New coral associated fungi found at Curacao

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ABSTRACT

One of the most biologically diverse ecosystems on earth are tropical coral reefs. It is the habitat for one-third of the marine fish species for at least part of their life cycle. Hard corals are the major structural builders of this system. Nowadays, knowledge of the microbiome of corals is mainly focused on bacteria and symbiotic zooxanthellae. Thus, the importance of fungal coral interactions remains unrevealed which may be both beneficial and pathogenic. Only recently more marine fungi have been studied due to an increase in corals affected by pathogenic fungi. We extended these studies by cultivating endolithic fungi from healthy and diseased coral *Sidrastrea siderea,* as well as healthy corals of the species *Agaricia agaricites, Montastraea annularis, Madracis mirabilis, Stephanocoenia intersepta* and *Montastraea cavernosa* from the reef of Curacao in the Caribbean Sea. Sterilisation by UV light turned out to be a successful method to kill microorganisms at the surface of the coral without affecting the fungi from the internal part. A total of 90 colonies originating from internal parts of corals were purified on plates based on visual inspection, while 4 species were found based on DNA sequencing. Newly found coral associated fungi were *Aspergillus tamarii, Penicillium solitum, Aspergillus flavus* or *Aspergillus oryzae*, *Aspergillus tubingensis*, *Aspergillus penicillioides*, *Penicillium citrinum*, and *Penicillium westlingii*. *Aspergillus versicolor*, *Aspergillus sydowii,* were identified in this study as well as in previous studies. Notably, a higher diversity of fungi was cultured from healthy than from diseased *S. siderea* coral. The identified fungi and what is known from these species suggest that coral associated fungi act as pathogens, bio-eroders, bio-borers, but may also be beneficial as being part of the coral microbiome. In the latter case it may produce mycotoxins, antibacterial, and / or fungal metabolites to protect the coral and its microbiome. In future studies, it would be interesting to combine metagenomics with the approach that was adopted in this study, especially since fungi play a bigger role in coral ecosystems than assumed so far.

Introduction

One of the most biologically diverse ecosystems on earth are tropical coral reefs. It includes for instance hard and soft-corals, sponges, crustaceans, worms, fish, bacteria, viruses, and fungi. They compete for resources such as food, space and sunlight. Most of the tropical coral reefs have hard corals as the major structural builders of this system[1]. Natural events such as tsunamis, volcanic eruptions, hurricanes as well as climate change cause shifts in these systems. Climate models predict an increase in the variability of weather; from year to year, and long-term effects, as a result from increasing anthropogenic CO_2 concentrations[2].

Tropical coral reef ecosystems are affected by the direct and indirect effect of human population growth[3]. Protection of coral reefs is not only important from a biodiversity point of view as they are also important for human food supply. 17% Of animal protein originates from fish with about one-third of the marine fish species using coral reefs during their lives[4]. Other major feedbacks between coral reefs and human kind are pollution and climate change. These proximal drivers have a negative effect on coral reef ecosystems, where ecosystem goods and services as ocean recreation and tourism, and more important coastal protection and food supply will be affected by these proximal drivers (**Figure 1**)[3,5].

Corals belong to the Class Anthozoans of the phylum Cnidaria. The largest order is covered by the Scleractinians that consists of the hard corals. Hard corals form the major coral reef structure. Other organisms as crustose coralline algae (CCA), calcium carbonate forming organisms, and sedimentary fill by all kind of organisms contribute too[1,6]. Hard corals consist mainly of polyps.
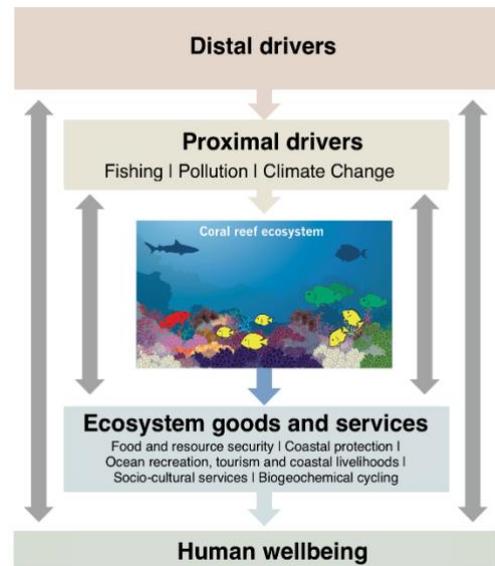
Figure 1; Feedback of human society and coral reefs. Distal drivers are traits in social systems that indirectly influence how people interact with coral reefs. Proximal drivers directly affect coral reef ecosystems (centre). Coral reefs provide important ecosystem services to people, which influence human wellbeing. Single-headed arrows indicate how the pathway flows from distal drivers to human wellbeing. Double-headed arrows show the complex linkages and feedbacks that also occur between the various components. Modified from Hughes et al. (2017) and Cinner et al. (2015).

Table 1; *Fungi associated with corals (modified from Kendrick et al. (1982) and Raghukumar and Ravindran (2012). Fungi from reference Kendrick et al. (1982) were fungi that were identified after culturing on mycological culture media[7,8]. * = found in healthy corals.*

Fungi	Coral host	Location	Reference number
Acremonium sp.	*Meandrina meandrites, Portites lutea*	Barbados (West Indies), Arabian Sea	7,9
Alternaria sp.	*Acropora formosa**	Great Barrier Reef (GBR)	10
Aspergillus-like	*Porites lobata, Porites lutea*	Moorea French Polynesia	11
Aspergillus restrictus	*Diploastrea heliopora*	GBR	7
Aspergillus sp.	*Portites lutea*	Arabian Sea	9
Aspergillus sydowii	*Acropora palmata, Montastrea annularis, Porites porites*	Barbados (West Indies)	7
Aspergillus versicolor	*Acropora sp., A. hyacinthus, Sidrastrea siderea, Goniastrea retriformis, Diplostrea heliopora, Porites australensis*	GBR	7
Asteromella sp.	*Diploastrea heliopora*	GBR	7
Aureobasidium sp.	*Portites lutea*	Arabian Sea	9
Aurobasidium pullulans	*Acropora formosa*	GBR	10
Bipolaris rostra	*Acropora palifera*	GBR	7
Chaetomium sp.	*Portites lutea*	Arabian Sea	9
Cladosporium sp.	*Portites lutea, Acropora formosa*	Arabian Sea, GBR	9,10
Cladosporium sphaerospermum	*Montastrea annularis, Porites sp.*	Barbados (West Indies), GBR	7
Class: Ascomycetes	-	Belize (Central America),	8,12
Corallochytrium limacisporum	*Acropora sp., Porites sp.*	Arabian Sea	13,14
Fusarium sp.	*Portites lutea*	Arabian Sea	9
Genus: *Koralionastes* (5 species not cultured)	-	-	15
Hormonema alopallonella	*A. hyacinthus*	GBR	7
Hormonema dematioides	*Acropora sp., Goniastrea retiformis, Porites australensis, Stylophora pistillata*	GBR	7
Humicola fuscoatra	*Acropora formosa, Cladocora caespitose*	GBR, Caribbean Sea and Indo-Pacific	10,16
Labyrinthula sp.	*Portites lutea*	Arabian Sea	9
Mycelial yeast	*Portites lutea*	Arabian Sea	9
Paecilomyces godlewski	*M. cavernosa*	Barbados (West Indies)	7
Paecilomyces lilacinus	*G. australensis*	GBR	7
Penicillium avellaneum	*Porites porites*	Barbados (West Indies)	7
Penicillium citrinum	*Acropora formosa*	GBR	10
Penicillium expansum	*Acropora palmata*	Barbados (West Indies)	7
Penicillium restrictum	*Porites australensis*	GBR	7
Penicillium stoloniferum	*Acropora palmata, Diploria labyrinthiformis*	Barbados (West Indies)	7
Phialophora bubaki	*Diploastrea heliopora, Goniastrea retiformis, Porites australensis*	GBR	7
Phoma sp.	*Acropora formosa**	GBR	10
Pichomyces chartarum	*Acropora sp.*	GBR	7
Scolecobasidium sp.	*Montipora tuberculosa, Porites lutea, Goniopora*	Andaman Islands, Bay of Bengal and Indian Ocean	14
Thraustochytrium motivum	*Various coral species*	Arabian Sea	14
Trichoderma sp.	*Cladocora caespitose*	Caribbean Sea and Indo-Pacific	16
Wallemia ichthyophaga	*Porites sp.*	GBR	7

Each polyp is made out of three tissue layers with the mesoglea being flanked by the epidermis and the endodermis[17]. A circle of tentacles around the mouth help the coral to capture and ingest plankton for food, clear away debris from the mouth, and act as the animals primary means of defence[6,17]. Stinging cells called nematocysts are located along the surface between the mouth and the tentacles. They secrete powerful and often lethal toxins. Corals use these cells to capture their prey, and facilitate coralline agonistic interactions[17].

Symbiotic zooxanthellae algae form a common symbiotic relationship with hard corals. They are located just underneath one mesoglea layer[17]. Zooxanthellae provide the coral with oxygen, glucose, glycerol and amino acids that are used as building blocks for proteins, fats, and $CaCO_3$. In return, corals give a protected environment, carbon dioxide for photosynthesis and nitrates and phosphates. The uptake of CO_2 produced by the coral by zooxanthella enhances biological limestone-secreting capacity of hard corals. $CaCO_3$ is the major chemical ingredient of calcareous sediments and limestones. The secreted $CaCO_3$ is typically of the calcite and/ or aragonite type[6,18,19]. Where calcite is a weaker structure then aragonite[18]. When corals 'bleach' the zooxanthellae expel. The coral colony will appear white because of disappearance of the pigments of the photosynthetic zooxanthellae[20]. Calcification rates will also decrease when photosynthesis of zooxanthellae is inhibited or when zooxanthella are expelled[20]. This is a likely threat for coral ecosystem structure. Zooxanthellae are described well in literature, and are part of the coral holobiont, a complex symbiosis between endosymbiotic alga and other microorganisms. The latter may contribute to coral defence by secretion of metabolites[10,21]. However, the importance of fungal coral interactions in relation to health of the holobiont remains unknown. Terrestrial fungi are investigated well in comparison with marine fungi, despite the fact that they are omnipresent in the marine environment[7,8]. Only a small fraction of marine fungi have been described so far with a total of 549 species out of an estimated 10,000 taxa[12,22]. They have been studied or identified in tidal salt marches, in mangroves, shorelines, living in rhizomes, marine wood or bark, as parasites and saprobes of algae, being part of submarine lichens and lichen like associations, being associated with marine animal diseases[7,8,10,12,22–26] and as part of corals. In the latter case, they are geographically and taxonomically widespread suggesting that these endolithic fungi play a greater role in ecology of coral reef systems than thought[7,10,27].

The importance of fungal coral interactions is unknown, but the number of coral species affected by pathogenic fungi has increased over the last decade[28,29]. Parasitic fungi may play a major role in degradation of calcareous substrates, including the hard coral framework[30]. Still, these fungal diseases are poorly understood[31]. Fungal diseases range from the black band/line disease on the coral *Montastrea annularis* caused by a lower marine fungus[32], the pink line disease on several coral species caused by *Scolecobasidium* sp.[14], to the fungal protozoan syndrome caused by *Trichoderma* sp., *Cladosporium* sp., *Penicillum* sp., *Humicola* sp. and the protozoan *Ciliate*[16] on soft coral *Gorgonian* sp. (subclass; Octocorallia, order Alcyonacea) and hard coral *Cladocora caespitose*[16]. An exception of these poorly investigated marine fungal diseases is Aspergillosis disease, caused by *Aspergillus sydowii* that is also a opportunistic pathogen of humans[33]. *A. sydowii* caused death of many Caribbean *Gorgonian* soft corals around 1990[31,34]. It erodes the skeletal framework and kills living tissue of the corals[16]. The affected *Gorgonians* are characterised by purple dense nodules. The sea snail *Cyphoma gibbosum* that feeds on Gorgonians was demonstrated to transmit *A. sydowii*[35]. Contra-intuitively, Toledo *et al.* (2007) showed a greater fungal diversity in healthy sea fans than in diseased ones[36].

A summary of fungi associated with hard corals is shown in **table 1**. Most of the associated coral fungi were harvested from diseased corals. *Phoma* sp. and *Alternaria* sp. were found to be associated with healthy corals only. Coral associated fungi have been identified by sequencing the nuclear ribosomal ITS region (18s RNA), determination by vision and using a microscope, metagenomics, and combinations thereof[7–10,14–16,26,31,32,34,36]. So far, the only autochthonous coral fungi are uncultured *Koralionastes* together with other unidentified Ascomycetes[8]. Some of the coral associated fungi are bio-eroders. Boring fungi may play an important role in the bio mineralization of coral skeleton, and therefore the coral reef structure. Harmless endolithic fungi can become opportunistic pathogens when the coral reef equilibrium is disturbed, initiated by environmental stressors[8]. Currently, effects of these penetrating fungal hyphae on corals, their mechanism of penetration and their role in enzyme processes are unkown[8]. Research about internal fungi, part of the corals holobiont, is scarce[8,11,27,37–42].

The Caribbean Sea is seen as a coral disease hotspot. Here, fungal cultivated diversity of the corals *Sidrastrea siderea, Agaricia agaricites, Montastraea annularis, Madracis mirabilis, Stephanocoenia intersepta* and *Montastraea cavernosa* from the reef of Curacao in the Caribbean Sea was studied[43]. Diseased pieces of *S. siderea* were also investigated. Fungi were identified microscopically and by using primers CMD5[44] (forward), V9G[45] (forward) and LS266[46] (reversed), for ITS and species related gene sequencing from internal parts of the corals.

Materials & Method

Coral harvesting

Coral samples were collected by Carmabi research station Curacao. A total of 33 samples were taken from *Sidrastrea siderea* (5 healthy and 4 diseased), *Agaricia agaricites* (5 healthy), *Montastraea annularis* (5 healthy), *Madracis mirabilis* (6 healthy), *Stemhphanocoenia intersepta* (5 healthy) and *Montastraea cavernosa* (3 unknown healthy or diseased), on Friday 16th of June 2017. The samples were collected at various sites at Curacao (12°07'08.6"N; 68°58'10.5"W), from approximately 7-meter depth (± 0.7 bar). At ± 7 meters depth, maximum light availability during the day is 1.08-1.35 Lux[47]. Fragments of approximately 2 x 2 cm were collected from colonies using hammer and chisel. Healthy and diseased colonies were sampled at similar locations, on the edge of the colony. Coral samples were kept in an aquarium for 6 days, which was connected to the ocean, prior to transport.

Coral transport

Samples were packed in tissue wetted with seawater. Each group of samples was packed in a plastic bag with 50 ml seawater. The plastic bags were packed in a cool box filled with a bottom of 5 cm original seawater of 28 °C and transported by plane. After transport, the cool box was stored for 7 days at 25 °C in a climate room (Snijders Scientific; MC785 Easy, room 2).

Media and growth

Coral samples were stamped on complete agar medium (CM)[48], CM with 36 g/l NaCl and 5 g/l CaCO$_3$ (CM+), minimal agar medium (MM)[48], MM with 36 g/l NaCl (MMNaCl), MM with 5 g/l CaCO$_3$ (MM CaCO$_3$) and MM with 36 g/l NaCl and 5 g/l CaCO$_3$ (MM+) (see **Table 2** for the exact composition). The enrichment of salt in medium was based on the sea surface salinity mean of Caribbean seawater of 36 psu (varies among the year)[49]. Media contained 50 µg ml^{-1} ampicillin and pH was set with NaOH, KOH and HCL, at 7.80 (local ocean water varies between 7.8 ± 8.2)[50,51]. After the pH was set, medium was autoclaved. Agar plates were stored at 4 °C and used within 3 weeks. Small agar plates were used for the diversity experiments (⌀5 cm, 10 ml) (see **Appendix I**), while big plates were used (⌀9 cm, 20 ml) for purification of the fungi and harvesting spores (see **Appendix II**). Fungi were grown at 25 °C in a dark box[8,37,52] in a climate room under aerobic conditions (annual sea surface temperature of the Caribbean Sea is 27 °C (±3°C)[49]). Air was saturated with water by putting tissues wetted with distilled water at the bottom of the box. 10 g of natamycin powder (50% natamycin, 50% lactose) was spread over the wetted tissues to prevent contamination between plates. Before the experiment, the box was cleaned with 50% ethanol and tissues with natamycin powder were replaced every 3 weeks. Samples were taken out of the plastic bags in a flow cabinet with sterile tissues and equipment. Every sample was stamped on six different agar plates (CM, CM+, MM, MMCa, MMNa, MM+), the so called 'unwashed treatment' (D). Stamping was done in a random order and added at **Appendix I**. Not all samples were stamped on the same day, a few were dead before these treatments (**Appendix IV; Table 1** and **2**). After stamping, samples were washed with sterile seawater (milli-Q with 36g/l Instant Ocean[53]), wiped off with sterile tissue wetted with sterile seawater, and stamped again on six new agar plates, called as 'washed treatment'(C). The coral samples were submerged in 50 ml sterile seawater tubes and incubated at 25 °C. Viability and bleaching were monitored every day by appearance following Manual for RSEC Coral Reef Projects[54]. The last coral died 33 days after harvesting. All samples were sterilised after death (**Appendix IV; Table 1** and **3**). The outside of the coral was sterilised by UV light (Chromatolux 2L, lamp; Svivania, G8W) for 2 min for each of the six sides. The UV light was placed right angled, 28.5 cm above the samples. After the UV treatment, the whole outer area of the coral was stamped on six different agar plates to confirm effective killing of the microbes at the surface, called 'UV treatment' (UV). First sample SIH2 was sterilised by UV light as a trial. When no growth was detected after three days, samples were then cut lengthwise with an electric saw (Zimmer, Plaster Saw type 2) followed by stamping six different agar plates, as so called 'cross-sectioned treatment'(L). In addition, internal parts of corals (1 ± 5 mm in diameter) were transferred to 1.5 ml tubes containing two sterile carbon balls. Material was homogenized in a Tissuelyser (Schwingmühle Tissulyser 2, 2008) with a 25-frequency s^{-1} for 15 min. The resulting powder was mixed with 1 ml of sterile water with 0.9% NaCl, and 0.15 ml of this suspension was plated out on each of the six different agar plates 'mortar treatment' (M).

Table 2; *Composition of media that were used for growing fungi. The basic ingredients of Complete medium and Minimal medium are based on de Vries et al. (2004)*[48].

Media	pH	Glucose	NaNO$_3$	KH$_2$PO$_4$	MgSO$_4$ (H$_2$O)$_7$	Agarose	Trypton	Casamino acids	Yeast extract	Yeast ribonucleic acids	NaCl	CaCO$_3$	Vishniac	Ampiciline
CM	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l	2 g/l	1 g/l	1 g/l	0.5 g/l			0.2 ml/l	1 ml/l
CM+	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l	2 g/l	1 g/l	1 g/l	0.5 g/l	36 g/l	5 g/l	0.2 ml/l	1 ml/l
MM	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l							0.2 ml/l	1 ml/l
MMNa	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l					36 g/l		0.2 ml/l	1 ml/l
MMCa	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l						5 g/l	0.2 ml/l	1 ml/l
MM+	7.80	5 g/l	6 g/l	1.5 g/l	0.5 g/l	15 g/l					36 g/l	5 g/l	0.2 ml/l	1 ml/l

Isolation of spores

10 ml Saline Tween mixture was used to harvest spores for each 9 cm Petri dish. Saline Tween mixture consisted of 200 ml sterile water with 0.9% NaCl, mixed with 2 ml 0.5% Tween. 1 ml Saline Tween was mixed with spores from one agar plate, which were put in 15 ml tubes. These tubes were centrifuged (Harrier, MSB080.CR.K.) for 10 min at 1000 RPM. The pellet was mixed with 2 ml of glycerol medium (87% glycerol, 2 volumes of 1% peptone in milli-Q) and 2 aliquots (1 ml each) was stored at -80 °C (Innova, C585-86).

Determination of fungi

Plates were checked for fungal grow after 3 and 8 days[12,55]. Fungi were purified by transferring them to fresh media and identified with the keys of Samson *et al*. (2010) using a binocular (Leica, DFC.420C) and light microscope (Zeiss, Axioskop 2 plus)[56]. Fungi isolated from the inside of corals were grown on Malt Extract agar (MEA)[56] and CZapek Yeast Autolysate agar (CYA)[57] for 7 days at 25 °C in the dark[58]. These plates were grouped and identified by Dr J. Houbraken from the Westerdijk institute. Plates were photographed at a distance of 30 cm right angled with a Nikon, D300S camera.

DNA isolation

A maximum of two fungi per group of successfully isolated fungi species, from treatments L and M, were used for DNA analyses.

Liquid shaken cultures inoculated with 50 µl isolated spores grown at 25 °C for 3 days at 200 RPM in 250 ml complete liquid medium containing 50 µg ml^{-1} ampicillin and 25 mM glucose. Mycelium was harvested by filtration, frozen in liquid nitrogen and stored at -20 °C. DNA isolation was done using an adjusted protocol from Abubakkar *et al.* (2012)[59]. Mycelium (2 grams wet weight) was homogenized by using a mortar and pestle under liquid nitrogen. The homogenate was transferred to a 50 ml Falcon tube containing 10 ml preheated (65 °C) extraction buffer (cethyl trimemethyl amonium bromide (CTAB) 20 g/l). The mycelium was suspended by inverting the tubes several times after which they were incubated for 60 min at 65 °C. 10 ml chloroform-isoamyl alcohol (24:1, v/v) was added, mixed to form an emulsion, and spun at room temperature for 15 min at 5309 rpm. The supernatant was transferred carefully to a new Falcon tube and an equal volume of chloroform-isomayl alcohol was added. After gentle mixing the emulsion, tubes were centrifuged again at room temperature for 15 min at 5309 rpm, this process was repeated twice. Supernatant was transferred again carefully, and 6 ml of pre-chilled (4 °C) 100% isopropanol was added, mixed gently by inverting the tube. Tubes were placed at -20 °C for 15 min. Precipitated DNA was pelleted by centrifugation at 6000 RPM for 10 min. The pellet was washed twice with 70% ethanol with intermediate centrifugation steps. Pellets were air-dried by placing them inverted on a paper towel. DNA was suspended in 200 µl 0.1 x TE buffer (1M Tris-HCl pH = 8.0; 0.5M EDTA, pH = 8.0) and 1 µl RNAse per 20 µl DNA solution was added. After incubation for 2 h at 37 °C 200 µl chloroform-isoamyl alcohol was added and mixed gently. The tube was spun for 10 min at 5309 RPM and the supernatant was transferred to a new Eppendorf tube. 1/10 volume of 3M NaCl was added, mixed, followed by adding 2 volumes of pre-chilled (4 °C 100% isopropanol). The sample was spun for 10 min at 8500 x g. The pellet was washed twice with 70% ethanol, air dried, and dissolved in 100 µl 0.1x TE buffer. DNA concentration was measured by OD 260 nm[60] by using a Nanodrop (spectrophotometer ND-1000). Quality of chromosomal DNA was checked by electrophoresis in a 1% agarose TAE gel. To this end, 20µl DNA sample was mixed with 4 µl orange-G. A 1 kb DNA ladder was used as reference.

PCR

Forward and reversed primers of β-tubuline, calmoduline and ITS (**Table 3**) were used to identify fungal strains. The PCR reaction mixture (25 µl) contained 0.5µl template (DNA isolate, 10 ng/µl), 2.5µl buffer (Taq inclusive MgCl$_2$), 2.5 µl 2mM dNTP's, 2.5 µl for each forward and reverse primer and 12 µl milli-Q. The amplification was carried out in a thermal cycler (Biometra, T300) using specific programs for *Aspergillus, Penicillium* and unidentified fungi (**Table 3**). In all cases, the PCR program started with an initial denaturing process of 120 seconds, followed by 35 denaturation, annealing and elongation cycles. The program ended with a final elongation at 72°C for 4 min. The amplified products were shown by electrophoreses on a 1% agarose gel, containing ethidium bromide.

Table 3; Thermal cycle programs used for amplification.

Indicated genus	Primer name and direction	Sequence	PCR program		
			Initial denaturing	Cycles, denaturing, annealing, elongation	Final elongation
Aspergillus	CMD5[44] forward	5'-CCG AGT ACA AGG ARG CCT TC- 3'	95 °C, 120 s	(35 times) 95°C, 60 s.; 56. 5°C, 60 s.; 72 °C, 60 s	72 °C, 240 s
	CMD6[44] reversed	5'- CCG ATR GAG GTC ATR ACG TGG-3'			
Penicillium	Bt2a[61] forward	5'-GGT AAC CAA ATC GGT GCT GCT TTC-3'	95 °C, 120 s	(35 times) 95°C, 60 s.; 59. 5°C, 60 s.; 72 °C, 60 s	72 °C, 240 s
	Bt2b[61] reversed	5'- ACC CTC AGT GTA GTG ACC CTT GGC- 3'			
Unidentified fungi	V9G[45] forward	5'-TTA CGT CCC TGC CCT TTG TA-3'	94 °C, 120 s	(35 times) 95°C, 60 s.; 55°C, 60 s.; 72 °C, 60 s	72 °C, 240 s
	LS266[46] reversed	5'-GCA TTC CCA AAC AAC TCG ACT C-3'			

DNA sequencing

PCR products of *Aspergillus* and *Penicillium* were cut out the gel and cleaned with a gel and PCR cleaning kit (NucleoSpin)[62] following the instructions of the manufacturer. All samples with a 230/260 ratio below 2 (from the nanodrop protein/DNA; 230/260), were cleaned again with NT3 of the kit. For *Aspergillus* PCR products, CMD5 forward[44] and CMD6 reversed[44] were used as primers. Bt2a forward[61] and Bt2b reversed[61] were used as primers for *Penicillium* PCR products. DNA sequencing was carried out by Macrogen (https://dna.macrogen.com). To this end, the company was supplied with more than 60 ng/ µl DNA per sample.

Data analyses

DNA sequences were blasted against the NCBI database (version 1.8.0). The ITS database of RefSeq dataset accessed from NCBI homepages was used to identify ITS primers. RefSeq database included *Aspergillus* and *Penicillium* genus, both where used for
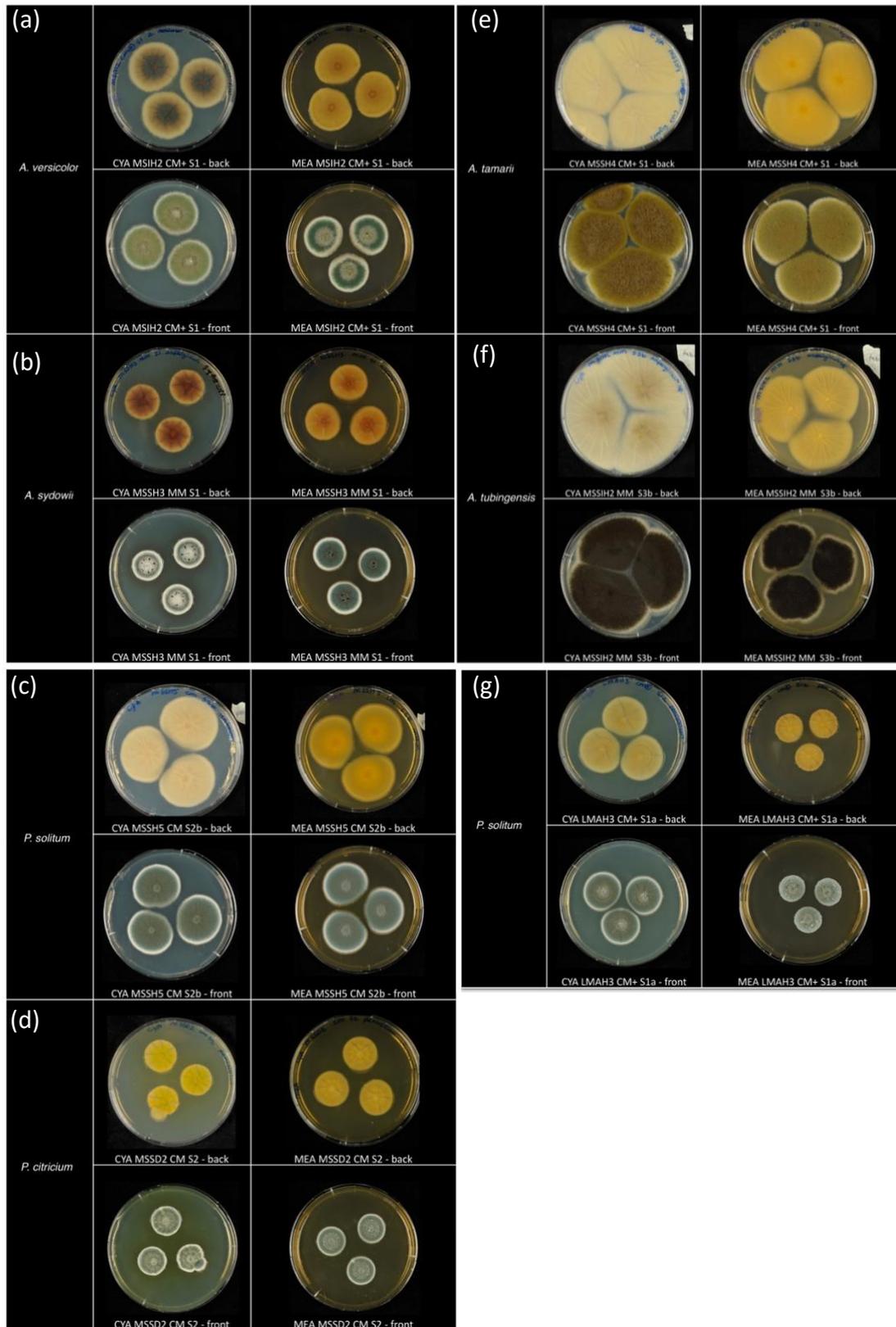
Figure 2*; Fungi associated with corals. Colonies were grown on CYA and MEA in the dark at 25 °C for 7 days. (a) A. versicolor, (b) A. sydowii, (c) P. solitum, (d) P. citrinum, (e) A. tamarii, (f) A. tubingensis, (g) P. westlingii. Left and right column represent bottom and upper part of the agar plate, respectively. Source codes under plate pictures refer to **Appendix II**.*

identifying species. Qualitative data was analysed by using Microsoft Excel (version 15.41) for MacOS Sierra (version 10.13.2). RStudio (version 1.0.153) for MacOS Siera was used for carrying out statistics, with custom scripts. Analysis of growth and stamp order was done with Chi-square tests in RStudio. A possible effect of media on growth was analysed with One-Way Anova test in RStudio. Both were tested with p<0.05. For data visualisation Microsoft Excel and Microsoft Powerpoint (version 15.41) for MacOS Sierra were used.

Results

33 Coral samples were stamped on 6 different agar media (CM, CM+, MM, MMCa, MMNa, MM+), using 5 different treatments (Unwashed (D), Washed (C), UV (UV), Cross-sectioned (L) and Mortar (M)). A total of 1041 different plates (173 CM, 162 CM+, 174 MM, 172 MMCa, 178 MMNa, 182 MM+) were used to detect fungal growth (see **Appendix I** for a complete overview). Stamping on the different media of treatments D, C, UV and L was done randomly. No significant difference between growth and stamp order was found (chi-test, p =0.95569). Out of 1041 plates, 227 fungal colonies were detected and transferred to purify these strains under the same medium conditions. From these 227 colonies, 179 were purified successfully (64 CM, 20 CM+, 50 MM, 22 MMCa, 10 MMNa, 13 MM+; see **Table 4**). No significant difference was found between medium and incidence of fungal growth (One-Way Anova, df = 5, p = 0.618) due to high variance in the different media groups. The 179 colonies were identified via visual inspection and microscopy. The identity of *Aspergillus* and *Penicillium* species from treatment L and M was confirmed by Dr J. Houbraken after growing the colonies on CYA and MEA agar plates in the dark at 25 °C for 7 days. Appearance of colonies of *A. versicolor*, *A. sydowii*, *P. solitum*, *P. citrinum*, *A. tamarii*, *A. tubingensis*, *P. westlingii* that had been grown in the dark on CYA and MEA are shown in **Figure 2**. *A. tubingensis* colony, as was identified by visual inspection by Dr J. Houbraken, may also be *A. acidus* or *A. niger.* Together, the colonies were identified to consist of 1 *Aspergillus flavus* or *Aspergillus oryzae*, 34 *Aspergillus tubingensis*, 11 *Aspergillus versicolor,* 24 *Aspergillus tamarii*, 14 *Aspergillus sydowii,* 1 *Aspergillus penicillioides*, 1 *Penicillium westlingii*, 21 *Penicillium solitum,* 1 *Penicillium citrinum,* 5 unidentified *Aspergillus* sp., 29 unidentified *Penicillium* sp., 1 predicted *Fusarium* sp., 2 putative Deuteromycota sp., and 34 unidentified fungi (which one without sporulation). DNA of 1 or 2 colonies was isolated of colonies that had been identified at the species or genus level (see **Appendix III**). Out of 34 DNA samples 11 sequences were obtained that resulted in identification after NCBI-blast; *A. sydowii* LT3001-2, *A. sydowii* FMR 14440, *A. tamarii* NRRL:427, *A. tamarii* SZMC 3077, *A. versicolor* LTBF 01-11 (initially identified as *A. nidulans),* *P. solitum* 20.01 (initially identified as *P. polonicum*) and *P. cordubense* CHR-4 (shown at **Table 5** and **6**). Samples which resulted in identification with forward primer as *P. cordubense* CHR-4, but with reversed primer in *P. solitum* 20.01, were assigned to *P. solitum* 20.01. This because all of these samples came from the same group, which were initially identified as *P. polonicum*.

Outside coral associated fungi
Unwashed treatment
A total of 56 out of 179 were isolated from the outside of all investigated coral species except from *A. agaricites. A. sydowii, A. tamarii, A. versicolor, A. tubingensis, Penicillium* sp., *Aspergillus* sp. and unidentified fungi were isolated based on visual inspection (**Appendix III**). The majority of isolated fungi (28) were isolated from CM medium (**Table 4**). Only one fungus, *Penicillium* sp., was isolated from MMNa medium and originated from *M. mirabilis* (**Table 6**, and **Appendix II**).

Table 4; *Summary of fungi isolated from each of the media. CM = complete medium, CM+ = complete medium medium with extra NaCl and CaCO₃, MM = minimal medium, MM+ = minimal medium with extra NaCl and CaCO₃, MMCa = minimal medium with extra CaCO₃, MMNa = minimal medium with extra NaCl.*

Fungi species	CM	CM+	MM	MM+	MMCa	MMNa
A. flavus or A. oryzae	1	0	0	0	0	0
A. penicillioides	0	1	0	0	0	0
A. sydowii	7	0	3	3	1	0
A. tamarii	6	4	19	3	2	0
A. tubingensis	18	0	14	0	2	0
A. versicolor	3	4	1	2	0	1
Aspergillus sp.	4	2	1	0	0	0
Deuteromycota	1	1	0	0	1	0
Fusarium sp.	0	0	1	0	0	0
P. citrinum	1	0	0	0	0	0
P. solitum	5	2	9	0	3	2
P. westelingii	0	1	0	0	0	0
Penicillium sp.	6	2	4	4	7	6
Unidentified	12	6	8	1	6	1
Total	64	20	50	13	22	10

Table 5; *Summary of fungi found after each of the treatments. D = unwashed treatment, C = washed treatment, UV = after 3ʳᵈ time UV treatment, L = cross-sectioned treatment and M = mortar treatment.*

Fungi species	D	C	UV	L	M
A. flavus or A. oryzae	0	0	0	0	1
A. penicillioides	0	0	0	0	1
A. sydowii	5	1	0	0	8
A. tamarii	7	2	0	0	15
A. tubingensis	25	4	0	1	4
A. versicolor	2	0	0	1	8
Aspergillus sp.	2	2	0	0	1
Deuteromycota	0	1	0	1	0
Fusarium sp.	0	0	0	1	0
P. citrinum	0	0	0	0	1
P. solitum	0	0	0	0	21
P. westlingii	0	0	0	1	0
Penicillium sp.	7	14	0	2	6
Unidentified	8	8	1	5	12
Total	56	32	1	12	78

Clean treatment

After cleaning samples with sterile seawater, 32 out of the 179 colonies were isolated from all coral species except from *M. cavernosa* (**Appendix II**). Based on visual inspection, one Deuteromycota was isolated from *M. annularis* on MMCa medium. Again, *A. sydowii, A. tamarii, A. versicolor, A. tubingensis, Penicillium* sp., *Aspergillus* sp. and unidentified fungi were isolated on CM medium (**Table 6**). Only one *A. sydowii* colony was found on CM medium that originated from healthy *S. siderea*. Despite the presence of ampicillin in the media, bacteria colonies were found. Undefined yeast and bacteria colonies were found on 119 plates out of 394 plates of D and C treatment (**Appendix I**).

UV treatment

UV treatment was successfully done for 27 samples. Six samples needed a second UV-treatment (see **Appendix I**). One sample of *M. cavernosa* even showed fungal growth after a third UV-treatment. A white unidentified colony was isolated from the outside, and grew on CM medium (**Table 4, 6** and **Appendix I**). No bacterial or yeast colonies appeared after UV treatment.

Internal coral associated fungi
Cross-sectioned treatment

Coral samples were cross-sectioned and stamped randomly on the 6 different media after the UV treatment. 12 out of 179 fungal colonies were from healthy *A. agaricites, M. annularis, S. intersepta* and diseased *S. siderea* and were identified based on visual inspection (**Appendix II** and **Table 6**). *A. tubingensis,* (putative) Deuteromycota, *Penicillium* sp., and unidentified fungi were isolated from the corals. No fungi were isolated from MM and MMNa medium (**Appendix I, II**). Most (5) fungi were isolated from CM+ medium (**Appendix I** and **II**). In contrast to outside (D, C, UV) treatments, *P. westlingii* and putative *Fusarium* were identified from the internal part of the corals. *P. westlingii* that grew on CM+ medium originated from *M. annularis* (**Table 4** and **6**). On the other hand, the *Fusarium* colony grew on MM medium and originated from diseased *S. siderea*.

Mortar treatment

Plating grinded coral samples after cross-sectioned treatment resulted in 78 colonies out of the total of 179 colonies (**Appendix I** and **II**). These fungal strains originated from *A. agaricites, M. mirabilis, M. annularis, S. siderea* (healthy and diseased) and *S. intersepta* (**Table 6**). The majority of the fungi grew on MM medium (23) and CM medium (21) (**Table 4** and **Appendix II**). *A. tubingensis, A. sydowii, A. tamarii, A. versicolor, Penicillium* sp., *Aspergillus* sp., and unidentified fungi were isolated from grinded coral, as identified by visual inspection (**Appendix I** and **II**). Two *A. sydowii* colonies from M treatment were verified by NCBI-blast. They had the closest match with strains LT3001-2, and FMR 14440 (**Table 5**). Similarly, the identity of the 3 *A. tamarii* colonies were confirmed by blasting with a closest match with strains NRRL 427 and SZMC 3077. *A. versicolor* was 3 times confirmed and identified as strain LTBF 01-11(see **Table 5**). Neither found on the outside treatments nor cross-sectioned treatment were *A. penicillioides, P. citrinum, P. solitum,* and *A. flavus* or *A. oryzae*. *A. penicillioides* was isolated from *M. annularis* and grew on CM+ medium (see **Table 6**). *P. citrinum* was isolated from a diseased *S. siderea* and grew on CM medium (see **Table 6**). The group *Penicillium* sp. from outside treatments may include *P. solitum*. *P. solitum* was isolated from one diseased *S. siderea* CM+ medium. A total of 14 separate *P. solitum* colonies were isolated from healthy *S. siderea* on all media types except MM (see **Table 6,** and **Appendix II**). 6 *P. solitum* colonies were isolated from *S. intersepta* on CM, MM, MMNa media (**Appendix II**). Note that *P. solitum* strain 20.01 was a match for one sample forward and reversed primer, but for two samples only a match for forward primer. The reversed primer has had a better match with *P. cordubense* CHR-4 (see **Table 5**). *A. flavus* or *A. oryzae,* grew on CM media and was isolated from a healthy *S. siderea* (see **Table 6**).

Table 6; Summary of fungi identified at the DNA level. Coral sample first letter is treatment (M = mortar treatment), SS = S. sidederea, the number represents number of coral piece, D= diseased, H=healthy, CM = complete medium, MM = minimal medium, CM+ = complete medium with extra NaCl and CaCO₃, MM+ = minimal medium with extra NaCl and CaCO₃, MMCa = minimal medium with extra NaCl, MMCa = minimal medium with extra CaCO₃. Matching F-primer explains the used matching forward primer, and mating R-primer explains which reversed primer is used and have had the best match with the strain highest match.

Fungi species	Strain highest match	Coral sample	Used F-primer	Used R-primer
A. sydowii	LT3001-2	M SSD2 MMCa	VG9	LS266
	FMR14440	M SSD2 CM	Calmd4	No signal
A. tamarii	NRRL27	M SSD2 CM	VG9	LS266
	NRRL27	M SSH5 MM+	VG9	LS266
	SXMC 3077	M SSH4 CM+	Calmd4	No signal
A. versicolor	LTBF 01-11	M SSH5 CM+	VG9	LS266
	LTBF 01-11	M SSH5 MM	VG9	LS266
	LTBF 01-11	M SSH5 MMNa	VG9	LS266
P. solitum	20.01	M SSD2 CM+	VG9	LS266
	20.01	M SSH3 CM	VG9	Match with *P. cordubense*
	20.01	M SSH3 MM	VG9	Match with *P. cordubense*
P. cordubense	CHR-4	M SSH3 CM	Match with *P. solitum*	LS266
	CHR-4	M SSH3 MM	Match with *P. solitum*	LS266

*Table 7; Summary of fungal species isolated from six different coral species (all were considered healthy, indicated otherwise as; H = healthy, D= diseased), per treatment (D =unwashed, C = clean, L= cross-sectioned, M= mortar, UV = after 3ʳᵈ time UV treatment), and on which media the isolated fungi were found (CM= complete medium, CM+ = complete medium with extra CaCO3 and NaCl, MM= minimal medium, MMCa = minimal medium with extra CaCO3, MMNa = minimal medium with extra NaCl and MM+= minimal medium with extra CaCO3 and NaCl). Aspergillus sp. and Penicillium sp. of the cross-sectioned and mortar treatments were identified by Dr J. Houbraken from the Westerdijk institute. 4 Fungi species were identified via DNA analysis (**Appendix III**).*

Fungi species	Coral origin	Found at treatment(s)	Grew on media	Total
A. flavus or A. oryzae	*Sidrastrea siderea (H)*	M	CM	1
A. tubingensis	*Agaricia agaricites, Madracis mirabilis, Montastraea annularis, Montastraea cavernosa, S. siderea (H), Stephanocoenia intersepta*	D, C, L, M	CM, CM+, MM, MMCa, MM+	34
A. penicillioides	*M. annularis*	M	CM+	1
A. sydowii	*M. annularis, S. siderea (H, D), S. intersepta*	D, C, M	CM, MM, MMCa, MM+	14
A. tamarii	*M. mirabilis, M. annularis, S. siderea (H, D), S. intersepta*	D, C, M	CM, CM+, MM, MMCa, MM+	24
A. versicolor	*S. siderea (H, D), M. annularis, S. intersepta*	D, L, M	CM, CM+, MM, MMNa, MM+	11
Aspergillus sp.	*M. cavernosa, S. siderea (H), S. intersepta*	D, C, M	CM, MM	5
Deuteromycota	*A. agaricites, M. annularis*	C, L	CM, MMCa	2
Fusarium	*S. siderea (D)*	L	MM	1
P. citrinum	*S. siderea (D)*	M	CM	1
P. solitum	*S. siderea (H, D), S. intersepta*	M	CM, CM+, MM, MMCa, MMNa	21
P. westlingii	*M. annularis*	L	CM+	1
Penicillium sp.	*A. agaricites, M. mirabilis, M. annularis, S. siderea (H, D), S. intersepta*	D, C, L, M	CM, CM+, MM, MMCa, MMNa, MM+	29
Unidentified	*a. agaricites, M. mirabilis, M. annularis, M. cavernosa, S. siderea (H, D), S. intersepta*	D, C, L, M, UV	CM, CM+, MM, MMCa, MMNa, MM+	33
Unidentified (no sporulation)	*S. siderea (H)*	D	CM	1
Total				179

Discussion

This study focussed on isolation and identification of endolithic fungi from the corals *Sidrastrea siderea, Agaricia agaricites, Montastraea annularis, Madracis mirabilis, Stephanocoenia intersepta* and *Montastraea cavernosa* from the reef of Curacao in the Caribbean Sea. Sterilisation by UV light turned out to be a successful method to kill microorganisms at the surface of the coral without affecting the isolation of fungi from the internal part. A total of 90 colonies originating from internal parts of corals were purified on plates. DNA was extracted from representatives of *Aspergillus* and *Penicillium* species groups based on visual inspection. To this end, DNA was extracted from 32 colonies, resulting in 11 successful identifications of *A. tamarii, A. sydowii, A. versicolor* and *P. solitum* based on blastn search of ITS and calmodulin sequences. This relatively low number is probably due to DNA contamination with phenolic compounds that are known to inhibit sequencing[59]. In the future DNA sequencing should confirm the identification of other species that were identified by visual and microscopic inspection. These included *A. flavus* or *A. oryzae, A. tubingensis, A. penicillioides, P. citrinum, P. westlingii* as well as one or more *Aspergillus* sp., one or more *Penicillium* sp., two predicted *Fusarium* sp., two putative Deuteromycota sp., and unidentified species. It should be noted that fungi were isolated that could grow at least on one of the 6 media that were used. The number of endolithic fungi may be higher if some of the species cannot grow under these conditions. In future studies, it would be interesting to combine metagenomics with the approach that was adopted in this study. Metagenomics could hint which media could be used to isolate certain fungi. The same can be done with samples of seawater originating from the reef of Curacao. Presence of endolithic fungi in the sea water would suggest vertical transmission, whereas their absence would be indicative for horizontal transmission.

Treatment methods

Surface located fungi may be part of the microbiome of the coral but may also be a contaminant from sea water or resulting from transport and processing of the samples. Surface located fungi were isolated from corals that had either or not been washed or had been washed and UV treated. The latter samples were also used to isolate endolithic fungi. To this end, these corals were cut and stamped on plates, and after homogenized followed by using the homogenate as an inoculum. The unwashed coral samples resulted in 56 colonies, while washing with water resulted in 32 colonies. A similar fungal composition was found before and after washing except for 2 species. *A. versicolor* was not found after cleaning the outside of the coral. In contrast, a Deuteromycota sp., which was only found after washing with sterile seawater. The fact that washing still resulted in fungal growth after stamping suggests a tight association with the coral. Future research should indicate which of the 88 colonies indeed originate from the microbiome and which result from contamination.

Yeast and bacteria grew on 119 out of 394 agar plates after stamping washed and unwashed coral surfaces despite the presence of 50 µg ml^{-1} ampicillin. Incidence of antibiotic-resistance is high in Curacao. Resistance of bacteria like *Escherichia coli* has been found for instance for ampicillin, oxytetracycline, trimethoprim and chloramphenicol[63]. The resistance was related to agricultural and medical practice[63]. Sewage systems are sometimes connected directly to the ocean[64], resulting in disposal of antibiotic resistant microorganisms. UV treatment turned out a successful method to eliminate outside growth of microorganisms. After one UV treatment, 27 samples showed no growth from the outside. After a subsequent treatment 5 out of the remaining 6 samples showed no growth. A single colony of an unidentified species was observed even after a 3rd UV treatment of a coral. This shows that one should always confirm whether an endolithic fungus indeed originates from the inner part of the sample and is not a contamination of the outer part. Grinding coral samples after UV treatment followed by using this as an inoculum resulted in more colonies than cutting the coral followed by stamping the inner surface. This resulted in 78 and 12 fungal colonies, respectively. Yet, these methods may be complementary since *P. westlingii* and putative *Fusarium* sp. was only found by stamping the inner surface. We cannot exclude that this is a matter of chance.

Fungi and medium preferences

The media CM, CM+ (includes CaCO$_3$ and NaCl), MM, MMCa (includes CaCO$_3$), MMNa (includes NaCl) and MM+(includes CaCO$_3$ and NaCl) were used to grow fungi. A total of 179 colonies originating from corals were purified on plates. Out of these 179 colonies, 64 colonies were derived from the CM agar plates, followed by 50 MM agar plates. The minority, with 10 colonies were derived from MMNa medium. This suggests that most isolated fungi do not need NaCl for their growth or are even sensitive to high salt. Sensitivity for salt may be indicative for a true endolithic fungus. *P. westlingii* and *A. penicilliodes* were only found on CM+ agar plates. The extra NaCl or CaCO$_3$, may inhibit or slow down growth of fast growers. Therefore, slow growers, which were still capable of growing on these media, or even were stimulated to grow were more competitive and could be isolated. No relation between isolation of colonies and stamp order (from medium to medium) was found. Besides, fungal species isolated more than one time grew always on different media. For that reason, stamping of coral can be used in the future to detect viable fungi. In combination with metagenomics, other media may be used to detect other viable fungi that have not yet emerged in this study. None of the fungi identified by visual inspection were found to be only associated with the outside of the coral samples. However, some of the unidentified fungi were only found at the surface. No isolated fungi were found specifically from the inner part of the corals. In general, these fungi were found in more than one coral species. *A. flavus* or *A. orzae, A. penicillioides, P. citrinum, P. westlingii* and *Fusarium* were found one time only; and thus, within a single coral species. *P. solitum* was isolated from more than one coral. A combination with metagenomics should confirm the existence of coral specific endolithic fungi.

Internal coral associated fungi and capabilities

The fungi that were isolated from the inner part of the corals have not been reported to be associated with bio-boring or bio-erosion. **A. flavus** or **A. orzae** was isolated from a healthy *S. siderea* sample and grew on CM agar. Both fungi are capable of producing mycotoxin cyclopiazonic acid (CPA)[56]. CPA is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR). No inhibition of Ca^{2+} activities in association with corals is known yet, but this

would be interesting to examine in the future. *A. penicillioides* originated from *M. annularis* and was isolated from CM+ media. This species is known to be a dry-tolerant fungus[56] and this may explain its growth on medium in the presence of salt. Possible, *A. penicillioides* grows in the "inner" part of the coral that is exposed to sea water. The combination of its pathogenicity in other environments[33,65] and the fact that *P. citrinum* was only isolated from a diseased *S. siderea* on a CM plate suggests that *P. citrinum* is a pathogen for corals too. On top of that, this fungus has antibacterial activity against *Staphylococcus aureus,* and antifungal activity against *Gaeumannomyces granins*[66]. Its optimum growth temperature of 37 °C may enhance the presence of this fungus in corals in the future when sea water temperature further increases. *P. westlingii* was isolated from the coral skeleton of *M. annularis* and grew on CM+ (includes $CaCO_3$ and NaCl). The combination of this media and place where this fungus was found, suggests that growth of *P. westlingii* is enhanced by $CaCO_3$, NaCl or a combination of these two. Therefore, *P. westlingii* could be a bio-eroder or bio-borer in coral. *P. westlingii* is known to produce the mycotoxin citrinin, which causes apoptosis, and which may be instrumental in penetration of the coral[67]. Yet, the fungus seems not to have a negative impact on coral health. A large number of 22 **P. solitum** individuals originated from the inside of healthy and diseased *S. siderea* and *S. interceptae*. *P. solitum* was isolated from all media except MM+. Transferring a colony on MM+ agar would confirm if *P. solitum* is unable to grow on MM+. This was unexpected because *P. solitum* is known to be a salinity-tolerant species[68] found for instance in marine sediments of Antarctica[68]. For these reasons, this fungus may be able to have a life stage outside corals, suggesting a vertical transmission. Additionally, *P. solitum* produces polygalacturonase (soften plant cell walls)[68,69], but the effect on corals is unknown.

Fusarium has been found in marine environment before, but never in association with corals[8]. In this study, a *Fusarium* sp. from an internal diseased *S. siderea* was isolated. *Fusarium* has the ability to produce fumonisins[70]. Fumonisins are structurally similar to sphingosine, sphingolipids plays an important role in signal transmission and cell recognition, and can cause death[70]. Fumonisins are expected to have a similar effect on corals too.

Fungi found at the surface and inner parts of corals
A similar fungal composition of A. *sydowii, A. tubingensis, A. tamarii, A. versicolor, Aspergillus* sp., *Penicillium* sp., Deuteromycota sp., and unidentified sp. were found on the surface and internal part of the corals. Both *A. sydowii* and *A. versicolor* are coral borers[7]. Corals can respond to fungal boring by covering the fungal holes with aragonite[7,71]. Microborers erode about 350 grams of $CaCO_3$ per square meter per year[72]. Therefore fungal boring plays an important role in bio-mineralization of coral skeleton[71]. Further effects of these penetrating fungal hyphae, mechanisms and enzymes involved remain unidentified[8,71]. It may be a similar mechanism (mechanical and physiological) to bio-boring sponges. Several of these bio-boring sponges show endosymbiotic relationship with dinoflagellate zooxanthellae (of the invaded coral), which enhance bio-erosion in light conditions[73–75]. Seawater chemistry may affect the saturation state at the site where the coral aragonite is dissolved. Then, corals lose their foundation, and thereby their habitat. Together with ocean acidification, bio-boring and erosion probably affect coral ecosystems even more[2,73].

Besides being a coral borer, *A. sydowii* is known as a parasitic fungus and caused death of many Gorgonians by Aspergilliosis. In this research, *A. sydowii* was found to be associated with *M. annularis, S. siderea* (healthy and diseased), and *S. intersepta*. Indeed, *A. sydowii* was found in healthy corals before[76]. The fact that I found *A. sydowii* both on the surface and internal parts of the corals suggests a vertical transmission of this fungus. Together, *A. sydowii* may not only be a pathogen or bio-eroder but also may be a simple commensal or even be a beneficial part of the microbiome. *A. sydowii* exhibits antibacterial activity against bacteria such as *E. coli* [66]. Moreover, it produces metabolites like cholin-O-sulfate (osmolite), synodic-acids, sydowinins and sydowins[33,66], which may affect the microbiome of corals either positively or negatively. In this study, **A. versicolor** was associated with healthy and diseased *S. siderea, M. annularis*, and *S. interceptae*. It is remarkable that *A. versicolor* was not found on MMCa media, because it is known to be a bio-borer. In general, *A. versiolor* is able to make mycotoxin CPA[33,77] and produces antibiotics affecting for instance *Staphylococcus epidermis*, *E. coli,* and *Staphylococcus aureus*[66]. Possibly, *A. versicolor* plays an important role in protecting corals from harmful bacteria, where bio-boring is a minor negative effect. **A. tamarii** was isolated from *M. mirabillis, M. anularis,* healthy and diseased *S. siderea*, and *S. interceptae*. The high incidence of isolation (24), and its presence in five out of six coral species suggests ubiquitous presence in corals. Similar to *A. versicolor, A. tamarii* is known to be halo-tolerant but was not found on MMNa. *A. tamarii* is known to be a pathogen and commensal, grows well at high temperatures and produces the mycotoxin CPA[33,77]. Therefore, *A. tamarii* may benefit from future increase in water temperature and may have a negative impact on future coral reefs. All agar plates had a pH of ± 7.8 which is considered high for fungi, but near the pH of seawater[51]. This turned out to be beneficial for **A. tubingensis** which can tolerate high pH[78]. *A. tubingensis* was isolated most (i.e. 34 times), originating from *A. agaricites, M. mirabilis, M. anularis, M. cavernosa,* healthy *S. siderea,* and *S. intersepta*. Oddly, none of the fungal colonies were isolated from media with extra salt (CM+, MMNa and MM+). This suggest horizontal transmission of *A. tubingensis* in marine environment. A growth experiment of *A. tubingensis* on media with extra salt should confirm this. The ability of *A. tubingensis* to dissolve phosphates in soil, and thereby reducing alkalinity may also take place in the coral environment. Reducing alkalinity is unfavourable for the calcification of corals. But, the buffer capacity of water may undo this process. Another negative effect on corals can be the production of ochratoxin, an immunosuppressant, produced by *A. tubingensis*[33,70,79,80]. Because there is a possibility that corals may suppress their useful immunoreactions by this ochratoxin. However, the fact that *A. tubingensis* was only found on and in healthy corals suggests that this fungus has a positive role in the corals microbiome.

Various **Aspergillus** sp., **Penicillium** sp., and unidentified fungi sp. were found on different media from different coral sources. Most *Aspergillus* sp. and *Penicillium* sp. produce ochratoxin[78], which as mentioned above could have a role in the coral. Only 2 isolated Deuteromycota were isolated from the surface of *M. annularis* CM medium and from the inside of *A. agaricites,* growing on MMCa medium. **Deuteromycota** was found 2 times in association with corals, and is found as boring fungi in molluscs in marine envirionment[8,12]. This suggests that Deuteromycota bore corals too.

Healthy versus diseased *S. siderea* associated fungi
In this study, healthy corals showed a higher diversity of fungi when compared to diseased corals. This corresponds to a study of healthy sea fans, which showed a greater fungal diversity than that of diseased ones[8]. A. *flavus* or A. *oryzae, A. tubingensis,* unidentified *Aspergillus* sp. and an unidentified fungus were found on healthy *S. siderea*, but not on diseased samples. These fungi may have a positive effect on *S. siderea*. *A. tubingensis* was found on all

healthy coral samples and did not appear on plates of diseased *S. siderea*. This implies that *A. tubingensis* play a positive role in coral microbiome. In contrast with healthy *S. siderea*, *Fusarium* sp. and *P. citrinum* were found on diseased individuals. These fungi may impact corals, and its microbiome negatively.

Conclusion

Fungi may play a bigger role in marine environment then assumed. Newly found fungi in association with corals in this study were *A. flavus* or *A. oryzae*, *A. tamarii, A. tubingensis, A. penicillioides, P. citrinum, P. solitum,* and *P. westlingii*. Previous found fungi in the marine environment and now found in association with hard corals are *A. sydowii, A. versicolor, Aspergillus* sp., *Penicillium* sp., Deuteromycota sp., *Fusarium* sp., and unidentified sp. Most fungi produce mycotoxins and antibacterial components. Their impact on corals should be studied in future studies. This impact may change with the effects of climate change.

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