbiota on the health and resilience of coral holobionts, we apply an indicator species approach aiming (1) to unravel the natural short-term dynamics of mucus-associated prokaryotic communities coupled to the cyclical aging and release of SML of *Porites astreoides* and (2) to follow the successional steps taking place after disruption of the coral's microbiome.

Materials and methods

Species, study location and sampling approach

*Porites astreoide*s Lamarck 1816, a common coral species on Caribbean reefs (Bak, 1975), exhibits the typical mucus aging behavior of poritid corals (Figure 1a and Supplementary Figure S1) and was chosen as a model species. Fieldwork was conducted between February and March 2014 on Curaçao (former Netherlands Antilles). Samples were collected by SCUBA diving at 5 m depth on the reef flat of the CARMABI Buoy One reef location (12°7.46'N, 68°58.31'W). *In situ* light irradiance (765 ± 178 µmol photon $m^{-2} s^{-1}$ at noon) and temperature ($26.6\,^{\circ}C \pm 0.4\,^{\circ}C$) were measured at 5 m depth with a Hydrolab DS5 Sonde and with HOBO Pendant Temperature/Light Data Loggers, respectively, for the entire duration of fieldwork. Our approach consisted of two parts: an *in situ* description of the 'natural dynamics of coral mucus-associated prokaryotes' related to the cyclical change between new and aged mucus over time, and a manipulative approach in which colonies were removed from the reef, their microbiome disturbed in the aquarium, and 'prokaryotic mucus re-colonization after antibiotics disturbance' monitored after re-introduction of the colonies to the reef environment (Figure 1).

Natural dynamics of coral mucus-associated prokaryotes

To assess *in situ* the microbial dynamics in the SML of *P. astreoides*, seven colonies were regularly monitored by collecting mucus samples with sterile cotton swabs ('swabbing') every second to third day for a total period of 2 months (one sample per colony per day; see Figure 1a). On each sampling occasion, the mucus aging state was visually categorized as either 'new' or 'aged' following published approaches (Coffroth, 1991). At the same time, 50 ml of seawater were collected into Greiner tubes at approximately 20 cm distance from one of the sampled corals and later filtered onto 0.2 µm Millipore GTTP filters (Cork, Ireland). Sediment was collected from the upper sediment layer next to sampled corals on three distinct occasions during the fieldwork period. Immediately after each dive, all the samples were flash frozen in liquid nitrogen and kept at $-80\,^{\circ}C$.

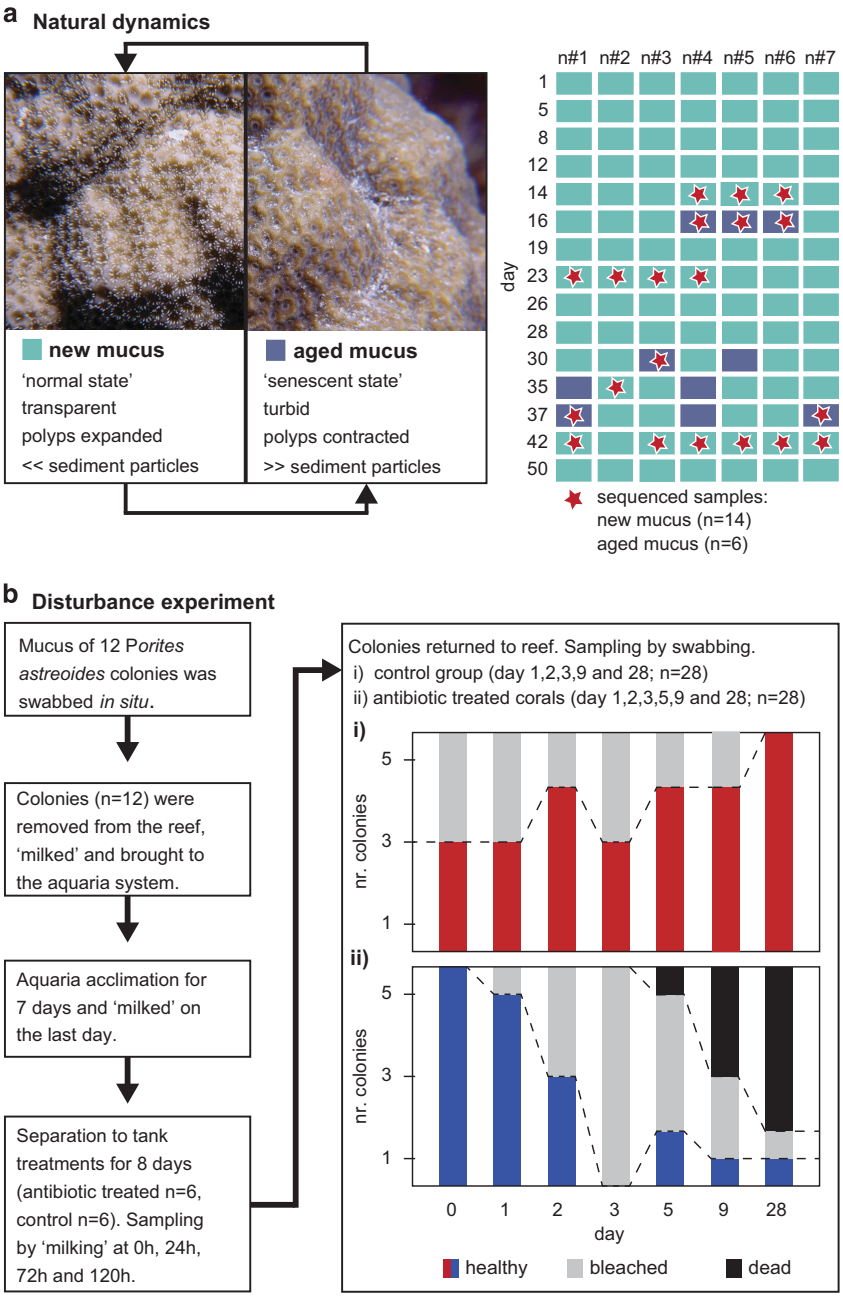

Figure 1 Diagram depicting the two experimental approaches. (**a**) Natural dynamics of coral mucus-associated prokaryotes: surface mucus of seven *Porites astreoides* colonies was regularly sampled *in situ* over a 2-month period and mucus aging state ('new mucus' versus 'aged mucus') was visually assessed (following Coffroth, 1991). Insert depicts an *in situ* impression of the SML for the same *P. astreoides* colony at two different time points: with new mucus (left side) and with a conspicuous and aged mucus sheet (right side). A selection of new ($n=14$) and aged mucus ($n=6$) samples was later used for 16S ribosomal RNA (rRNA) gene amplicon sequencing. (**b**) Prokaryotic mucus re-colonization after antibiotics disturbance: mucus of 12 *P. astreoides* colonies exhibiting new mucus was sampled *in situ* and whole colonies later removed from the reef and brought to the aquaria system of the CARMABI station. Six colonies were incubated in a mix of antibiotics and the other six colonies were incubated as controls. After 8 days in the aquaria, corals were again sampled and thereafter brought back to the reef and installed on a rack (day 0). Over the next 28 days, the health of the colonies was visually assessed and mucus samples were regularly collected. No visual signs of mucus aging were observed throughout the experiment. The 16S rRNA gene was sequenced for all collected mucus samples ($n=80$) to follow microbial community composition during re-colonization of mucus after antibiotics disturbance.

Twelve other conspecific colonies exhibiting either conspicuously aged ($n=6$) or clearly new mucus ($n=6$) were removed from the reef on 2 consecutive days to collect large quantities of mucus through air exposure, thereafter referred to as 'milking' (Garren and Azam, 2010). Removed corals were of similar size (diameter of approximately 10 cm) and all colonies were 'milked' for 5 min before being brought back to the reef. Released mucus (range: 8–10 ml) was collected into sterile 50 ml Greiner tubes before fixing with formaldehyde (2% final

concentration) at 4 °C for at least 4 h. In all, 1–2 ml of each fixed sample was then passed through an 0.2 µm GTTP filter (Millipore; supported by a 0.45 µm HAWP filter, Millipore). This filter was used to enumerate microbial abundance by epifluorescence microscopy (minimum of 30 fields of view and 300 cells) after 4′,6-diamidin-2-phenylindol (DAPI) staining. The microbial abundance associated with mucus is given below per mucus volume (Garren and Azam, 2010).

Prokaryotic mucus re-colonization after antibiotics disturbance

To assess the impact of antibiotic treatment and subsequent microbial re-colonization on coral health, 12 additional *P. astreoides* colonies (diameter of approximately 6 cm) with conspicuous new mucus were freshly collected from the reef and transferred to the reef-water flow-through aquaria system of the CARMABI station (Figure 1b and Supplementary Figure S2). Before removal of the colonies, their SML was sampled by 'swabbing', and used as baseline for comparison with the microbial parameters determined in the subsequent experiments.

After an acclimation period of 7 days, coral colonies were transferred to transparent plastic beakers filled with aquarium seawater (600 ml) that was continuously agitated. Beakers were kept in the flow-through system at stable temperature conditions (26.5 °C ± 0.3 °C). Six of these colonies were incubated in an antibiotic mix (Supplementary Table S1) added in a dilution of 1:100 to seawater replaced every 12 h for a total incubation period of 8 days.

Corals were 'milked' for a period of 2 min directly after removal from the reef, after the 7 days acclimation phase ($t = 0$ h), and three times during the incubation ($t = 24$, 72 and 120 h). Before 'milking', each colony was rinsed with 0.2 µm-filtered aquarium seawater to remove loosely attached prokaryotes from the coral surface. Mucus samples were fixed with formaldehyde (2% final concentration) and processed to monitor prokaryotic abundance in the SML as described for the natural dynamics experiment.

After the aquaria incubation, all colonies, the antibiotic-treated and control colonies, were brought back to the CARMABI Buoy One reef and installed on a rack in an alternating order (Supplementary Figure S2). Colonies were 'swabbed' directly after the re-introduction, at the following 3 consecutive days and then every second to third day for a total period of 28 days. In addition, the health status of each coral was visually monitored for occurrence of bleaching, lesions and mortality over the study period. Coral health was in general categorized in the three following groups: healthy—no visual signs of bleaching or necrosis, bleached—visual observation of bleaching and/or necrosis, dead—>80% of tissue loss. Throughout the disturbance experiment (incubation and re-introduction), none of the colonies exhibited visual signs of mucus aging.

DNA extraction, 16S ribosomal RNA gene sequencing and taxonomic annotation

DNA was extracted from 120 samples representing a selection of swabbed mucus (Figure 1), seawater and sediment samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions.

DNA extracts were sent on dry-ice to the IMGM laboratories GmbH (Martinsried, Germany) for sequencing a 728-bp fragment of the 16S ribosomal RNA (rRNA) gene (see Supplementary Information) using 454 GL FLX+ technology (Roche, Martinsried, Germany). Barcoded 16S rRNA gene PCR amplicons were denoised in Acacia (version 1.52.b0; Bragg *et al*., 2012) and analyzed using Qiime (version 1.8.0; Caporaso *et al*., 2010a). Obtained sequences were clustered into $OTUs_{0.02}$ (operational taxonomic units) based on ⩾ 98% sequence similarity excluding singletons. Representative $OTUs_{0.02}$ were picked and aligned with PyNAST (version 1.2.2, Caporaso *et al*., 2010b) using the Greengenes database (version 13.5). Taxonomy was assigned using the Ribosomal Database Project Classifier (version 2.2, Wang *et al*., 2007) with a confidence minimum of 85%. OTU tables, based on the taxonomic hierarchical levels were generated in Qiime. Diversity estimates were calculated using R (R Development Core Team, 2008). To take the different sequencing efforts into account, 801 sequences were randomly picked from each sample as suggested by Qiime and chloroplast OTUs were removed. The rarefied OTU table based on family level and their relative abundances per sample was used for further analyses. Demultiplexed 16S rRNA gene raw reads and respective metadata are available in the NCBI SRA database (http://www.ncbi.nlm.nih.gov/sra/) under accession number SRP069317.

Statistics

The effect of sampling group on prokaryotic abundance as well as on diversity indices (Shannon Weaver Index, richness and evenness) was tested with repeated-measures analysis of variance (rANOVA) and further pairwise comparisons with the Tukey HSD test at 95% confidence level. Data were log transformed if required. Venn diagrams were used to depict numbers of unique, shared and ubiquitous OTUs.

The variation in prokaryotic community composition among different sampling groups (beta-diversity) was visualized by non-metric multidimensional scaling ordination (nMDS; after 10 000 permutations) based on Bray–Curtis dissimilarity matrices. Differences were further tested with analysis of similarity (ANOSIM; 10 000 permutations). Furthermore, the homogeneity of multivariate dispersions was tested using a resemblance-based permutation test (PERMDISP) before confirming differences in community structuring between sampling groups by applying a permutational multivariate analysis of variance

(PERMANOVA) using Bray–Curtis dissimilarity matrices (Anderson *et al*., 2006).

The explanatory power of factors such as habitat, time (that is, sampling day) and treatment on the observed prokaryotic community assembly was determined by canonical correspondence analysis (CCA) and the significance of factors was further verified using an ANOVA-like permutation test based on 1000 permutations.

OTUs contributing up to a cumulative value of 70% of the total divergence in prokaryotic community assembly among different sampling groups were shown by similarity percentages (SIMPER).

In this study the indicator value analysis (IndVal; De Cáceres and Legendre, 2009) was applied to detect prokaryotic families significantly associated ($P<0.05$, when both specificity and fidelity have probabilities >0.5) to different sampling habitats: new mucus, aged mucus, seawater and sediment. Specificity is the probability that the specific taxon belongs to the habitat group given the fact that the species has been found, whereas fidelity is the probability of finding the taxon in assemblages belonging to the habitat group. Prokaryotic families identified by IndVal, here assumed to represent prokaryotic indicators of each particular habitat, have the highest probability of occurrence for that particular habitat. IndVal has been used in the past to identify strict habitat specialists of particular coral compartments (Li *et al*., 2014).

All statistical tests and graphs were compiled in R (R Development Core Team, 2008).

Results

Sequencing and sample overview

A total of 639 196 reads were retrieved from the 110 successfully sequenced and further analyzed samples (see Table 1 for details) and clustered into 353 OTUs (based on family level). The required minimum of 801 sequences per sample led to the removal of one mucus and two sediment samples. Observed richness within sampling groups was about two-thirds of the estimates obtained with Chao (Table 1).

Natural dynamics of coral mucus-associated prokaryotes

Each *P. astreoides* colony exhibited an aged mucus sheet on up to two occasions during the 2 months of fieldwork (Figure 1a). Mucus aging, however, was neither synchronized among different colonies nor related to any of the measured environmental parameters. Aged mucus remained for up to 3 days on the coral's surface before it was released into the surrounding environment. Prokaryotic abundance in the SMLs of *P. astreoides* colonies ranged from $3.2\pm0.5\times10^5$ cells ml^{-1} in newly produced mucus to $8.1\pm0.6\times10^5$ cells ml^{-1} in aged mucus sheets, representing a twofold increase in cell abundance (ANOVA, $P<0.01$). Mucus (aged and new), seawater and sediment prokaryotes comprised in total 255 OTUs, of which 32 OTUs were ubiquitously present in all habitats (Supplementary Figure S3). Approximately 18% and 21% of the OTUs associated with aged and new mucus layers, respectively, were unique to that particular SML aging state (Supplementary Table S2 and Supplementary Figure S3). Furthermore, mucus shared about two times more members with the prokaryotic community associated with sediments than with ambient seawater. Alpha diversity, richness and evenness were highest in aged mucus and sediment samples (Table 1), albeit statistically not significant (see Supplementary Table S3).

Prokaryotic community composition revealed by nMDS ordination (Figure 2) was structured based on

Table 1 Overview of number of samples, number of 16S rRNA gene sequences retrieved and corresponding diversity indices (average ± s.d.) for each sampling group within two experiments (natural dynamics and disturbance experiment)

Sampling group	*Total no. of samples*	*No. of sequences*[a]	*Richness*	*Evenness*	*Shannon index*	*OTUs in total*[a]	*Chao estimate*[a]
Natural dynamics							
New mucus	14	4882 ± 2534	50 ± 20	0.49 ± 0.19	1.95 ± 0.38	174	256
Aged mucus	6	5867 ± 2770	82 ± 29	0.81 ± 0.05	3.48 ± 0.90	178	248
Seawater	6	7709 ± 3758	28 ± 8	0.59 ± 0.15	2.07 ± 0.52	66	88
Sediment	4	923 ± 116	70 ± 12	0.80 ± 0.05	3.40 ± 0.30	137	210
Disturbance experiment							
Initial composition	12	5941 ± 2962	54 ± 12	0.55 ± 0.14	2.23 ± 0.72	170	244
Aquaria treated	6	3837 ± 2436	50 ± 12	0.75 ± 0.07	2.33 ± 0.45	109	155
Aquaria control	6	4122 ± 3249	84 ± 19	0.81 ± 0.05	3.32 ± 0.47	176	230
1–3 Days treated[b]	18	7000 ± 3728	67 ± 17	0.68 ± 0.10	2.84 ± 0.54	201	316
1–3 Days control[b]	18	5494 ± 1563	81 ± 18	0.71 ± 0.11	3.42 ± 0.34	231	309
4–28 Days treated[b]	10	5528 ± 3499	46 ± 20	0.70 ± 0.13	2.64 ± 0.67	138	186
4–28 Days control[b]	10	8003 ± 2583	71 ± 15	0.78 ± 0.06	3.17 ± 0.43	183	315

All data are based on a rarefied OTU table at the family level from which chloroplast-affiliated OTUs were removed.
Abbreviations: OTU, operational taxonomic unit; rRNA, ribosomal RNA.
[a]'No. of sequences', 'OTUs in total' and 'Chao estimate' were calculated from the original non-rarefied OTU data set (including chloroplasts).
[b]1–3 and 4–28 days represent the number of days after re-introduction on the reef. Data were pooled for each of those two periods.

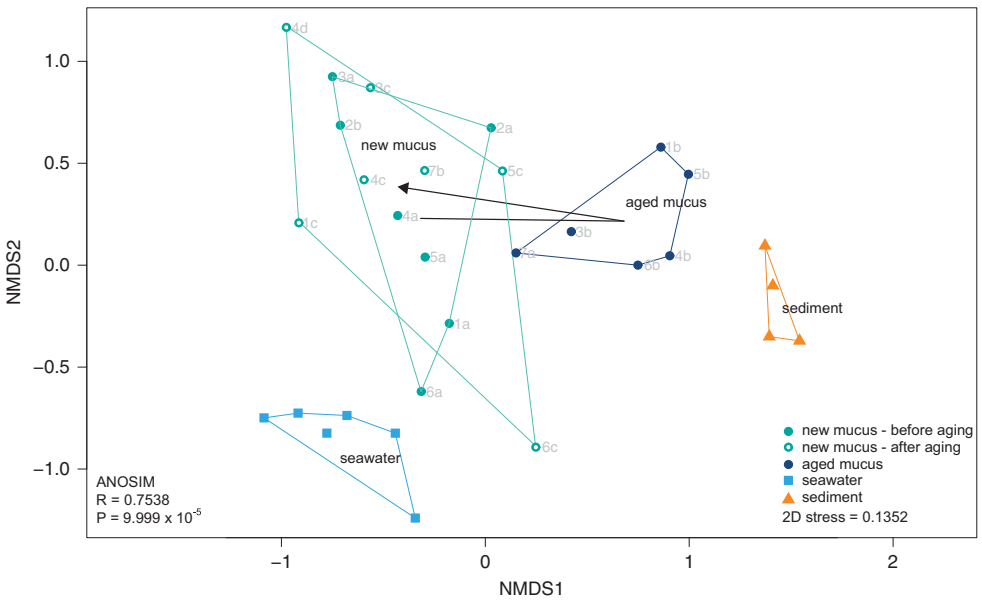

Figure 2 Natural dynamics of coral mucus-associated prokaryotes: non-metric multidimensional scaling (nMDS) plot of the Bray–Curtis-based dissimilarity matrix of prokaryotic communities colonizing the mucus of *Porites astreoides* and the adjacent reef environment (sediment and ambient water). Mucus samples are divided into 'new mucus' (samples that were collected up to 2 weeks before and after an aging event) and into 'aged mucus'. Arrow indicates shift in the centroid of the mucus community before aging, in aged mucus and after aged mucus was released. Each code depicted in the figure corresponds to an individual colony (1–6) throughout the experiment in a chronological order (a–d).

habitat (ANOSIM, $R=0.7538$, $P<0.001$). Communities present in aged mucus were intermediate in their composition between those typically found in new mucus and in sediments. However, after the release of the aged mucus layers, the prokaryotic community reverted to its initial state in new mucus. A homogeneous multivariate dispersion among sampling groups (PERMDISP, $P>0.05$, Supplementary Table S4) allowed applying PERMANOVA to further confirm this habitat-based structuring of prokaryotic communities ($P<0.001$, Supplementary Table S5). CCA (Supplementary Figure S4) depicted that 81% of the variation in the prokaryotic community assembly was explained by the factor habitat ($P<0.001$), whereas the effect of time and colony was negligible ($P>0.05$).

The relative abundances of those OTUs responsible for the observed changes in community structure between aged and new mucus (Supplementary Table S6) were compared (Supplementary Figure S5). *Endozoicimonaceae* was the most abundant bacterial family in new mucus of *P. astreoides* (41.6% ± 32.4% of the overall prokaryotic abundance), followed by *Pelagibacteraceae* (12.4% ± 8.8%) and *Oxalobacteraceae* (7.3% ± 9.9%). In contrast, aged mucus layers exhibited a low relative abundance of *Endozoicimonaceae* (4.1% ± 6.5%), however, an up to 10-fold increase was observed in *Verrucomicrobiaceae* (9.4% ± 4.1%), *Vibrionaceae* (7.6% ± 6.0%), *Flammeovirgaceae* (7.0% ± 3.8%) and *Rhodobacteraceae* (6.4% ± 2.4%) compared with new mucus. The archaeal domain generally exhibited a very low relative abundance in new and aged mucus (1.1% ± 1.7% and 1.4% ± 1.2%, respectively; Supplementary Figure S6) and did not significantly contribute to the variation in community structure between mucus aging stages.

Prokaryotic mucus re-colonization after antibiotics disturbance

Antibiotic-treated colonies exhibited no visible signs of health deterioration during the 8 days of aquaria incubation. Control colonies, in contrast, were negatively affected, as half ($n=3$) of them showed bleaching and necrosis during the incubation period (Figure 1b). Prokaryotic abundance in mucus of the colonies was highly variable, with an average of $3.9 \pm 4.5 \times 10^5$ cells ml^{-1} before the experiment ($n=12$). After 24 h of incubation with antibiotics (n = 6), prokaryotic abundance was only $5.4 \pm 4.6 \times 10^4$ cells ml^{-1} (Figure 3) representing a reduction to 14% of the original *in situ* abundance (rANOVA, $P<0.001$; Tukey HSD, $P<0.01$, see Supplementary Table S7). There was no apparent change in the overall alpha diversity of the coral mucus community (Table 1) concomitant with this reduction. This low abundance remained relatively constant until the end of the incubation period. In contrast, prokaryotic abundance in mucus of control colonies ($n=6$) remained relatively high ($4.1 \pm 1.7 \times 10^5$ cells ml^{-1} at 24 h) throughout the entire incubation period (see Figure 3 and Supplementary Table S7), accompanied with a significant increase in alpha diversity (rANOVA, $P<0.001$; Tukey HSD, $P<0.01$; see Supplementary Table S8–S10) compared with the original *in situ* alpha diversity.

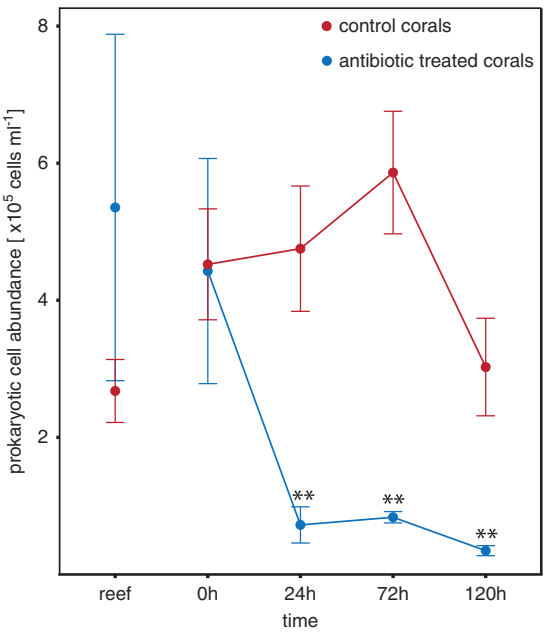

Figure 3 Prokaryotic cell abundance in coral mucus of *Porites astreoides* throughout the aquaria incubation for antibiotic-treated and non-treated control colonies. Samples were collected directly after corals were removed from the reef (reef), after 8 days acclimatization to the aquaria environment (0 h) and 24, 72 and 120 h after the beginning of incubation with antibiotics. Antibiotic treatment resulted in a significant decrease (Tukey HSD; $^{**}P<0.01$) in prokaryotic abundance relative to *in situ* values (reef). Error bars indicate ± s.d.

Once coral colonies were brought back to the reef they exhibited health response patterns opposite to those observed in the aquaria incubation (Figure 1b). Within the first 3 days, all antibiotic-treated colonies ($n=6$) showed rapid health deterioration and exhibited clear signs of bleaching, with only two surviving the following weeks. Non-treated corals, in contrast, recovered from aquarium incubations within 28 days of re-introduction to the natural reef environment (Figure 1b).

In total, 39% of the prokaryotic OTUs originally associated with mucus were present throughout the antibiotic treatment, as well as after re-introduction of treated corals into their natural habitat (Supplementary Figure S7a). Here, the families *Endozoicimonaceae* and *Oxalobacteraceae* were numerically dominant (Supplementary Figure S8). The control group, however, harbored 58% of the initial number of OTUs until the end of the incubation experiment (Supplementary Figure S7b). Furthermore, the prokaryotic community present in mucus of the control colonies (see Table 1) exhibited a significant increase in alpha diversity once transferred back to the reef (rANOVA, $P<0.001$; Tukey HSD, $P<0.01$ see Supplementary Table S8–S10). Re-introduced antibiotic-treated colonies, in contrast, were not significantly different in their alpha diversity from the original *in situ* alpha diversity (Tukey HSD, $P>0.5$, see Supplementary Table S8–S10).

Prokaryotic succession in coral mucus, as visualized by nMDS ordination (Figure 4 and Supplementary Figure S9), revealed contrasting successional paths for antibiotic-treated and non-treated corals (ANOSIM, $R=0.5082$, $P<0.001$). Initially, during the aquarium incubation, both groups diverged from the natural community typical of new mucus layers on healthy corals. After being deployed back on the reef, antibiotic-treated corals exhibited a major change in their community assembly within the first 24 h becoming more similar to that found in sediments and in aged mucus layers. Within the next days, their community became similar to the one of aged mucus layers and of unhealthy-looking colonies such as the control group during the aquaria incubation. This shift in the prokaryotic community happened concomitantly with an increase in bleaching and mortality. After 28 days, the only two surviving colonies harbored a community similar to that in new mucus. The control group, however, after suffering of necrosis and bleaching during aquarium incubation, rapidly regained a prokaryotic community similar to the original one and concurrently exhibited again a healthy appearance (Figure 4).

Differences in the successional path of antibiotic-treated and control corals throughout the monitoring period were revealed by multivariate community composition analysis (PERMDISP, $P>0.05$, Supplementary Table S11, PERMANOVA, $P<0.01$, Supplementary Table S12). Moreover, CCA (Supplementary Figure S10) attributed 67% of the variation in the prokaryotic community structure to differences in treatment (antibiotics versus control) and time (ANOVA-like permutation test, $P<0.001$ and $P<0.01$, respectively), whereas the colony effect was negligible ($P>0.05$).

Out of the prokaryotic families responsible for the divergence among treatment groups (SIMPER, Supplementary Table S13), *Endozoicimonaceae* was dominant in mucus (relative abundance up to 80%) of corals in their natural environment (Supplementary Figure S8). However, it decreased in relative abundance once corals suffered from bleaching and necrosis when kept in the aquaria but also in the reef. Within 24 h after re-introduction to the reef, the mucus of antibiotic-treated corals became dominated by *Verrucomicrobiaceae* (35.9% ± 20.2%) and *Vibrionaceae* (13.5% ± 14.7%). Within the next days, *Rhodobacteraceae*, *Oceanospirillaceae*, *Vibrionaceae*, *Flammeovirgaceae*, *Verrucomicrobiaceae* and *Colwelliaceae* dominated the mucus of antibiotic-treated colonies. Mucus of the control group became evenly colonized by various prokaryotic families such as *Verrucomicrobiaceae, Vibrionaceae, Rhodobacteraceae, Alteromonadaceae, Colwelliaceae, Pelagibacteraceae* and *Synechococceae* (Supplementary Figure S8). Archaea did not contribute to significant community variation and

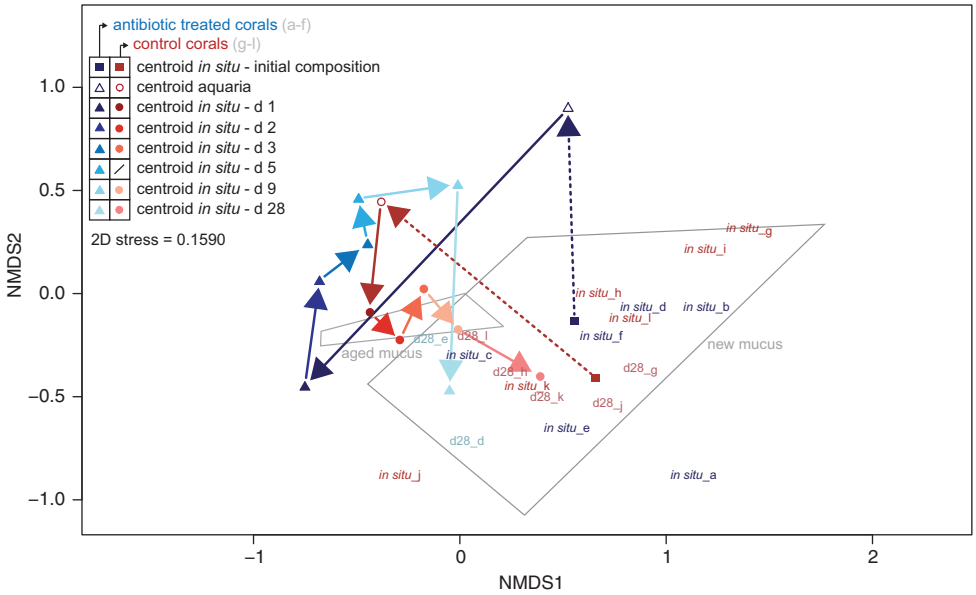

Figure 4 Prokaryotic mucus re-colonization after antibiotics disturbance: non-metric multidimensional scaling (nMDS) plot of the Bray–Curtis-based dissimilarity matrix of prokaryotic communities colonizing the mucus of *Porites astreoides* and their shifts after a disturbance event. The successional path, here represented by arrows, was reconstructed based on the position of group centroids and follows each group of corals (antibiotic-treated, a–f; control, g–l) at a particular time (indicated by color gradient). The starting point '*in situ* - initial community composition' represents the original *in situ* state, followed by the shift in the community composition in the aquaria incubation (dashed arrows) and finally describing the successional path over 28 days observed in the field after the two experimental groups of corals were brought back to the reef (normal arrows). Prokaryotic community structure of individual colonies is only given for mucus samples collected before the experiment started (*in situ*_a–l) and on the last day of the experiment (d28_a–l). Polygons indicate the relative position of prokaryotic communities associated with different mucus aging stages ('new mucus' versus 'aged mucus') characterized during the natural dynamics experiment and are shown as reference. Individual sample points throughout the whole experiment are given in detail in Supplementary Figure S9.

showed very low relative abundance in mucus throughout the disturbance experiment (overall 1.25% ± 4.28%) with the exception of two samples collected at the end of the antibiotic treatment (34.33% and 17.48%, respectively; Supplementary Figure S11).

Prokaryotic indicators associated with the coral mucus microbiome

Identified prokaryotic indicators were distinct among the three habitats but individual members not strictly confined to the respective habitat (Figure 5 and Supplementary Table S14). *Oxalobacteraceae* and *Endozoicimonaceae* were identified as prokaryotic indicators associated with new mucus of *P. astreoides*. Together they accounted for 50% of all prokaryotes found in new coral mucus *in situ*. Similarly high contributions to the mucus microbiome were observed in the population of corals used for the disturbance experiment (before collection), as well as after treatment with antibiotics and, to a minor extent, within colonies surviving the disturbance experiment after 28 days. Indicators for new mucus, however, showed a reduction in their relative abundance in aged mucus, whose typical indicators consisted of *Verrucomicrobiaceae*, *Vibrionaceae*, *Flammeovirgaceae*, *Rhodobacteraceae* and *Alteromonadales*. Furthermore, mucus of disturbed corals showed increased relative abundances of aged mucus indicators, particularly dominant in antibiotic-treated colonies after re-introduction to the reef environment. Prokaryotic indicators identified for seawater and sediment did not exhibit major variations in dominance throughout the experiment, with the exception of a high contribution of typical seawater OTUs to the microbiome of control colonies at the end of the experiment.

Discussion

Natural dynamics of coral mucus-associated prokaryotes

Although the cyclic aging and shedding of the SML in colonies of *Porites* sp. is fairly well documented (see Coffroth, 1991 and Supplementary Information), the associated dynamics of its mucus-dwelling prokaryotic community remained to be resolved. We demonstrate that the prokaryotic community undergoes significant changes throughout the mucus aging cycle, both in terms of cell abundance and community composition. Generally, the SML of *P. astreoides* was dominated by the bacterial families *Oxalobacteraceae* and *Endozoicimonaceae*, the latter of which has commonly been found in healthy corals (Apprill *et al.*, 2013; Lema *et al.*, 2014;

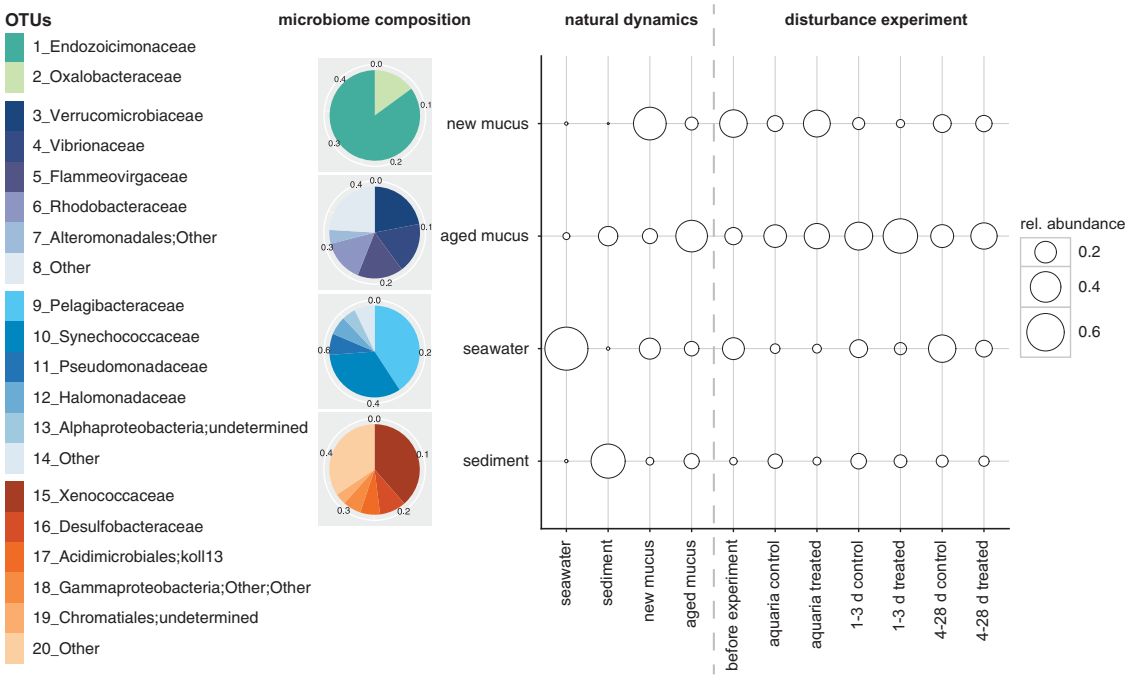

Figure 5 Prokaryotic indicator assemblages identified for the microbiome of SML of *Porites astreoides* ('new mucus' versus 'aged mucus'), the adjacent seawater and the sediment, and their abundance distribution throughout the natural dynamics and the disturbance experiment. Pie charts represent the relative abundance of indicator prokaryotic families (given on the left) that are significantly associated with a particular microbiome as revealed by the indicator value analysis (IndVal). Numbers around pie charts depict, for each habitat studied, the proportion of the total community represented by the indicator assemblages. The balloon plot on the right displays the relative abundance of the identified indicator assemblages in the various natural habitats and throughout the disturbance experiment. Note that prokaryotic indicator assemblages identified for a particular habitat are not restricted to that habitat and are also present (in lower abundance) in other habitats.

Meyer *et al*., 2014) and suggested to have coevolved with specific host species (Bayer *et al*., 2013).

In contrast, aged mucus sheets exhibited a high relative abundance of *Verrucomicrobiaceae*, *Flammeovirgaceae, Rhodobacteraceae* and *Vibrionaceae* (see Figure 5 and Supplementary Figure S5). The latter two bacterial families include coral pathogens and are commonly associated with coral diseases (Ben-Haim *et al*., 2003; Sunagawa *et al*., 2010). After the detachment of the aged mucus sheet, however, the microbial community reverted to its original composition within 3–5 days (see Figure 2), supporting the idea that periodical mucus shedding in poritid corals generates a natural, rather deterministic fluctuation of the mucus-dwelling prokaryotic community, taking place within a temporal scale of weeks. As the prokaryotic indicator assemblages associated with aged mucus sheets showed surprisingly high similarity to the community associated with mucus of disturbed coral colonies (Figures 4 and 5), some of which died off, we propose that periodic mucus shedding (Coffroth, 1991) is an important mechanism supporting coral health by periodically removing undesirable prokaryotes from the surface of the colony leading to the maintenance of a beneficial mucus microbiome.

Once coral mucus is detached from the colony, it functions as particle trap before sinking to the seafloor, where it acts as energy and nutrient source for benthic organisms in coral reefs (Wild *et al*., 2004; Naumann *et al*., 2009). Alongside, aged mucus sheets frequently harbor sediment particles (Figure 1a), suggesting that prokaryotes colonizing upper sediment layers in coral reefs could 'hitch a ride' to the nutrient-rich mucus layer via sediment resuspension. Thus, sediments may serve as a 'seed-bank' for coral mucus-associated microbes as proposed by Carlos *et al*. (2013). Both, the resuspension of sediment particles (and its associated microbes) onto the coral's surface and the rapid sedimentation of detached coral mucus would lead to a high connectivity between the microbial communities of aged mucus and coral reef sediments, and consequently, may explain the similarity in their prokaryotic community composition (Figure 5). In contrast, microbial communities of the ambient seawater exhibited only minor overlap with those found in mucus. This confirms earlier findings (Rohwer *et al*., 2001; Frias-Lopez *et al*., 2002) and stresses the importance of the SML as a selective medium for the microbial pool in the adjacent seawater.

Prokaryotic mucus re-colonization after antibiotics disturbance

Antibiotic treatment led to a significant reduction of prokaryotic abundance in the SML and concurrent changes in community composition. Although the

full extent of the influence of antibiotics on the holobiont's fitness remains elusive, no visual cues of a negative impact on coral health were noted. This confirms previous reports of minimal effect of antibiotic treatment on zooxanthellae photosynthetic efficiency and host tissue protein content (Gilbert *et al.*, 2012). These findings suggest that coral hosts are not strictly dependent on their mucus-associated prokaryotic symbionts, at least for such short periods of up to 8 days and in the absence (or deactivation) of pathogens.

Control colonies kept in the aquaria without the addition of antibiotics exhibited obvious signs of bleaching and necrosis and underwent a significant shift in their mucus prokaryotic community (Figure 4). The bacterial family *Endozoicimonaceae*, which showed the highest relative abundances in newly produced SMLs and is associated with mucus of healthy *P. astreoides* colonies (Meyer *et al.*, 2014), showed a significant decrease in its relative abundance within the control group. Concomitant with this decrease, many bacterial groups such as *Rhodobacteraceae*, *Verrucomicrobiaceae*, *Colwelliaceae*, *Oceanospirillaceae* and *Flavobacteriaceae* increased in their (relative and absolute) abundance (see Figure 3 and Supplementary Figure S8). These findings are consistent with a previous study attributing the expression of lesions in *P. astreoides* colonies to the loss of *Endozoicimonaceae* and the proliferation of an opportunistic bacterial community (Meyer *et al.*, 2014). Compositional shifts in the microbial community associated with the SML have been observed under stressful environmental conditions (Thurber *et al.*, 2008). Based on these findings, the observed health deterioration of the untreated control group may have been caused by aquaria conditions leading to a destabilization of the natural mucus community. This interpretation is in agreement with the hypothesis that disturbances in the dynamic equilibrium of the coral's native microbiota may result in health deterioration (Lesser *et al.*, 2007).

Both, antibiotic-treated and control colonies, once brought back to their natural habitat, exhibited rapid changes in their mucus-associated prokaryotic community (Figure 4 and Supplementary Figure S8). For the microbe-depleted (treated) colonies, the increase in *Vibrionaceae* was dominated by *Vibrio* sp., a genus harboring well-known coral pathogens such as *Vibrio shilonii* (Kushmaro *et al.*, 1997) and *Vibrio corallilyticus* (Ben-Haim *et al.*, 2003; Garren *et al.*, 2014). The latter is reported to use coral-produced sulfur compounds as a cue to target stressed corals (Garren *et al.*, 2014). Curiously, a recent study has shown that *P. astreoides* (among other coral species) produces copious amounts of the organic sulfur compound dimethylsulfoniopropionate, particularly upon stress (Frade *et al.*, 2015). Furthermore, *Verrucomicrobiaceae, Flammeovirgaceae* and *Rhodobacteraceae*, other families associated with necrotic and diseased *P. astreoides* colonies, have been found to be overrepresented in poritid corals suffering from White Band Disease (Séré *et al.*, 2013; Roder *et al.*, 2014). Although non-treated colonies slowly recovered from tissue lesions and regained a mucus community very similar to their original community, antibiotic-treated colonies suffered from mortality (from day 3 onward) and exhibited increased relative abundances of bacterial families described as early colonizers of marine biofilms, such as *Rhodobacteraceae* and *Oceanospirillacae* (O'Toole *et al.*, 2000; Sweet *et al.*, 2011b). Although we cannot exclude other synergistic effects on the host's health, these results suggest that microbe-depleted SML provides an open niche, which gets rapidly colonized by opportunistic bacteria. Thus, we hypothesize that the re-establishment of both coral health and the native prokaryotic community after disturbance of the *P. astreoid*es holobiont depends on the initial degree of disruption of the microbiome. Total recovery of control colonies under *in situ* environmental conditions in contrast to mortality of microbe-depleted colonies suggests that, upon disturbance, the remnant prokaryotic community in the mucus/tissue may act as 'seed-bank'.

The mucus microbiome and its influence on coral health and survival

Host-associated bacteria form unique microbiomes highly adapted to a particular host niche (Ainsworth *et al.*, 2015). Although being generally considered to comprise commensals, microbiomes can fulfill important biological needs of their hosts, for example, immune development or nutrient acquisition (Round and Mazmanian, 2009; Shin *et al.*, 2011). We identified *Endozoicimonaceae* and *Oxalobacteraceae* as significant indicators for the mucus microbiome of healthy *P. astreoides* colonies (Figure 5). The reduction in the relative abundance of these microbiome members observed before coral necrosis and bleaching suggests that the loss of beneficial bacteria can result in a serious health threat for the holobiont, often associated with an increase in opportunistic and potentially pathogenic bacteria (Meyer *et al.*, 2014). Although our study does not aim at determining the metabolic function of particular microbiome members, it reveals that an intact mucus microbiome may function as a barrier against potentially harmful bacteria. This defense barrier could be based on commensal-like prokaryotes, which prevent harmful colonization by successfully outcompeting pathogens (Reid *et al.*, 2001) and/or depend on stimulating host immune response via the recognition of commensal-derived signals such as microbial-associated molecular patterns (Mackey and McFall, 2006). Our results suggest that the mucus microbiome acts as defense barrier against pathogenic microbes, therefore facilitating

homeostasis and contributing to the survival of the coral holobiont.

In summary, we demonstrate that the previously documented periodical mucus aging and shedding cycle in *P. astreoides* is provoking predictable shifts in the mucus microbiome, leading to changes between a beneficial community and a potentially opportunistic/pathogenic one. The periodical release of mucus seems to be part of a life strategy that supports the maintenance of a beneficial mucus microbiome and the resilience of coral health in shallow water habitats characterized by frequent sediment resuspension. However, severe disruption of the natural microbial community upon external stress could negatively and irreversibly affect the fate of the coral holobiont. Further investigations on the functional capacities of the mucus prokaryotic community are warranted to better understand the role of the mucus microbiome in the dynamic equilibrium of the coral holobiont. Finally, we have shown that specific bacterial members can be used as indicators of coral microbiome disruption, paving the way to the development of early diagnostic tools to monitor the health status of corals.

Conflict of Interest

The authors declare no conflict of interest.

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