Bacterial Response to Elevated Dissolved Organic Carbon in Coral Reef Ecosystems

Dissertation
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

dem Fachbereich Biologie / Chemie
der Universität Bremen
vorgelegt von

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December, 2016
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SUMMARY

Tropical coral reefs are globally affected by ocean warming and acidification. These factors can interact with local stressors such as overfishing and marine pollution. For instance, several human activities are considered to directly or indirectly increase the runoff of dissolved organic carbon (DOC) to coral reefs. Direct inputs of DOC are primarily caused by sewage and fish farm effluents, whereas indirect inputs are caused by excessive exudation by macroalgal-dominated (i.e., degraded) coral reefs as a consequence of eutrophication and overfishing. Dominant chemical species of the labile fraction of allochthonous (e.g., sewage) and autochthonous (e.g., macroalgal exudates) include neutral sugars that are shown to have highly detrimental effects on coral reefs through the stimulation of bacterial growth and the accumulation of suspended particles (i.e., aggregate formation). The prolonged exposition to particle aggregates has substantial implications for the food web structure, microbial processes and carbon cycling. In spite of evidence linking high labile DOC and coral reef degradation, no previous efforts have evaluated the combined effects of high DOC and global factors such as ocean acidification (i.e., high dissolved inorganic carbon, or DIC), affecting benthic and pelagic bacterial communities and organic carbon transformations on coral reefs.

Therefore, one objective of this thesis is to determine possible interactions between high DOC and DIC concentrations on aggregate formation and bacterial community diversity in coral- versus calcifying algal-dominated mesocosms over 42 days (experi-
The results of this experiment show that high DOC concentrations resulted in increased aggregate formation rates, which were further enhanced in a combined DOC + DIC enrichment. Furthermore, the bacterial community structure drastically shifted when exposed to high DOC in all treatments. However, the strongest shifts in bacterial community structure were observed after combining DOC + DIC enrichments in algal mesocosms. Differences in the total aggregated volume and shifts in the bacterial community composition in algal- compared to coral-dominated mesocosms support the notion that benthic community composition affects the availability of DOC involved in shaping bacterial diversity and particle aggregate formation. The results of this experiment suggest that bacterial communities associated with algal-dominated (i.e., degraded) reefs will be more susceptible to lose key functional groups under the combined effect of ocean acidification and DOC enrichment than bacterial communities associated with coral-dominated reefs.

Furthermore, previous results have shown that high sugar concentrations select for presumed opportunistic pathogenic bacteria that carry numerous virulence factors in their genomes. Corals are believed to be harmed by these opportunists. In spite of this assumption, there is a clear knowledge gap of how high sugar concentrations modulate microbial metabolic strategies (e.g., commensals to pathogens), and their putative promotion of negative interactions with corals. The main objective of this thesis is therefore the evaluation of the individual effects of sugar enrichment on coral reef bacterioplankton diversity, carbon catabolism and virulence factor expression. To address this objective, coral reef water incubations enriched with the most dominant monosaccharides (glucose, galactose, mannose, and xylose) found in allochthonous and autochthonous DOC were carried out (experiment 2). The contribution of each sugar to shifts in the bacterial community structure and gene expression profiles of dominant microbial populations were determined through 16S rRNA gene amplicon sequencing, metagenome binning and metatranscriptomics. Evidence of metabolic
shifts and expression of virulence factors such as metalloproteases, siderophores and toxins were identified in members of the bacterial Genera *Alteromonas*, *Erythrobacter*, *Oceanicola* and *Alcanivorax*. These findings suggest potential mechanisms associated with a shift from commensal to pathogenic lifestyles in microbial population genomes.

Overall, the experiments presented in this thesis further our understanding of the negative effects of high labile DOC enrichment on coral reefs. The overall picture of this thesis show that DOC is a crucial driver of biogeochemical organic carbon transformations and bacterial activity on tropical coral reefs. Finally, this thesis highlights the contribution of benthic community composition (i.e., coral- vs. algaedominated) and ocean acidification to this processes.
keine Studien welche die Effekte von DOC in Kombination mit Ozeanverauerung (in der Form von gelöstem inorganischen Kohlestoff, oder DIC) untersucht. Konkret gibt es zum Beispiel keine Hinweise darauf, wie benthische und pelagische Bakteriengemeinschaften und organische Kohlenstoff-Transformationen in Korallenriffen beeinträchtigt werden.


Hohe Konzentrationen von DOC können die Präsenz und Vermehrung von potentiell opportunistischen Bakterien fördern, die eine Vielzahl von Virulenzfaktoren in ihren Genomen beherbergen. Man nimmt weithin an dass solche bakteriellen Opportunisten die Gesundheit von riffbildenden Korallen beeinträchtigen können. Es

ACKNOWLEDGEMENTS

I would like to start by thanking my two extraordinary mentors Astrid and Chris. You two mentored me through the most incredible and fruitful research experience. Astrid, thank you for believing in me. Even when I had the craziest ideas, you always supported them enthusiastically until they became this dissertation. Chris, thank you so much for hosting me in your group and making me a part of the Voolstra lab family. My time in your group has been an exciting experience full of joy, friendship and lots of learning. Thank you Chris for being such an amazing advisor, always providing me with your guidance, kindness, and unconditional support.

Thank you to the rest of my committee members, Christian Wild and Nicole Dubilier, for being supportive through this challenging process and for not chastising me about reading this thesis in such short time. Christian, thank you for including me many times in your group activities and always making me feel welcomed. You gave me invaluable suggestions and feedback in these formative years of coral reef research in the cold Bremen. Nicole, thank you for your inspirational lectures during the MarMic, and for supporting me during my first experiences at teaching (Virus tutorials). My gratitude is also extended to the MarMic program, especially Christiane Glöckner for helping me through all the procedures during my Ph.D. I thank all the MarMic staff and fellow students of my MarMic class.

I would like to sincerely thank all of my past and present lab and field mates at ZMT and KAUST, Jenny, Claudia, Nils, Hauke, Fritz, Till, Anna, Inken, Yustian,
Nataly and Antho, for their support and the hundreds of laughs and curses we shared together. Matt, Fauzi and Seb, thank you for sharing pieces of your bioinformatics knowledge with me, and Camille, Creig, Achim and Mustafa for your assistance in the lab. Special thanks to Claudia and Nils. Thank you guys for sharing this challenging journey with me. Even though hard at times, it was a fun and an undoubtedly productive time. I am looking forward for more to come! Special thanks to Topo, Jenny, Jime, Carol and Edinson for being incredibly supportive during these years. You have always been there for me, and no matter what I asked for, you always helped me with no hesitation. Thank you guys!

Pursuing my Ph.D. far away from old friends and family would not have been possible without their immense love and constant encouragement. Last but not least, I like to acknowledge with enormous and deep thanks to my amazing boyfriend Phil. Thank you for your lovely support and for teaching me every day a little bit about everything in life.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examination Committee Approval</td>
<td>ii</td>
</tr>
<tr>
<td>Copyright</td>
<td>iii</td>
</tr>
<tr>
<td>Summary</td>
<td>iv</td>
</tr>
<tr>
<td>Zusammenfassung</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xvii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xix</td>
</tr>
<tr>
<td>1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.1 State of the art</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Coral reefs: threatened hotspots of diversity</td>
<td>2</td>
</tr>
<tr>
<td>1.1.2 Inorganic nutrient enrichment and coral reef phase shifts</td>
<td>3</td>
</tr>
<tr>
<td>1.1.3 Dissolved organic carbon and its fundamental role in coral health</td>
<td>4</td>
</tr>
<tr>
<td>1.1.4 Elevated DOC concentrations and their role in aggregate formation</td>
<td>8</td>
</tr>
<tr>
<td>1.1.5 Bacterial metabolism under elevated DOC</td>
<td>8</td>
</tr>
<tr>
<td>1.2 Thesis Proposal</td>
<td>12</td>
</tr>
<tr>
<td>1.2.1 Knowledge gaps and aims</td>
<td>12</td>
</tr>
<tr>
<td>1.2.2 General approach</td>
<td>13</td>
</tr>
<tr>
<td>1.2.3 Thesis outline</td>
<td>14</td>
</tr>
<tr>
<td>1.2.4 Publication overview</td>
<td>14</td>
</tr>
<tr>
<td>2 The formation of aggregates in coral reef waters under elevated concentrations of dissolved inorganic and organic carbon: A mesocosm</td>
<td>2</td>
</tr>
</tbody>
</table>
approach

2.1 Abstract ........................................... 18
2.2 Introduction ........................................ 19
2.3 Methods .............................................. 22
   2.3.1 Origin and preparation of organisms ............... 22
   2.3.2 Setup of mesocosms ................................ 23
   2.3.3 Background parameters and DIC/DOC enrichment .... 23
   2.3.4 Carbon pool analyses ............................... 25
   2.3.5 Rolling tank experiments ............................ 26
   2.3.6 Bacterioplankton respiration and abundance ........ 27
   2.3.7 Statistical analysis ................................ 27
2.4 Results ................................................ 28
   2.4.1 Carbon chemistry background parameters ............ 28
   2.4.2 Effects of elevated DIC concentrations .......... 30
   2.4.3 Effects of elevated DOC concentrations .......... 30
   2.4.4 Combined effects of elevated DIC and DOC concentrations . . 33
   2.4.5 Comparison between algae and coral mesocosms .... 35
2.5 Discussion ............................................. 35
   2.5.1 Effects of high DIC on in-vitro particle aggregation ...... 35
   2.5.2 Effects of high DOC on in-vitro particle aggregation ...... 36
   2.5.3 Combined compared to independent effects ........ 37
   2.5.4 The influence of reef communities on particle and aggregate
       formation ............................................... 37
2.6 Ecological perspective ................................ 38
2.7 Conclusions .......................................... 39
2.8 Supplementary information ............................. 40

3 Microbiomes of algae-dominated coral reefs are more susceptible to
combined than individual effects of ocean acidification and dissolved
organic carbon enrichment ........................................ 43
   3.1 Abstract .......................................... 43
   3.2 Introduction ........................................ 44
   3.3 Results ............................................. 46
      3.3.1 Chemical parameters measured in coral reef mesocosms .... 46
      3.3.2 DNA sequencing .................................... 46
      3.3.3 Bacterial diversity in coral reef mesocosms ............ 47
3.3.4 Changes in bacterial diversity over time .......................... 47
3.3.5 Identification of key OTUs affected by simulated future ocean conditions .................................. 51
3.4 Discussion ......................................................... 53
3.5 Conclusions ......................................................... 57
3.6 Experimental Procedures ........................................... 57
3.6.1 Experimental design .............................................. 58
3.6.2 DOC measurements .............................................. 58
3.6.3 Water column sampling and nucleic acid extraction ............... 59
3.6.4 Sediment sampling and nucleic acid extraction ....................... 59
3.6.5 Sequence data and statistical analysis ................................ 60
3.6.6 Sequence Data deposition ......................................... 61
3.7 Supplementary information ........................................... 62

4 Excess labile carbon promotes the expression of virulence factors in coral reef microbial populations

4.1 Abstract ............................................................... 71
4.2 Introduction ............................................................ 72
4.3 Materials and methods .................................................. 75
4.3.1 Water sampling and incubations .................................... 75
4.3.2 Cell density, DOC concentrations and efficiency calculations ... 76
4.3.3 Nucleic acid extraction and isolation ............................... 76
4.3.4 16S-based diversity analysis ....................................... 77
4.3.5 Metagenome binning .................................................. 78
4.3.6 RNA mapping and gene expression analysis ......................... 79
4.3.7 Data deposition ........................................................ 80
4.4 Results and discussion .................................................. 80
4.4.1 Microbial diversity and DOC consumption .......................... 80
4.4.2 Metagenomic binning and microbial population genomes .......... 84
4.4.3 Sugar transporter diversity and microbial metabolism ................ 86
4.4.4 Expression of microbial interaction genes .......................... 91
4.4.5 Virulence Potential in Opportunistic Pathogens ...................... 92
4.4.6 Expression of aggregation genes to promote virulence potential .... 95
4.4.7 Expression of genes related to iron uptake to promote virulence potential ........................................ 95
4.4.8 Expression of toxins and proteases to promote virulence potential .... 96
## 4. Energy-dependent regulation of virulence factor gene expression

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.9 Energy-dependent regulation of virulence factor gene expression</td>
<td>98</td>
</tr>
<tr>
<td>4.4.10 Environmental sensing-dependent regulation of virulence factor</td>
<td>99</td>
</tr>
</tbody>
</table>

## 4.5 Conclusions

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 Conclusions</td>
<td>100</td>
</tr>
</tbody>
</table>

## 4.6 Supplementary information

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6 Supplementary information</td>
<td>102</td>
</tr>
</tbody>
</table>

## 5  Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Outline</td>
<td>110</td>
</tr>
<tr>
<td>5.2 Who are the unseen players fueling the positive coral degradation feedback on coral reefs?</td>
<td>111</td>
</tr>
<tr>
<td>5.3 Bacterial metabolic shifts and their ecological implications</td>
<td>112</td>
</tr>
<tr>
<td>5.4 Excess of labile carbon mediates virulence factor expression</td>
<td>112</td>
</tr>
<tr>
<td>5.5 Two sides of the same coin</td>
<td>114</td>
</tr>
<tr>
<td>5.6 The other face of DOC enrichment on coral reefs</td>
<td>116</td>
</tr>
<tr>
<td>5.7 Perspectives</td>
<td>117</td>
</tr>
<tr>
<td>5.8 Conclusions</td>
<td>118</td>
</tr>
</tbody>
</table>

## References

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>119</td>
</tr>
</tbody>
</table>
xvi
LIST OF FIGURES

1.1 Major sources and sinks of organic carbon on coral reefs. ............. 5
1.2 The dissolved organic carbon (DOC), Disease, Algae, Microorganism
    (DDAM) positive feedback loop. .................................... 7
1.3 Virulence factor regulation under high sugar concentrations. ........ 11

2.1 Effects of elevated DIC enrichment on DOC and TEP concentrations
    as well as total aggregated volume. .................................. 24
2.2 Effects of elevated DOC enrichment on DOC and TEP concentrations
    as well as total aggregated volume. .................................. 31
2.3 Combined effects of elevated DOC and DIC enrichment on DOC and
    TEP concentrations as well as total aggregated volume. ............ 32
2.4 Mesocosm set-up. ....................................................... 40
2.5 Suspended particulate matter (SPM) measurements. ................. 41
2.6 Variation of bacterial oxygen uptake rates and cell density in the water
    column. ................................................................. 41

3.1 Microbial diversity across samples and time in coral reef mesocosms. 48
3.2 Temporal changes in abundant families in coral and algae mesocosms. 49
3.3 Bray–Curtis dissimilarities of bacterial communities over time. .... 50
3.4 Venn diagram showing the number of unique and overlapping kOTUs
    (0.03 sequence difference) with a significant contribution to the treat-
    ment variation in each of and between samples. .................... 52
3.5 Changes in coral reef bacterial OTU relative sequence abundances per
    potential functions. ................................................. 53
3.6 DOC concentrations in coral reef mesocosms. ........................... 62
3.7 Relative abundance of abundant phyla in coral and algae mesocosms. 63
3.8 Relative abundance of genera at time points where significant different
    bacterial compositions were found. .................................. 64
3.9 Phylogenetic diversity of the OTUs with significant contribution to the enriched DIC effect. .................................................. 65
3.10 Phylogenetic diversity of the OTUs with significant contribution to the DOC enrichment effect. ............................................ 66
3.11 Phylogenetic diversity of the OTUs with significant contribution to the combined effect. .................................................. 67

4.1 Impact of high sugar concentrations on coral reef planktonic microbial diversity. ................................................................. 83
4.2 Sugar transporter diversity and expression in microbial population genomes. ................................................................. 88
4.3 Expression of genes involved in different glycolytic pathways. ........ 90
4.4 Interaction and virulence factor genes in microbial population genomes. 93
4.5 Expression of genes involved in pathogenesis in potential opportunistic pathogens. ............................................................ 94
4.6 Schematic representation of the experimental setup: coral reef water amended with monosaccharides. ................................. 102
4.7 Gene expression patterns in microbial population genomes. .......... 103
4.8 Expression of bacterial interaction genes in selected microbial population genomes. .......................................................... 104
4.9 Calcium-binding motifs of RTX homologs found in the population genome MB_1 (Oceanicola sp.). ............................................. 105

5.1 Proposed strategies used by opportunistic pathogens. ............... 115
LIST OF TABLES

2.1 Mesocosm water chemistry after 42 days of incubation. .............. 29
2.2 Significance values for pairwise comparisons between variables. ...... 34

3.1 Mesocosm water chemistry after 42 days of incubation. .............. 68
3.2 Number of samples and total reads obtained before and after quality check. .................................................. 69
3.3 Adonis analysis of the Bray–Curtis dissimilarities for bacterial OTU community structure in relation to habitat, time and treatment effects. 69
3.4 Pairwise ANOSIM between group vs. control treatments. .............. 69

4.1 Microbial planktonic growth efficiencies after 48h sugar incubations. . 81
4.2 Microbial population genomes retrieved and assembled in this study. . 85
4.3 Gene ontology (GO) terms used for functional annotation of microbial population genomes. ............................. 102
4.4 Taxonomic assignment of microbial population genomes using average nucleic acid identities (ANI), average amino acid identities (AAI), SpecI, PhyloPythiaS, and CheckM marker lineages. ............... 106
4.5 Extracellular proteases and toxins expressed in potential opportunistic pathogens (POPs). .............................. 107
4.6 Log2 fold change of selected regulatory, metabolism, and chemotaxis genes. ............................................... 108
Chapter 1
Chapter 1

Introduction

1.1 State of the art

1.1.1 Coral reefs: threatened hotspots of diversity

Coral reefs are often called “the rainforests of the sea” because, even though covering less than 1% of the ocean floor, they support a diversity comparable to the 20-times larger Amazon forests [1, 2, 3]. Due to this enormous biodiversity, coral reefs are considered one of the most economically important ecosystems on the planet. By providing ecological goods and services through fisheries, coastal protection, building materials, bioprospection new biochemical compounds and tourism, they contribute an estimated $375 billion annually to the global economy [4, 5]. Despite their ecological and economical importance, coral reefs have been recognized as one of the most vulnerable ecosystems [6] and evidence supports the unprecedented worldwide decline of coral reefs [7, 8]. Decades of coral reef research demonstrate that coral reefs are susceptible to the effects of global climate change (global stressors) such as ocean warming [9, 10, 11] and acidification [12, 13, 14]. Local stressors include natural disturbances such as hurricanes, floods, low-tides [15, 16], and also anthropogenic activities which impact coastal coral reefs increasingly, such as overfishing [17, 18].
sedimentation [19, 20] and land-based nutrient pollution [21, 22].

1.1.2 Inorganic nutrient enrichment and coral reef phase shifts

Coral reef ecosystems have long been considered a paradox, because in spite of the oligotrophic nature of the waters in which they thrive, they have the highest gross primary production (300-5000 g C m\(^{-2}\) yr\(^{-1}\)) of all marine ecosystems [23, 24, 25]. Indeed, the main benthic primary producers, the reef-building corals, are highly adapted to the life at such low nutrient concentrations [26]. Maintaining low nutrient concentrations is critical for balancing the cycling of nutrients and limiting the growth of successful competitors [27, 28, 29]. Extensive data in the last decades show that turf algae, macroalgae, and cyanobacteria benefit from episodic nitrogen and phosphorus enrichment in near-shore reefs [30, 31, 29, 32]. The subsequent dominance of non-coral benthic primary producers is commonly associated with substantial decreases in coral cover. This regime or ‘phase-shift’ has been reported in the Caribbean [33], Eastern Africa [34], Eastern Pacific [35] and Australia [36]. Other factors contributing to these benthic community shifts towards algal-domination include the massive reduction of herbivorous reef fish by overfishing, and the indirect effects of climate change [37, 33, 38, 17, 39, 40]. While the importance of top-down (grazing pressure) and bottom-up (nutrients) factors in regulating coral-algal competition are well documented, much less is known about the specific mechanisms by which macroalgal overgrowth is killing corals. Some of the proposed mechanisms include shading [41, 42], abrasion [43, 44], and inhibition of coral recruitment [45]. However, in the last decade, a possible correlation between algal dominance and the prevalence of coral diseases has gained substantial attention from the scientific community [33, 44, 46]. This potential correlation is explained by the Dissolved organic carbon, Disease, Algae, Microorganism (DDAM) model that will be explained below.
1.1.3 Dissolved organic carbon and its fundamental role in coral health

New organic carbon enters coral reef ecosystem autochthonously by exudation of photosynthates by primary producers and allochthonously in the form of particulate organic carbon (POC) (suspended cells) and dissolved organic carbon (DOC) (carbohydrates, lipids, and proteins) [47]. Recycling mechanisms of organic carbon species into the different coral reef compartments is shown in Fig. 1.1. Human-derived activities such as sewage and fish-farms play an essential role in increasing the runoff of organic carbon and therefore causing an impact on neighboring coral reefs [48, 49, 50]. For instance, average DOC concentrations in shrimp and fish ponds from NE Hainan, China average around 800µM, and can peak at more than 1,700µM [51], corresponding to more than 15 times the average DOC concentrations on coral reefs (29.0±0.6 to 72.0±1.4µM [52]). These aquaculture ponds reportedly have DOC and POC export loads of 2220 and 3931 t yr$^{-1}$, respectively, causing severe organic carbon enrichment on coral reefs located up to ~ 4 km away [51]. However, the primary source of new organic carbon entering most coral reefs is introduced by the exudation of benthic organisms as DOC [47, 53]. Among the reef primary producers, turf algae, macroalgae, and cyanobacterial mats are the major contributors of DOC release [54, 55, 56, 57]. Macroalgae exhibit the highest daily net DOC release rates (7.25 ± 0.58 mmol m$^{-2}$ d$^{-1}$) followed by turf algae (4.30 ± 0.72 mmol m$^{-2}$ d$^{-1}$), while corals release very little in comparison (1.32 ± 0.72 mmol m$^{-2}$ d$^{-1}$) [54, 57, 58]. Differences between algae and coral exudates however are not limited to the rates of exudation, but also to the carbohydrate composition. Algal exudates are composed mainly of glucose, with higher proportions of labile DOC that can be utilized faster by heterotrophic bacteria [59, 55, 60, 61, 62]. In contrast, coral exudates are less labile with a similar carbohydrate composition to the water column and that of offshore DOC pools [63, 64].
Consequentially, algal compared to coral exudates are removed from the water column at higher microbial consumption rates, resulting in an increased carrying capacity of water for microbial biomass and oxygen depletion [65, 55, 57, 66]. The effects on coral reefs can be severe. For instance, corals suffer from higher mortality rates when exposed to high DOC concentrations or algal exudates [66, 67, 68]. The proposed mechanism for coral death is thought to be anoxia and the accumulation of toxic substances produced during microbial metabolism [66, 67, 68, 69].

The evidence mentioned above served as the fundamental base for Barott and Rohwer [70] to propose the Dissolved organic carbon, Disease, Algae, Microorganism (DDAM) model to explain the link between the increasing abundance of benthic algal cover on coral reefs and the emergence of coral disease (see Fig. 1.2). The DDAM is a positive feedback loop that starts with the increased (macro) algal cover caused by human-derived stressors such as overfishing and nutrient enrichment (eutrophication).
Overfishing results in the reduction of herbivorous reef fishes that control macroalgal cover, thereby promoting their dominance on a reef. Increased nutrient availability on the other hand will stimulate increased macroalgal growth and primary production. As a consequence, more photosynthate (labile DOC) is exuded and rapidly taken up by bacteria. The labile DOC then fosters bacterial growth and oxygen removal, and promotes the accumulation of toxic bacterial metabolites. In addition, recent studies show that algal exudates have an impact on bacterioplankton communities. For instance, increases in copiotrophs, such as Gammaproteobacteria (Enterobacteriaceae and Vibrionaceae) are observed in algal exudate treatments. These bacterial community shifts select for known opportunistic pathogens that carry numerous virulence factors in their genomes [52, 61]. These opportunistic pathogens can reduce host resistance, providing space for algae to grow and become dominant on the reef. In contrast, in coral-dominated reef waters, bacterial communities are more diverse and the widespread Alphaproteobacteria is dominant [52, 61].
Figure 1.2: The dissolved organic carbon (DOC), Disease, Algae, Microorganism (DDAM) positive feedback loop. 1. Human-derived local factors such as overfishing and eutrophication affect reef health by promoting the growth of benthic algae and cyanobacteria. 2. Benthic algae and cyanobacteria release DOC rich in monosaccharides in a similar composition as found in fish farm effluents and sewage. 3. DOC stimulates microbial growth and respiration rates. 4. Corals are harmed through hypoxia and unknown microbial interactions.
1.1.4 Elevated DOC concentrations and their role in aggregate formation

One of the various consequences of elevated DOC concentrations on coral reefs is the formation of larger sinking particles, or particle aggregates [71]. The formation of these particle aggregates from DOC generally involves acidic polysaccharide gel-like particles termed transparent exopolymer particles (TEP) [72, 73, 74]. Macroalgal exudates, presumably the dissolved carbohydrate fraction, are a source of TEP precursors. These may coagulate into TEP [75, 76], which serves as a “glue” promoting aggregate formation by adhering POC into larger particles [74, 77]. Although DOC is the most dominant form of organic carbon in most marine ecosystems, large sinking particles constitute an often small but biologically relevant contribution to the total organic carbon (TOC) pool [78, 79]. In coral reef waters, the particulate fraction can be trapped by coral mucus and remineralized by benthic bacterial communities to fuel secondary production [80, 81, 82]. High levels of suspended particulate matter can, however, directly affect light penetration and sedimentation rates, and may thereby promote coral mortality [21, 83, 84, 85, 86].

1.1.5 Bacterial metabolism under elevated DOC

The bacterioplankton community is a major contributor to the total DOC uptake on coral reefs (removing up to 50 mmol of C m$^{-2}$ day$^{-1}$), and therefore an essential trophic bridge to deliver energy and nutrients to higher trophic levels via the microbial loop [87, 88, 89]. From these complex mixtures of DOC, dissolved monosaccharides are one of the growth-limiting resources and important substrates that sustain heterotrophic bacterial growth in oligotrophic environments such as coral reef waters [55, 61, 90]. Some free-living heterotrophic bacteria have a versatile metabolism for the uptake of many different sugars as a sole source of carbon and energy [91, 92].
This metabolic versatility is favored by a tight connection between complex regulation cascades that allow the expression of metabolic pathways and environmental cues such as sugar availability. The metabolic pathways for sugar utilization are inducible in the presence of the sugar, and include transporters, enzymes involved in sugar uptake, as well as numerous regulatory elements controlling positive and negative feedback [93, 94]. Sugar-inducible pathways are a common strategy to conserve resources when the sugar is absent, and its regulations vary enormously through bacteria, resulting in contrasting populations behaviors [95, 96]. Sugar uptake in bacteria involves an enormous diversity of transporters, including the major facilitator superfamily (MFS), the bacterial phosphotransferase system (PTS), and the ATP-binding cassette (ABC) superfamily [97, 98]. Many of these families are tightly linked with global regulators of gene expression as is the case of PTS, and the levels of the second messenger cyclic adenosine monophosphate (cAMP) in a mechanism called carbon catabolite repression (CCR) [93]. The CCR is a complex regulatory network observed in most free-living heterotrophic bacteria that allows the uptake of the preferential carbon source while inhibiting the uptake of non-preferred carbon sources. CCR controls the expression of 10% of all bacterial genes, most of them involved in transport and metabolism, plus a large fraction of genes directly or indirectly involved in bacterial interactions with the environment [99, 100]. As an example of an indirect interaction, CCR supports the competition between bacterial populations by not only controlling the carbon source to be assimilated but also the rate of assimilation [93, 99]. The increased energy entering from carbon metabolism trigger responses observed at high population density such as bacterial cell-to-cell communication or quorum sensing [101]. On the other hand, a more direct interaction is the expression of virulence factors regulated by CCR and the energetic status of the cell [93, 102, 103]. These virulence factors are frequently directed to allow the bacterium to gain access to new sources of nutrients and at the same time
promoting negative interactions with other organisms. A relationship between CCA elements and virulence factor expression has been evidenced for cytotoxins \[104\], adhesins \[105\] bacterial secretion systems contained in pathogenicity islands \[106\] and extracellular proteases \[107, 108\] among others. The molecular mechanisms by which sugar metabolism promote virulence factor expression in represented in Fig. 1.3.
Figure 1.3: Virulence factor regulation under high sugar concentrations. Sugar-dependent regulation starts with the sugar sensing and transportation through the phosphotransferase system (PTS) system. The activation of the PTS-dependent cytoplasmic protein (EAII) will determine levels of cyclic AMP (cAMP) and thereby the activity of the cAMP receptor protein (CRP), required for activation of virulence phenotypes in several species. Quorum sensing circuits are an example of a sugar-independent regulation of virulence factors in response to increases in cell-population density. In this mechanism LuxI-type proteins produce autoinducer molecules that accumulate as cell density increases. When autoinducer molecules reach threshold concentration, the activation of the transcription factor, LuxR-type protein, leads to the expression of several genes including virulence factors. Proteins belonging to the PTS-dependent regulation of virulence factors are shown in red, to quorum sensing circuits in green, general carbon catabolic pathways in grey and virulence factors in blue.
1.2 Thesis Proposal

1.2.1 Knowledge gaps and aims

Coral reefs are affected by local and global disturbances simultaneously and most of the studies have focused on the response to single factors rather than the synergies among them [5, 109]. High DOC concentrations have a clear impact on bacterial activity and aggregate formation, and are known to negatively affect coral reef health. Despite their critical importance in coral reef degradation, no previous efforts have evaluated the combined effects of high DOC and global factors such as ocean acidification (i.e., high dissolved inorganic carbon, or DIC), affecting benthic and pelagic bacterial communities and organic carbon transformations on coral reefs (Aims 1 and 2). Furthermore, even though harmful algal cover increases on many coral reefs worldwide, there are no baseline data comparing algal- versus coral-dominated coral reef communities under such conditions. This shortcoming has to be addressed to gain a better understanding of coral reefs under changing environmental conditions.

While labile DOC is commonly a diverse ‘cocktail’ comprised by multiple components, recent studies have reported that the dominant chemical species of the labile fraction of allochthonous (e.g., sewage) and autochthonous (e.g., macroalgal exudates) DOC on coral reefs include the neutral monosaccharides glucose, mannose, xylose, and galactose [60, 61, 110]. It is known that this DOC ‘cocktail’ can have highly detrimental effects on coral reefs. The individual contribution of these sugars, however, to shifts in community structure and metabolic responses of coral reef bacterioplankton has not been assessed previously (Aim 3).

Under high DOC concentrations, corals presumably are harmed by low dissolved oxygen (DO) concentrations and the promotion of dominant opportunistic pathogens.
However, there is no evidence of how DOC modulates microbial metabolic strategies (e.g., commensals vs pathogens) and potentially promotes negative interactions with corals (Aims 4 and 5). Therefore, understanding microbial DOC uptake and how it drives cell activity (i.e., DOC uptake, growth rates, and carbon metabolism) and virulence factor expression is crucial to properly link the impact of high DOC concentrations to coral disease. Under consideration of these knowledge gaps, the aims of this thesis are to (1) investigate the individual and combined effects of DOC and ocean acidification on aggregate formation in coral-vs. algae-dominated reef mesocosms (2); investigate the individual and combined effects of DOC and ocean acidification on benthic vs. pelagic bacterial diversity in coral reef mesocosms; (3) evaluate the individual effects of enrichment with the neutral monosaccharides glucose, mannose, xylose, and galactose on coral reef bacterioplankton diversity; (4) characterize carbon metabolism mechanisms in dominating microbial populations under DOC enrichments; and to (5) evaluate virulence factor expression in those dominant bacterial populations in the different DOC enrichment.

1.2.2 General approach

Experiment 1 consisted of a controlled mesocosm setups at the MARine Experimental Ecology facility (MAREE) of the Leibniz Center for Tropical Marine Ecology (ZMT). The treatments corresponded to individual and combined enrichments of DIC (900 μatm), as an ocean acidification treatment, and high concentrations of glucose (150 μM glucose), as a high DOC treatment. Aggregate formation (aim 1) as well bacterial community diversity (aim 2) were evaluated over 42 days in mesocosms dominated by either benthic calcifying algae or by hard corals.

Experiment 2 consisted of coral reef water incubations independently enriched with glucose, mannose, galactose and xylose (500 μM). The seawater was collected on the exposed sites of the mid shore reef al Fahal (22°18.22.6N 38°57.494E) located in
the Central Red Sea off the Saudi Arabian coast. Bacterial diversity was evaluated through 16S rRNA amplicon sequencing (Aim 3) and gene expression of bacterial populations was assessed by combining metagenomic binning and metatranscriptomics (Aims 4 and 5).

1.2.3 Thesis outline

This thesis consists of a general introduction (chapter 1), three chapters presenting the research of the PhD (chapters 2-4), and a general discussion 5. Chapters 2-4 are either published or intended for publication as independent research articles in international peer-reviewed journals. Additional co-authored publications that are related to the thesis, but not included in it are listed below and the abstracts are presented at the end of the thesis.

1.2.4 Publication overview

Chapter 2 has been published in full in the journal *Marine Chemistry*:


This project was initiated by Anny Cárdenas (20%), Dr. Astrid Gärdes (40%) and Prof. Christian Wild (40%). The experimental design for this study was developed by Anny Cárdenas (80%), Dr. Friedrich Meyer (5%), Dr. Astrid Gärdes (10%) and Prof. Christian Wild (5%). Sampling was conducted by Dr. Friedrich Meyer (5%) and Anny Cárdenas (95%). Sample processing was done by Anny Cárdenas (95%) and Dr. Hauke Schwieder (5%) and data analysis was conducted by Anny Cárdenas. The manuscript was written by Anny Cárdenas with improvements from
all contributing authors.

Chapter 3, in full is under review for publication in the journal *Environmental Microbiology*:

2. **Anny Cárdenas**, Alban Ramette, Astrid Gärdes. Microbiomes of algae-dominated coral reefs are more susceptible to combined than individual effects of ocean acidification and dissolved organic carbon enrichment. Under review for publication in the journal *Environmental Microbiology*.

This project was initiated by Anny Cárdenas (50%) and Dr. Astrid Gärdes (50%). The experimental design for this study was developed by Anny Cárdenas (70%) and Dr. Astrid Gärdes (30%). Sampling and sample processing was conducted by Anny Cárdenas. Data analysis was conducted by Anny Cárdenas and corrected by Dr. Alban Ramette. The manuscript was written by Anny Cárdenas with improvements from Dr. Alban Ramette and Dr. Astrid Gärdes.

Chapter 4 in full is under review for publication in the journal *ISME*.


This project was initiated by Anny Cárdenas (80%) and Dr. Astrid Gärdes (20%). The experimental design for this study was developed by Anny Cárdenas (80%) and Prof. Christian Voolstra (20%). Sampling and sample processing was conducted by Anny Cárdenas (90%), Dr. Claudia Pogoreutz (5%) and Nils Rädecker (5%). Data analysis was conducted by Anny Cárdenas (80%) with the guidance of Dr. Matthew
J. Neave (5%), Dr. Mohamed Fauzi and Haroon (5%) for Linux and python programming and Prof. Christian Voolstra (10%) for results analysis. The manuscript was written by Anny Cárdenas with improvements from all contributing authors.

Other contributions:


   The dissertation author was involved in sampling and sample processing, DNA extraction and 16S rRNA amplicon sequencing data analysis and manuscript improvements.


   The dissertation author was involved in Dissolved Organic Carbon (DOC) sampling and sample processing, DNA extraction and 16S rRNA amplicon sequencing data analysis and manuscript improvements.


   The dissertation author was involved in processing nutrient analysis from the water samples and manuscript improvements.
Chapter 2
Chapter 2

The formation of aggregates in coral reef waters under elevated concentrations of dissolved inorganic and organic carbon: A mesocosm approach


2.1 Abstract

The transformation of dissolved organic carbon (DOC) to particulate organic carbon is the major mechanism through which large sinking organic particles are formed
in aquatic systems. Global stressors, such as high concentrations of dissolved inorganic carbon (DIC) due to ocean acidification, as well as local stressors, such as high DOC concentrations due to coastal eutrophication, can significantly affect the formation and settling of aggregates and thereby the marine biogeochemical carbon cycle. Increasing aggregate formation rates can contribute to the mortality of benthic organisms in coral reef ecosystems, but relevant knowledge is scarce. Therefore, the present study addresses this issue and studies the individual and combined effects of high DIC (900 μatm) and DOC (150 μM glucose) on organic matter dynamics as well as the formation of organic aggregates in a manipulative study over 42 days using 24 mesocosms dominated by either benthic calcifying algae or by hard corals. Organic aggregates in terms of transparent exopolymer particles (TEP) concentrations and total aggregated volume were measured. Results showed lower TEP concentrations and aggregated volume under high DIC concentrations. By contrast, under DOC enrichment higher rates of aggregate formation and microbial oxygen uptake were observed. Surprisingly, the highest aggregate formation rates were observed under the combined DIC and DOC enrichment. Furthermore, benthic organisms influenced the availability of DOC resulting in higher aggregate formation in coral compared to calcifying algae mesocosms. These experiments simulate future ocean conditions in coastal ecosystems where elevated DOC concentrations could aggravate the effect of high DIC on aggregate formation. In coral reef ecosystems, this may have important consequences on benthic organisms.

2.2 Introduction

Coral reef decline occurs as a direct response to a single stressor or as a consequence of different global and local stressors acting simultaneously [5, 111, 112]. Ocean acidification (OA) represents one of the most significant long-term threats to coral
reefs. Experimental evidence suggests that a doubling of pre-industrial pCO₂ has reduced coral growth and calcification by up to 40% due to the reduction of aragonite formation [14]. Local and regional threats generally include overfishing and land-based pollution [109, 113]. These threats are generally linked to some of the main causes of coral reef degradation, such as algal overgrowth, increased disease prevalence [114, 115, 116], bleaching [117, 118], and sedimentation [21, 119]. Elevated DOC may cause different pathologies and increase the rate of coral mortality [66, 67]. Negative effects of high DOC concentrations on coral reef health have been linked to enhanced bacterial growth rates and activities that cause coral death by oxygen depletion and accumulation of toxic substances [65, 68, 120]. Exogenous inputs of DOC in coral reefs are mainly derived from sewage, terrestrial run-off and marine fish farms [21, 48, 121]. For instance, the global flux of riverine DOC has been calculated as high as 7.8 × 10¹⁴ g Cyr⁻¹ [122]. However, DOC inputs have spatial and temporal variations, being higher in areas exposed to river discharges and during the wet season when precipitation is higher and storm events are more frequent [123, 124]. High concentrations of DOC can also enter the coral reef system in the form of exudates released by the benthic community, mainly from fleshy macroalgae and predominantly in the form of dissolved carbohydrates [60, 61, 120]. For instance, macroalgal blooms represent large amounts of continuous organic matter loads which can increase DOC concentrations up to 1000 μmol L⁻¹ [66]. Coral reefs are thus profoundly impacted by any activity linked to macroalgal overgrowth, as is the case of inorganic nutrient enrichment and overfishing (Smith et al., 2006). Although DOC is the most dominant form of organic carbon in most marine ecosystems, large sinking particles constitute a biologically important, though often small, percentage of the total organic carbon [78, 79]. In coral reef waters, the particulate fraction may be trapped by coral mucus and is mainly remineralized by benthic bacterial communities in order to fuel new production [80, 81, 82]. However, high levels of suspended particulate matter
directly affects light penetration and sedimentation rates and may subsequently promote coral mortality \[21, 84, 85, 86\]. The formation of large sinking particles from DOC generally involves acidic polysaccharide gel-like particles termed transparent exopolymer particles (TEP) \[72, 125\]. Abiotic TEP formation requires TEP-precursors present in some DOC pools, and depends on turbulence, ion density and concentration of inorganic colloids \[74, 126, 127\]. However, TEP can also be directly produced from cell exudates of numerous organisms, particularly from phytoplankton cells as well as macroalgae, coral and bacteria \[77, 128, 129, 130\]. Local and global stressors may significantly alter biogeochemical carbon cycling. For instance, declines in pH due to DIC enrichment cause changes of TEP properties and abundance, presumably because of alterations in total alkalinity (TA). This can lead to higher downward carbon exportation \[131, 132\]. Mesocosm experiments examining phytoplankton blooms under elevated DIC show an increased CO\(_2\) uptake and subsequent TEP exudation of phytoplankton cells \[133, 134, 135\], which can stimulate particle aggregation and acceleration of sedimentation \[127, 131, 136\]. However, there are no studies focused on understanding particle aggregation under high DIC in coral reef environments. DOC drives the microbial loop and further transformations into large aggregates. For example, anthropogenic activities can result in high loads of organic matter in coastal ecosystems, and these are often related to large quantities of particulate organic matter in the form of large suspended particles \[48, 121, 137\]. Experimental evidence also suggests increases in aggregate formation in eutrophic coastal waters, most likely due to the increased phytoplankton growth and subsequent DOC release \[138, 139, 140\]. However, there is no evidence supporting the effects of high DOC on aggregate formation in coral reef systems, despite its importance in coral mortality related to increased sedimentation \[21, 141, 142\]. This study explores the individual and combined effects of elevated DIC and DOC concentrations on aggregate formation processes in the overlying water column in coral reef mesocosms. Furthermore, it intends to compare
the effect of these stressors working on two groups of key players in coral reefs specifically, benthic calcifying algae and hard coral communities. Since aggregates and TEP always reach high concentrations in elevated nutrient scenarios of eutrophication, such as after phytoplankton blooms or upwelling events [139, 143], higher concentrations of TEP and higher aggregate formation in DIC and DOC treatments were expected. For this study we used replicated mesocosms deployed with calcifying algae or hard coral communities. The following future ocean scenarios were manipulated over 42 days: Elevated DIC concentrations (DIC), elevated DOC concentrations (DOC), the combined effect of elevated DIC and DOC (combined), and a control treatment. During the course of the experiment, the following parameters were measured: DOC, TEP and suspended particulate matter (SPM) concentrations, total aggregated volume, bacterioplankton cell density and oxygen uptake.

2.3 Methods

2.3.1 Origin and preparation of organisms

The experiments were carried out at the MARine Experimental Ecology facility (MAR-REE) of the Leibniz Center for Tropical Marine Ecology (ZMT). Coral and algal fragments were taken from existing colonies at the MAREE. The fragmentation was done 45 days prior to the starting point of the experiments and fragments were acclimatized for 15 days. The algal species used in the experiments were Halimeda cuneata, Halimeda opuntia, Halimeda macroloba, Halimeda copiosa and Amphiroa foliacea. Coral species used were Acropora millepora, Pocillopora damicornis, Seriatopora hystric, Stylophora pistillata and Acropora muricata.
2.3.2 Setup of mesocosms

Mesocosms were assembled as an open system using two-compartment tanks with a total volume of 264 L and a constant flow between compartments 2.1. Mesocosms were filled with reverse osmosis and ion exchange resin (Dowex) prepared water with the addition of artificial sea salts. Every tank contained 20 liters of oolite live sand (Ocean Direct) with a fraction size ranging from 200 μm to 1000 μm as a substrate. Of 24 tanks, twelve were set up with algae and twelve with coral fragments, with the following conditions: 3 tanks for DIC enrichments, 3 tanks for DOC enrichment, 3 tanks for the combined treatment and 3 tanks to perform the control treatment. Every mesocosm contained between 48 and 52 fragments comprising 5 species of algae or coral. Every tank contained a protein skimmer with 10% of the water being exchanged every week to ensure complete homogenization while minimizing material accumulation. Water was sampled from the lower compartment, directly at the inflow point of the upper compartment using polypropylene beakers (sulfuric acid-cleaned and artificial seawater-leached).

2.3.3 Background parameters and DIC/DOC enrichment

To obtain elevated DIC conditions, CO$_2$ was supplied constantly via aeration with 1000 μatm CO$_2$ pre-mixed gas (gas mixing system, HTK Hamburg, Germany) at a flow rate of 25 L min$^{-1}$ in the DIC and combined treatments. Control and DOC tanks were also aerated at the same flow rates in order to avoid differences in microbial and chemical activity due to bubbling although using 400 μatm CO$_2$. In DOC and combined treatments, DOC enrichment was achieved by adding 1.5 μM h$^{-1}$ glucose using peristaltic pumps and a glucose stock solution of 30 mmol L$^{-1}$ exchanged once a week. Total alkalinity was maintained constant using 40 g L$^{-1}$ CaHCO$_3$ stock solution by peristaltic pumps. The carbonate chemistry was monitored by measuring
Figure 2.1: Effects of elevated DIC enrichment on DOC and TEP concentrations as well as total aggregated volume. DOC concentrations in (A) algae and (B) coral mesocosms. TEP concentrations in (C) algae and (D) coral mesocosms. Total aggregated volumes in rolling tank experiments from (E) algae and (F) coral mesocosms. Aggregate volume per tank was calculated every 24 hours during a total period of 72 hours from particle numbers and their corresponding size assuming spherical shapes. The value shown on the graph represents the maximum value over the 72 hours. Error bars denote ± SD. Asterisks indicate significantly different means compared to control at p < 0.05 (Holm–Sidak method).
in situ pHNBS, temperature, oxygen saturation and salinity using a multi-probe, (WTW 3430, Germany). TA was measured by end-point titration using the TitroLine alpha plus Titrator (SI Analytics, Germany) using 0.5 M HCl and certified reference material (Batch 111, CRM Andrew Dickson, Scripps Institution of Oceanography). The complete carbonate system was calculated from pHNBS and TA using the CO₂ Sys Excel Macro [144] with the KSO₄ constants of Dickson [145] and K₁ and K₂ from Mehrbach et al. [146] refitted by Dickson and Millero [147].

2.3.4 Carbon pool analyses

Changes in concentration of DOC, TEP, and SPM were measured over time to follow changes in organic carbon pools during the various future ocean scenarios. For DOC analysis 20 mL of water were collected weekly from each mesocosm, giving a total of 192 samples. Samples were filtered through 0.45 μm pore size Minisart-GF filters (Sartorius, Gottingen, Germany). The filtrate was collected in 25 mL pre-rinsed polyethylene HDPE bottles and acidified using 100 μL of a 32% HCl solution (pH below 2). The acidified samples were stored at −20 °C in the dark until processing. DOC concentrations were measured using the high-temperature combustion method [148] with a TOC-VCPh TOC analyzer (Shimadzu, Mandel, Canada). For calibration and quality control purposes, 10 ppm Fluka TOC standards (Sigma-Aldrich, Steinheim, Germany), artificial seawater Hansell standards (Hansell Laboratory, RSMAS/University of Miami) and ultrapure water blanks were run in the analysis. The DOC concentrations were the average of 3 injections from each sample. The standard deviation between the 3 injections was below 1%. When a higher deviation occurred, the sample was repeated. TEP concentrations were quantified using the semi-quantitative spectrophotometric determination method [149, 150]. From every mesocosm, a total water sample of 300 mL, in subsamples of 50 to 100 mL, was filtered through 0.4 μm polycarbonate filters (Whatman, Maidstone, England) and processed in triplicate at
constant vacuum of 120 mm Hg. Filters were stained with Alcian Blue, rinsed with ultrapure water and stored at −20 °C until further analysis. A total of 144 filters were incubated in 10 mL-glass tubes containing 6 mL of 80% H₂SO₄ and rotated every 30 min. After 2.5 hours of incubation, the filters were removed and the solution was measured spectrophotometrically at 787 nm. Retained Alcian Blue was calibrated using the standard polysaccharide Gum Xanthan (75 mg L⁻¹ solution). TEP were expressed as micrograms of Xanthan equivalents per liter (μg Xeq. L⁻¹).

To measure SPM, 47-mm GF/F filters with particle retention of 0.7 μm were combusted at 400 °C and subsequently weighed. Sample volumes of mesocosm water between 500 and 1500 mL were filtered through the GF/F filters, dried overnight at 40 °C and weighed again. Triplicates were taken for a total of 96 samples. SPM was determined as the difference in weight normalized by the filtered volume.

2.3.5 Rolling tank experiments

To understand the effect of elevated DIC and elevated DOC concentrations on aggregate formation, aggregation experiments were set up using mesocosm water at different incubation times in rolling tank experiments. Rolling tank experiments mimic continued sinking of particles by rotating the liquid against gravity with constant turbulence facilitating aggregate formation [151]. To fill up 1.1-liter plexiglas cylindrical tanks in triplicate, 4-liter water samples (one mesocosm per treatment) were used. Tanks were placed on rolling tables for 7 days at 1.5 rpm and 26.5 °C in the dark (Shanks and Edmondson, 1989). Aggregate formation was monitored by counting and classifying the aggregates into 5 size classes (< 1 mm, 1 – 3 mm, 3 – 5 mm, 5 – 10 mm and 10 – 20 mm) every 24 hours. The potential of mesocosm water to form aggregates over time was thus determined and expressed as total aggregated volume, calculated from the number of aggregates and their diameters assuming spherical shapes. Aggregate formation rates were calculated from the change in total
aggregated volume over time. Additionally, oxygen sensor spots (FireSting O$_2$, Pyro Science GmbH, Germany) were placed inside each one of the rolling tanks to monitor oxygen concentrations over time, as an estimate of bacterial activity as aggregates were being formed. Internal O$_2$ concentrations were followed daily.

2.3.6 Bacterioplankton respiration and abundance

To determine if elevated DOC concentrations had an effect on bacterioplankton populations, total bacterial cell numbers and bacterial oxygen demand (BOD) were estimated. For total cell counts, 9 mL of water samples from each mesocosm (168 samples in total) were incubated with 1 mL of 37% formaldehyde for 3 hours at room temperature (22 °C). Samples were filtered through 0.2 μm polycarbonate filters (Whatman, Maidstone, England) covered in aluminum foil and stored at −20 °C until further analysis. Filters were washed with sterile ultrapure water and 70% ethanol, and cut into smaller pieces. From every filter, 3 small pieces were mounted with Roti ®-Mount FluorCare DAPI. The number of bacterial cells was counted in 10 randomly selected fields of views per sample at 1000× magnification using an Axioskop 40 epi-fluorescence microscope (Carl Zeiss, Gttingen, Germany). Oxygen consumption rates were determined through incubations in 1 L-airtight-jars filled with mesocosm water. Jars were sealed and incubated in the dark at 26.5 °C for 24 hours. Dissolved oxygen concentrations were determined with a WTW 3430 multi-parameter system (Weilheim, Germany). Oxygen consumption rates in the water column were calculated as the difference in oxygen concentrations before and after the incubation, divided by the incubation time.

2.3.7 Statistical analysis

Average values are given by the statistical mean and its standard variation (SD). Data were normally distributed and of equal variance. A two-way ANOVA with repeated
measurements was used to determine significant differences in total aggregated volume, bacterial cell numbers, BOD as well as DOC, TEP and SPM concentrations between treatments over time. Pairwise multiple comparison procedures were carried out using the Holm–Sidak method within the software Sigma Plot V12.5 (SPSS Inc, Chicago, IL). Statistical significance was accepted for $p < 0.05$.

### 2.4 Results

#### 2.4.1 Carbon chemistry background parameters

Chemical parameters for algae and coral mesocosms are summarized in Table 2.1. There were no significant differences between treatments in temperature (overall average: $26.2 \pm 0.8 ^\circ C$), salinity (overall average: $35.8 \pm 0.7 ^\circ C$) and TA (overall average: $2238.5 \pm 267.5 \, \mu$mol kg seawater$^{-1}$). Observed pH values were significantly lower in the DIC-enriched treatments. $\text{HCO}_3^-$ and aragonite values were significantly lower in DIC and combined treatments compared to the control in algae mesocosms.
Table 2.1: Mesocosm water chemistry after 42 days of incubation. Average values (± SD) are given for each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Salinity (NBS)</th>
<th>pH (μmol kg⁻¹)</th>
<th>TA (μmol kg⁻¹)</th>
<th>DIC (μatm)</th>
<th>HCO₃⁻ (μmol kg⁻¹)</th>
<th>Ω</th>
<th>Aragonite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Algae mesocosms</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.8 ± 0.4</td>
<td>35.6 ± 0.3</td>
<td>8.130 ± 0.024</td>
<td>2143 ± 72</td>
<td>456 ± 56</td>
<td>1732 ± 140</td>
<td>2.75 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>DIC</td>
<td>26.0 ± 0.1</td>
<td>35.7 ± 0.3</td>
<td>7.991 ± 0.062</td>
<td>2206 ± 114</td>
<td>854 ± 115</td>
<td>2348 ± 41</td>
<td>2.19 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>25.9 ± 0.2</td>
<td>35.6 ± 0.5</td>
<td>8.097 ± 0.046</td>
<td>2320 ± 86</td>
<td>604 ± 82</td>
<td>2130 ± 196</td>
<td>2.82 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>26.6 ± 0.3</td>
<td>35.8 ± 0.6</td>
<td>7.933 ± 0.035</td>
<td>2195 ± 158</td>
<td>1170 ± 120</td>
<td>2823 ± 156</td>
<td>2.01 ± 0.14</td>
<td></td>
</tr>
<tr>
<td><strong>Coral mesocosms</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.6 ± 0.1</td>
<td>36.2 ± 0.4</td>
<td>8.183 ± 0.016</td>
<td>2401 ± 89</td>
<td>423 ± 33</td>
<td>1833 ± 107</td>
<td>3.66 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>DIC</td>
<td>25.2 ± 0.1</td>
<td>36.1 ± 0.3</td>
<td>8.085 ± 0.022</td>
<td>2333 ± 47</td>
<td>581 ± 45</td>
<td>1896 ± 55</td>
<td>2.79 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>25.5 ± 0.4</td>
<td>36.1 ± 0.5</td>
<td>8.114 ± 0.029</td>
<td>2430 ± 111</td>
<td>520 ± 18</td>
<td>1924 ± 65</td>
<td>3.27 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>25.3 ± 0.6</td>
<td>36.2 ± 0.2</td>
<td>8.07 ± 0.048</td>
<td>2310 ± 87</td>
<td>553 ± 64</td>
<td>1861 ± 61</td>
<td>2.87 ± 0.29</td>
<td></td>
</tr>
</tbody>
</table>
2.4.2 Effects of elevated DIC concentrations

In the DIC treatment, DOC concentrations varied between 281.8 ± 3.7 and 328.8 ± 38.3 μM in algae mesocosms and 160.8 ± 24.6 and 299.1 ± 122.1 μM in coral mesocosms with no significant differences compared to the control (Fig. 2.1A-B). TEP concentrations ranged from 517.7 ± 65.2 to 797.3 ± 14.2 μg Xeq. L⁻¹ in algae mesocosms and from 141.7 ± 20.9 to 371.6 ± 84.5 μg Xeq. L⁻¹ in coral mesocosms. Concentrations of TEP were significant lower in the DIC treatment compared to the control in the last sampling point (42 days after the enrichment) in algae and coral mesocosms (Fig. 2.1C-D). SPM increased with time. In algae mesocosms, SPM concentrations ranged between 22.6 ± 0.6 μg mL⁻¹ and 33.5 ± 3.3 μg mL⁻¹ with no significant differences compared to the control (Fig. 2.2). In coral mesocosms SPM concentrations ranged from 18.8 ± 0.2 μg mL⁻¹ to 24.8 ± 0.6 μg mL⁻¹ in the DIC treatment, being significantly lower compared to the control (Fig. 2.2). Significantly lower aggregate formation rates were observed under high DIC treatments compared to all other treatments. The highest aggregated volume in DIC treatment was observed 35 days after the enrichment in algae mesocosms (8.4 ± 3.1 cm³) and 14 days after the enrichment in coral mesocosms (6.7 ± 1.9 cm³) (Fig. 2.1E-F). DIC enrichment did not have significant effects on cell density or microbial oxygen uptake (Fig. 2.3).

2.4.3 Effects of elevated DOC concentrations

Initial DOC concentrations in algae mesocosms were 688.3 ± 145.9 μM and reached 1448.0 ± 89.2 μM 21 days after the enrichment (Fig. 2.2A). In coral mesocosms initial concentrations were 201.8 ± 10.8 μM with a maximum of 365.5 ± 90.6 μM after 21 days of incubation (Fig. 2.2B). TEP concentrations were always significantly higher than the control mesocosms (P < 0.01). Initial TEP concentrations in algae mesocosms were 719.6 ± 82.8 μg Xeq. L⁻¹ and increased to maximum values of 1733.1 ± 226.8 μg
Figure 2.2: Effects of elevated DOC enrichment on DOC and TEP concentrations as well as total aggregated volume. DOC concentrations in (A) algae and (B) coral mesocosms. TEP concentrations in (C) algae and (D) coral mesocosms. Total aggregated volumes in rolling tank experiments from (E) algae and (F) coral mesocosms. Aggregate volume per tank was calculated every 24 hours during a total period of 72 hours from particle numbers and their corresponding size assuming spherical shapes. The value shown on the graph represents the maximum value over the 72 hours. Error bars denote ± SD. Asterisks indicate significantly different means compared to control at $p < 0.05$ (Holm–Sidak method).
Figure 2.3: Combined effects of elevated DOC and DIC enrichment on DOC and TEP concentrations as well as total aggregated volume. DOC concentrations in (A) algae and (B) coral mesocosms. TEP concentrations in (C) algae and (D) coral mesocosms. Total aggregated volumes in rolling tank experiments from (E) algae and (F) coral mesocosms. Aggregate volume per tank was calculated every 24 hours during a total period of 72 hours from particle numbers and their corresponding size assuming spherical shapes. The value shown on the graph represents the maximum value over the 72 hours. Error bars denote ± SD. Asterisks indicate significantly different means compared to control at p < 0.05 (Holm–Sidak method).
Xeq. L$^{-1}$ by day 28 (Fig. 2.2C). In coral mesocosms, initial TEP concentrations were significantly lower than in algae mesocosms, (142.6 ± 43.9 μg Xeq. L$^{-1}$). Highest TEP concentrations in coral mesocosms were observed 21 days after the enrichment (1216.9 ± 132.4 μg Xeq. L$^{-1}$) (Fig. 2.2D). In algae mesocosms, SPM increased over time, reaching a peak value of 42.7 ± 4.3 μg mL$^{-1}$ by day 42 (Fig. 2.5A). In coral mesocosms, a significant increase in SPM was observed from 14 to 35 days after the enrichment with maximum concentrations of 82.9 ± 3.5 μg mL$^{-1}$ (Fig. S2B). Aggregate formation was significantly higher (Table 2.2) in DOC treatments compared to controls in both algae and coral mesocosms. Aggregate volume and formation rates increased especially during high TEP concentrations in the mesocosms (days 14 to 35) (Fig. 2E-F). In rolling tank experiments from algae mesocosms, the highest aggregated volume formed was 16.0 ± 4.9 cm$^3$ at high TEP concentrations (Fig. 2.2E). In rolling tank experiments from coral mesocosms, the highest aggregated volume formed was 35.0 ± 7.0 cm$^3$ in the DOC treatment (Fig. 2.2F). Cell numbers in the DOC treatment were not significantly higher in coral and algae mesocosms compared to the controls (Table 2.2, Fig. 2.6C-D). However, microbial oxygen uptake was significantly higher in the DOC treatment compared to the control treatments in both algae and coral mesocosms (Fig. 2.6A-B).

2.4.4 Combined effects of elevated DIC and DOC concentrations

DOC concentrations in algae mesocosms in the combined treatment were significantly lower than in the DOC treatment alone (Table 2.2). However, in coral mesocosms the combined treatment had no significant differences compared to the DOC treatment. The highest DOC concentrations observed were 1351.2 ± 47.8 μM by day 21 in algae mesocosms and 390.8 ± 25.1 μM in coral mesocosms by day 14 (Fig. 2.3A-B). Initial TEP concentrations in algae mesocosms were 760.3 ± 39.6 μg Xeq. L$^{-1}$ and
Table 2.2: Significance values for pairwise comparisons between variables. Pairwise multiple comparison procedures were done by the pairwise comparison Holm–Sidak method using the software Sigma Plot V12.5 (SPSS Inc, Chicago, IL). Underlined values represent statistically significant differences compared to control treatments (P < 0.05).

<table>
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<td>Aragonite</td>
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increased to maximum values of 1265.0 ± 82.2 µg Xeq. L⁻¹ by day 28 (Fig. 2.3C). In coral mesocosms, initial TEP concentrations were significantly lower than in algae mesocosms (392.1 ± 79.7 µg Xeq. L⁻¹), but reached the highest observed TEP concentration (2750.7 ± 299.9 µM) 21 days after the enrichment (Fig. 2.3D). In algae mesocosms, the highest values of SPM observed were 50.8 ± 1.6 µg mL⁻¹ after 35 days of enrichment (Fig. 2.5A). In coral mesocosms the SPM increased significantly from 14 to 35 days after the enrichment with maximum concentrations of 108.5 ± 7.7 µg mL⁻¹ by day 28 (Fig. 2.2B). In both algae and coral mesocosms the highest aggregate formation was observed in the combined treatments especially from 14 to 35 days after the enrichment (Fig. 2.3E-F), corresponding to high TEP abundance. The highest aggregated volume observed was 20.7 ± 2.6 and 25.0 ± 4.2 cm³ for algae and coral mesocosm rolling tank experiments, respectively. Bacterial oxygen uptake was only significantly higher in algae mesocosms compared to the control (Fig. 2.6A). Cell density values did not differ significantly from the control (Table 2.2).
2.4.5 Comparison between algae and coral mesocosms

The most remarkable difference between algae and coral mesocosms was the DOC concentration. In algae mesocosms, DOC concentrations were more than three times as high as for coral mesocosms (Fig. 2.2A-B and Fig. 2.3A-B). TEP concentrations were higher in the DOC treatments in algae, and in the combined treatment in coral mesocosms (Fig. 2.2C-F and 2.3C-F). Furthermore, there were no significant differences in bacterial numbers and oxygen uptake between algae and coral mesocosms.

2.5 Discussion

2.5.1 Effects of high DIC on in-vitro particle aggregation

DIC enrichment is commonly associated with increased photosynthesis, exudation rates and TEP concentrations [134, 152]. It has previously been suggested that changes in both TA and pH may have an effect on TEP properties [132]. In the present study, TEP concentrations were affected by DIC enrichment, remaining always significantly lower compared to the control mesocosms, even when TA remained constant. In the DIC treatment, even if the photosynthesis was increased and resulted in a larger transitory organic carbon pool, these photosynthates seemed to be mainly used to fuel benthic bacterial biomass rather than contribute to the TEP pool in the water column. Therefore, contrary to what was expected, exogenous glucose seemed to be the main contributor to TEP formation rather than increases in DIC.

Mari [131] showed that a low pH reduces TEP stickiness, decreasing aggregate formation and therefore causing a decrease in the downward flux of carbon. Similarly, the lowest aggregate volumes were observed in the DIC treatment in this study, suggesting that TEP stickiness could have been affected by changes in pH. Nonetheless, other changes in TEP properties cannot be excluded and would require further
2.5.2 Effects of high DOC on in-vitro particle aggregation

Glucose, which was used in the present study to enrich DOC concentrations, not only represents one of the most abundant monosaccharides in coastal waters with anthropogenic influences [110, 153] but is also a frequently dominant monosaccharide in natural exudates of several phytoplankton and macroalgae species [154, 155]. Our results suggest that high DOC concentrations may trigger aggregate formation through the formation of TEP. Since a large fraction of algae and coral-derived DOC is composed of carbohydrates, which in turn are the main components of TEP, it may be that cell exudates may contribute to the DOC pool and therefore TEP precursors.

TEP concentrations did not increase steadily as has been observed in other mesocosm experiments [135, 156]. A relationship exists between bacterial cell abundance and TEP concentrations, indicating that production and/or consumption of TEP in our experiments were coupled with microbial cell density. Due to the small differences in bacterial cell numbers and oxygen uptake over time, we assume that only a small amount of DOC was taken up by the bacterioplankton community and a larger proportion of DOC was either taken up by benthic bacterial communities or spontaneously assembled into TEP and SPM as has been shown in other studies [77, 126]. However, oxygen consumption was significantly higher in the DOC-enriched treatments (Table 2.2), suggesting that the addition of a carbon source favored heterotrophic activity in the system. Peaks in respiration coincided with points in time of higher aggregate formation. This indicates that suspended particle matter represents an important source of nutrients for the plankton community.
2.5.3 Combined compared to independent effects

Our results, in agreement with previous studies [84, 136, 143], suggest TEP as one of the main factors driving aggregate formation. High concentrations of TEP in the mesocosms were always consistent with high aggregation rates in the rolling tank experiments. However, the largest aggregate volumes were observed in the combined treatment, even when the highest concentrations of TEP were measured in the DOC treatments. One possible explanation is that the slightly lower pH in the combined treatment compared to the DOC treatment, is enhancing aggregate formation in an unknown way. For instance, Mari [131] showed that a low pH increased TEP buoyancy, causing flotation rather than sinking of aggregates. As a consequence, TEP with longer residence time in the water column may further enhance aggregate formation. In our study, aggregate formation in the combined treatment was five times higher than in the individual treatments. Nevertheless, it is still not possible to accurately relate how the combined effect of elevated DIC and elevated DOC concentrations are enhancing aggregate formation, and should be addressed in future studies.

2.5.4 The influence of reef communities on particle and aggregate formation

Previous results have shown either slight or no significant differences in DOC concentrations released by benthic calcifying algae (Halimeda opuntia) compared to corals (Porites lobata) [56, 57]. However, present results showed severe differences in DOC concentrations between algae and coral mesocosms (Fig. 2.2A-B and 2.3A-B). Higher DOC concentrations in algae mesocosms are suggesting that in coral mesocosms DOC was either rapidly transformed into particulate organic matter (TEP and particle aggregates occurred earlier in coral mesocosms) or taken up directly by corals, as has
been shown before [157, 158, 159]. However, the activity of sediment microbial communities and communities hosted in the mucus layer of corals cannot be excluded, since they can significantly contribute to DOC uptake.

It is hypothesized that bacterial growth is triggered by the algae-derived DOC rather than coral-derived DOC [56, 57]. However, previous research demonstrates that calcifying algae release lower amounts and different compositions of exudates compared to their non-calcifying counterparts [60, 160]. We observed an increase in bacterial respiration in glucose-enriched treatments, but were unable to detect any significant differences in bacterial cell numbers and respiration rates between algae and coral mesocosms. This might suggest that calcifying algae release higher amounts of less labile DOC than corals, which in the end might yield the same oxygen uptake in the bacterial community. However, it is still necessary to consider DOC composition to understand all mechanisms involved in the transformations of organic carbon.

2.6 Ecological perspective

Elevated DOC concentrations are already one of the main concerns of coral reef health [56, 66, 67]. Several studies reported that enhanced bacterial growth results in oxygen depletion and accumulation of toxic substances which ultimately leads to an increased coral mortality [65, 66, 68, 161]. Unfortunately global and local stressors are not often considered simultaneously, thus neglecting the threats coral reefs face if exposed to multiple stressors. The combined effects of OA and increased DOC concentrations represent a more realistic scenario for many coastal ecosystems that are constantly under the effect of organic nutrient loads derived from anthropogenic activities. If proven in-situ, this could have profound biological implications in coral reef ecosystems as a consequence of higher aggregate formation and sinking rates, which in turn may enhance coral mortality caused by sedimentation. This could have
a stronger impact in reefs that are located in poorly flushed locations or those with high exposure to pollutants. Furthermore, our results indicate that coral and algae release DOC in different quantities and compositions, which in turn has an impact on the aggregate formation. In a community dominated by corals rather than calcifying algae, sedimentation could be faster. It is also important to include the effect of aggregate formation in coral reefs dominated by non-calcifying macroalgae in future studies, while considering that many coral reefs have already undergone phase-shifts from coral to non-calcifying macroalgae dominance [38, 162]. This algal overgrowth is known to elevate DOC levels and enhance microbial activity [57, 66, 67]. In the same way, this might trigger coral mortality associated with suspended particle matter and increased sedimentation rates.

2.7 Conclusions

Experiments reported here were designed to shed light on the effects of local and global stressors on different organic carbon pools, ranging from the dissolved to the particulate state in coral reefs. The findings in this work demonstrate that combined effects of OA, in terms of elevated DIC concentrations and elevated DOC concentrations on aggregate formation, are more severe than each stressor individually. Combined effects of DIC and DOC enrichments increased TEP concentrations and formation of aggregates. These experiments could represent possible scenarios for future conditions, especially in coastal ecosystems where elevated DOC concentrations exert greater impact that could increase the susceptibility to ocean acidification. An increase in aggregate formation will likely result in higher export of carbon to the deep sea. However, in coral reef ecosystems this may lead to negative consequences due to an increase in sedimentation of labile organic carbon that could result in harmful effects for reef organisms. Nonetheless, the impact of increased sedimentation rates due
to coral and algal-derived organic matter is not understood and needs to be addressed in further studies.

2.8 Supplementary information

The following are the supplementary data related to this article.

Figure 2.4: Mesocosm set-up. Tanks were composed of two chambers. The upper chamber with a total volume of 164 L and an area of 0.476m² contained fragments of either benthic calcifying algae or hard coral species. The lower chamber had a total volume of 100 L and contained the protein skimmer, filters and the supplier tubes.
Figure 2.5: Suspended particulate matter (SPM) measurements. (A) Algae mesocosms. (B) Coral mesocosms. Error bars denote ± SD. Asterisks indicate significantly different means compared to the control at p < 0.05 (Holm–Sidak method).

Figure 2.6: Variation of bacterial oxygen uptake rates and cell density in the water column. Bacterial oxygen uptake in (A) algae and (B) coral mesocosms. Bacterial cell densities in (C) algae and (D) coral mesocosms. Error bars denote ± SD. Asterisks indicate significantly different means compared to the control at p < 0.05 (Holm–Sidak method).
Chapter 3
Chapter 3

Microbiomes of algae-dominated coral reefs are more susceptible to combined than individual effects of ocean acidification and dissolved organic carbon enrichment

Anny Cárdenas, Alban Ramette, Astrid Gärdes. Microbiomes of algae-dominated coral reefs are more susceptible to combined than individual effects of ocean acidification and dissolved organic carbon enrichment. Under review for publication in the journal Environmental Microbiology.

3.1 Abstract

Coral reef-associated microorganisms respond fast to changing environmental conditions. Global and local stressors can result in rapid shifts in bacterial community
structure, having direct consequences on the ecological function and biogeochemical transformations. Here we evaluated the effect of elevated dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) on bacterial diversity in coral and macroalgal mesocosms. 16S-based diversity was compared between water and sediments in coral and algal mesocosms over a 42-day incubation period. DOC acted as the main driver of changes in bacterial beta-diversity, resulting in the concomitant proliferation of Enterobacteriaceae and Rhodobacteraceae in water samples from coral and algal mesocosms. DIC alone had a minor impact on community composition, but when combined with increased DOC concentrations, strong bacterial community shifts were observed in algal mesocosms. Furthermore, shifts in the bacterial community associated with increasing DOC are predicted to displace important functional groups such as diazotrophs and nitrite reducers in the water column. Our results suggest that increasing benthic algal coverage is an additional factor contributing to coral reef vulnerability to DIC and DOC enrichments via its modifying effects on bacterial diversity and functioning.

3.2 Introduction

Reef degradation is often associated with several interacting global stressors such as climate change and a variety of local stressors derived from anthropogenic activities such as overfishing and eutrophication. Atmospheric carbon dioxide (CO₂) levels have increased nearly by 100 ppm from the industrial revolution, overpassing 400 ppm and expected to continue rising [163, 164]. This increase in atmospheric CO₂ results in increasing dissolved inorganic carbon concentration (DIC) in the worlds oceans, causing ocean acidification (OA) [13]. High DIC concentrations are linked with the inability of corals, coralline algae and other calcifying reef organisms to form a skeleton [14, 165, 166]. Further, biochemical cycling, physiological responses and
growth rates are shown to be affected by elevated planktonic DIC concentrations in non-calcifiers as well [167, 168, 169, 170]. Reported results on changes in bacterial community diversity under high concentrations of CO$_2$ are inconsistent: while some mesocosm experiments find discrete or no shift in marine bacterial communities [171, 172], others report marked response to natural and manipulated CO$_2$ gradients [173, 174, 175]. Human activities are considered to directly or indirectly increase the runoff of DOC to the ocean, triggering multiple responses of marine biological communities [66, 67, 176]. For instance, in coral reef ecosystems, eutrophication and overfishing are known to have indirect consequences for coral reef health by promoting macroalgal overgrowth, which results in excessive release of DOC-rich exudates [66, 67, 68]. These macroalgal exudates are composed predominantly of carbohydrates, mainly glucose, with higher proportions of labile carbon that can be used with higher growth efficiency [59, 60, 177, 178]. In contrast, coral exudates are less labile with a more diverse carbohydrate composition [63, 64]. Therefore, macroalgaedominated reefs promote fast microbial growth of opportunistic copiotrophs, occasioning oxygen depletion and accumulation of toxic substances that feed the loop of reef degradation [65, 66, 68, 161]. Global and local stressors act on coral reefs in a synergistic, antagonistic or additive manner, affecting many biological processes in coral reef organisms [179, 180, 181]. Although the individual effects of DIC and DOC enrichment have been previously addressed, our study is the first to assess individual and combined effects of DOC and DIC enrichments on bacterial diversity in coral reef mesocosms. Moreover, considering that algae-dominated states are becoming more prevalent in coral reefs worldwide, we considered essential to evaluate differences in bacterial diversity associated with coral and algal mesocosms to represent a more realistic scenario for coral reefs in the near future.
3.3 Results

3.3.1 Chemical parameters measured in coral reef mesocosms

Temperature (overall average: $26.2 \pm 0.8 \, ^\circ C$), salinity (overall average: $35.8 \pm 0.7 \, ^\circ C$) and total alkalinity (overall average: $2238.5 \pm 267.5 \, \mu$mol kg seawater$^{-1}$) did not show significant variations across the mesocosms (Supplementary Table 3.1). Dissolved organic carbon concentrations (DOC) varied during the experiment (Supplementary Fig. 3.6), being significantly higher in glucose-enriched treatments ($P < 0.001$) and in algal mesocosms than in coral mesocosms ($P < 0.002$). Significantly lower pH values ($P < 0.05$) were observed in DIC-enriched mesocosms.

3.3.2 DNA sequencing

Barcode pyrosequencing of 121 bacterial community samples from coral and algal mesocosms resulted in 242,148 sequences spanning the hypervariable regions V3 to V6 of the 16S rRNA gene sequence. A total of 155,585 reads with a mean length of 629 bp were obtained after quality filtering and were further used for downstream analyses. The average number of reads obtained per sample before and after quality filtering and their respective length average are summarized in supplementary table 3.2. Rarefaction analysis, based on OTUs clustered at 3% sequence difference, indicated that the curves were not asymptotic (data not shown), suggesting that deeper sequencing might be required to estimate the overall bacterial diversity. Nevertheless, in our study where we mostly focus on changes in the community structure over time, and not in a thorough assessment of the absolute diversity in each sample, this issue is less of a concern.
3.3.3 Bacterial diversity in coral reef mesocosms

Bacterial richness was highly variable across the sampling period. Coral mesocosms harbored a higher richness than algal mesocosms, and sediment samples were also more OTU rich than water samples (Fig. 3.1A-B). OTU richness and Ace diversity index did not have significant differences between treatments over time. Changes in bacterial community composition were visualized by principal coordinate analysis of the Bray–Curtis dissimilarity matrix (Fig. 3.1C). Adonis analysis was performed to assess whether habitat (coral sediments, coral water, algal sediments and algal water), treatment (DIC, DOC, combined and control), and incubation time (0, 3, 7, 14, 21, 28, 35 and 42 days after enrichment) as well as their combinations of these factors have an effect in coral reef bacterial communities (Supplementary Table 3.3). The analysis showed that these factors and their combinations significantly ($P < 0.05$) impacted soil microbial communities with about 51% of the total variation explained by this model, suggesting that coral reef bacterial community structure was driven by habitat (27%) as the main factor, followed by treatment (3%) and time (1%). There was a clear shift towards similar bacterial communities in the DOC and combined treatment of water samples when comparing time points in the second half of the experiment.

3.3.4 Changes in bacterial diversity over time

Taxonomic diversity in coral reef mesocosms was analyzed at phylum, family and genus level. Sediment samples were characterized by the presence of Cyanobacteria, Planctomycetes and Bacteroidetes, while water samples had a higher proportion of Proteobacteria and the presence of Actinobacteria members (Supplementary Fig. 3.7). Most abundant families (relative abundance $> 2\%$) are depicted in Fig. 3.2. Rhodobacteraceae and Enterobacteriaceae increased noticeably in relative sequence abundance in water samples in DOC and combined treatments, while there was a re-
Figure 3.1: Microbial diversity across samples and time in coral reef mesocosms. (A) Observed OTUs, Chao and Ace index at different time points in the different treatments. (B) Observed OTUs, Chao and Ace index in water and sediment samples of coral and algal mesocosms. (C) First two principal coordinates from PCoA on the Bray–Curtis dissimilarity matrix representing bacterial community diversity across samples and time. Each point represents a sample colored by (AW) algal mesocosms water column (AS) algal mesocosms sediments, (CW) coral mesocosms water column, and (CS) coral mesocosms sediments. Shapes represent treatments. The variance explained by the PCs is indicated on the axes.
Figure 3.2: Temporal changes in abundant families in coral and algae mesocosms. Only families with relative abundances higher than 2% are shown. Letters in panels show most important changes in bacterial community composition compared to the control treatment.

Production of the Piscirickettsiaceae and an increase in the less abundant families (families with relative abundances lower than 2%) in sediment samples. We also found significant changes in community composition at the genus level between bacterial families (Supplementary Fig. 3.8).

Effect of elevated dissolved organic and inorganic carbon on coral reef mesocosms ANOSIM was performed to test for statistically significant differences (Supplementary Table 3.4) in OTU composition between treatments in each of the sample groups, namely algal water (AW), algal sediments (AS), coral water (CW) and
Figure 3.3: Bray–Curtis dissimilarities of bacterial communities over time. (AW) algal mesocosms water sample (AS) algal mesocosms sediment samples, (CW) coral mesocosms water sample, (CS) coral mesocosms sediment samples. Bray–Curtis dissimilarities were calculated from a square-root transformed abundance matrix and normalized to zero at the starting point of the experiment.

coral sediments (CS). Significant differences in the bacterial community structure under DIC enrichment compared to the control were found only in algal sediments ($R = 0.19, P = 0.017$). DOC enrichment was found to have significant effects ($R > 0.30, P < 0.01$) on bacterial communities in algal sediments and coral water samples. The combined effect of DIC and DOC enrichment had a significant effect ($R = 2.39, P < 0.009$) on the bacterial communities in water samples of algal mesocosms. Bray–Curtis dissimilarities of each treatment compared to the control were represented over time (Fig. 3.3). Bacterial community composition was strongly affected by the combined treatment in algal mesocosms and by DOC treatment in coral mesocosms.
3.3.5 Identification of key OTUs affected by simulated future ocean conditions

SIMPER analysis was performed to identify the contribution of individual OTUs (defined at 0.03 sequence difference) to the community variation between treatments. OTUs with a significant contribution to treatment variation were referred to as key OTUs (kOTUs) in our study. Out of a total of 433 OTUs, 328 kOTUs were found to contribute significantly to the variance between controls and DIC treatment, 98 kOTUs in the DOC treatment and 88 kOTUs in the combined treatment. Among these kOTUs, 12 were shared between all treatments, 39 were shared between DOC and combined treatment, 11 were shared between DIC and DOC and only 7 were shared between DIC and the combined treatment (Fig. 3.4).

Most of these kOTUs belonged to the Proteobacteria, Planctomycetes and Bacteroidetes phyla. The most abundant families in glucose-rich treatments were Rhodobacteraceae, Rhodospirillaceae, Alteromonadaceae and Flavobacteriaceae, while Rhodobacteraceae, Phycisphaeraceae, Pirellulaceae and Amoebophilaceae in the DIC treatment. The kOTUs shared between all treatments contained members of the genera *Planctomyces* (Planctomycetaeae), *Muricauda* (Flavobacteriaceae), *Microbulbifer* (Alteromonadaceae), *Rhodococcus* (Nocardiaceae) and *Mycoplasma* (Caulobacteriaceae). kOTUs shared between glucose-rich treatments belong to the genera *Alteromonas* (Alteromonadaceae), *Nautella* (Rhodobacteraceae), *Verrucomicrobiium* (Verrucomicrobiaceae), *Desulfobacter* (Desulfobacteraceae) and an evident high number of *Spirochaeta* (Spirochaetaceae) members. kOTUs shared between DIC-rich treatments belong to the genera *Lactobacillus* (Lactobacillaceae), *Cryocola* (Microbacteriaceae) and *Owenweeksia* (Cryomorphaceae). Representations of the phylogenetic relatedness of the kOTUs are provided in Supplementary Figs. 3.9-3.11.

Functional predictions revealed significant changes in the relative abundance of
Figure 3.4: Venn diagram showing the number of unique and overlapping kOTUs (0.03 sequence difference) with a significant contribution to the treatment variation in each of and between samples. Inner rings represent bacterial phyla and outer rings represent classes within each phylum.
groups with the ability of carry on major functions to the ecosystem. For instance, DOC enrichment is accompanied by a decrease in the abundance of known diazotrophs, nitrite, ammonia and sulfate reducers and sulfur oxidizers in bacterioplankton communities (see Fig. 3.5. However, in sediments samples, diazotrophs and nitrite reducers were more abundant in DOC-enriched samples than in the control treatments.

3.4 Discussion

Anthropogenic pressure on tropical coral reefs is expected to keep increasing, favoring macroalgal-dominance and elevated DOC [56, 182]. In addition, atmospheric CO$_2$ concentrations are expected to exceed 700 ppm by 2100 [14, 183]. We simulate these predicted scenarios in a crossed design by controlling individual and combined en-
richments of DIC (constant supply with 1000μatm) and DOC (added as 1.5μM h⁻¹ of glucose) to evaluate their effect on the bacterial diversity associated with coral reefs. DOC concentrations in coral reef oscillate between 29 to 75.5μM [52] for samples taken 25 cm above the reef surface at a water depth of 10 m. In our study the sampling distance was between 3 and 5 cm, resulting in higher average DOC values of 241μM and 298μM in the control treatment in coral and algal mesocosms respectively. This increment in DOC concentrations has been previously reported for reef bottom and the benthic surface contact water, where DOC concentrations can be as high as 2.3 mg l⁻¹ [184]. By comparing algae with coral mesocosms, we investigated future ocean scenarios in the so-called “phase shifts” that have changed many coral-dominated towards algae-dominated reefs [38, 185]. The transition to an algae-dominated state affects the ecosystem in several ways, possibly starting from the microbial scale by affecting bacterial metabolism and subsequent biogeochemical cycling and disease progression. Therefore, algae-dominated states are particularly important to consider in the field of coral reef microbiology since they represent a more realistic scenario for the near future coral reefs. Our results show profound differences in bacterial diversity between coral and algal mesocosms. These differences are suggested to be the consequence of dissimilar organic matter composition between algae and coral exudates [61]. Hass et al. [57] proposed a model that explains the influence of reef benthic community structure on bacterial metabolism. In their results, coral exudates promote an autotrophic microbial metabolism by increasing net O₂ production, while algal exudates promote heterotrophic microbial metabolism by increasing net O₂ consumption. In agreement with Hass et al., coral mesocosms were enriching over time with Bradyrhizobiaceae members, known to encompass several autotrophs and diazotrophs [186, 187]. In contrast, Enterobacteriaceae and Oxalobacteraceae, with numerous heterotrophs and highly virulent species, were enriched by the second half of the experiments in algal mesocosms.
To evaluate the effect of the treatments on changes in bacterial communities associated with coral reefs, we calculated Bray–Curtis dissimilarities between every treatment and controls and identified key OTUs (kOTUs) with a significant contribution to the effect of the treatment. Large Bray–Curtis dissimilarities in the DOC-associated communities and combined treatments suggested DOC as the main driver of bacterial diversity. Changes in phylotype composition over time (Fig. 3.3) and SIMPER analysis (Fig. 3.4, 3.9 and 3.11) indicated *Marivita, Nautella* (Rhodobacteracea) and *Erwinina* (Enterobacteracea) as possible opportunistic copiotrophs and r-strategists that quickly utilize resources in otherwise oligotrophic environments. *Marivitia* and *Nautella* fall into the Roseobacter clade, one of these major marine groups, making up to 25% of the bacterial biomass in coastal ecosystems [188]. This clade is known to rapidly respond to high organic carbon availability and has been reported in a wide diversity of environments including associations with marine algae and natural phytoplankton blooms [189, 190, 191], coral diseases [192, 193], and other marine animal diseases [194]. Members of the Escherichia/Shigella clade are able to utilize carbon sources in very efficient ways [195] and have been extensively studied for carrying diverse sets of pathogenicity islands and virulence factors regulated by environmental factors such as pH [196] and temperature [197]. Enterobacteriaceae members are often classified as human pathogens, and have also been associated to coral diseases as illustrated by *Serratia marcescens*, the etiological agent of the White Pox Disease [198, 199]. After this coral pathogen was introduced in coral reefs from sewage-polluted waters, high DOC concentrations played a fundamental role in the survival of this specie in seawater and in the activation of virulence mediated by quorum sensing [200, 201]. Therefore, elevated DOC concentrations are problematic in coral reefs for increasing the carrying capacity of the water and promoting virulence in a high-cell-density manner in opportunistic pathogens. In sediment samples, glucose-enriched samples caused a notable decrease of Piscirickettsiaceae members. The latter
have been studied extensively in the field of Salmonidae infections [202, 203], although members of this family are also generally present in marine sediments [204, 205, 206].

Bacterioplankton diversity under DIC enrichments revealed blooms of unclassified Rhodospirillacea over time. Even though the majority of the 297 kOTUs affected by the single effect of DIC were unclassified sequences, there were high numbers of known bacteria dominated by genera *Jannaschia* (Alphaproteobacteria), *Altererythrobacter* (Alphaproteobacteria) and *Propionibacterium* (Actinobacteria). A common feature of these genera is the capability to tolerate moderate acidic conditions [207, 208, 209]. Phylotype composition in sediment samples did not vary as much as in the water column and the major shifts associated high DIC concentrations were the increase in members of the genera *Erwinia* (Fig. 3.3 and 3.8). *Erwinia* members commonly occur in soils and many species are considered pathogens to plants and animals [210, 211].

When DIC and DOC enrichments were combined, the composition of bacterial communities from algal mesocosms had stronger shifts than in any of the single treatments. Such shift in the bacterial composition may be a consequence of increased algal stress that could have had an impact on the composition of released exudates. Although the chemical composition of algal exudates has been reported before [60, 61], no data is available on factors altering the chemical composition of macroalgal exudates. Interacting effects of DIC and DOC have also been reported for other processes in coral reefs such as particle aggregation [71], algal respiration, and dark calcification [181, 212]. In contrast, DOC treatment caused the strongest shifts in the bacterial community associated with coral mesocosms, indicating antagonistic effects between elevated DIC and DOC.

By assigning OTUs to putative functional categories, we could speculate about shifts in the potential community functions. For instance, glucose enrichment caused a drop of taxa known to accomplish important biochemical processes in coral reefs such as nitrogen fixation [213] and nitrite reduction [214]. Reduction of nitrogen fixation
has been previously reported for coral-associated dinoflagellates during DOC [215] and DIC enrichments [216]. Our results and previous results suggest that DOC and DIC enrichments could affect coral reef ecosystems by the loss of important microbial groups and promotion of heterotrophic metabolism leading to the alteration of biogeochemical cycling in the system.

3.5 Conclusions

Global and local stressors operate simultaneously on coral reef ecosystems and understanding the interaction between these stressors will always be challenging. By evaluating the interactions between DIC and DOC enrichments, we were able to estimate the effects of concurring OA and organic carbon enrichment in coral reef bacterial diversity. We show that bacterial community structure associated with algal mesocosms drastically shift after combining DIC and DOC enrichments. Nonetheless, in coral reef mesocosm, the combined DIC and DOC enrichment acted in an antagonistic manner, limiting the adverse effects of DOC enrichment. This indicates that reef benthic cover status has a profound implication on bacterial community diversity under natural and anthropogenic-derived stress. According to our results, bacterial communities associated with algal-dominated reefs will be more susceptible to the effect of OA and DOC enrichment, causing the loss of fundamental bacterial groups such as diazotrophs and nitrite reducers necessary for ecosystem functioning in the water column.

3.6 Experimental Procedures

Mesocosms setup Experiments were carried out at the MARine Experimental Ecology facility (MAREE) of the Leibniz Center for Tropical Marine Ecology (ZMT). Full details of the setup and maintenance of the mesocosms during this experiment are
provided by Crdenas et al., [71]. Briefly, 24 mesocosms were filled with reverse osmosis and ion exchange resin (Dowex™) prepared water with the addition of artificial sea salts. Oolite live sand (Ocean Direct™) with a size fraction between 200μm to 1000μm served as a substrate and bacterial inoculum. Every tank contained a protein skimmer and 10% of the water was exchanged every week to avoid material accumulation. Coral and algae specimens were fragmented 45 days before the starting point of the experiments from existing colonies at the MAREE. The algal community was represented by Halimeda cuneata, Halimeda opuntia, Halimeda macroloba, Halimeda caposia and Amphiroa foliacea. Coral community was represented by Acropora millepora, Pocillopora damicornis, Seriatopora hystrix, Stylophora pistilata and Acropora muralica.

3.6.1 Experimental design

From the total of 24 tanks, 12 were filled with algae and 12 with coral fragments in the following conditions: 3 tanks for DOC enrichments, 3 tanks for DIC enrichment, 3 tanks for the combined DIC-DOC enrichment and 3 tanks for the control treatment. DOC enrichment was done adding 10 mL 27.5 mg L⁻¹ glucose solution per hour. DIC enrichment was done by the constant CO₂ supply via aeration with 1000μatm at a flow rate of 25 L min⁻¹. Alkalinity was maintained constant by peristaltic pumps using 40 g L⁻¹ CaHCO₃ solution. Chemical parameters measurements and sampling for DNA extraction were done on a weekly basis for 42 days.

3.6.2 DOC measurements

Water subsamples from each mesocosm were filtered through 0.45μm pore size Minisart-GF filters (Sartorius, Göttingen, Germany). The filtrate was collected in 25 mL pre-rinsed polyethylene HDPE bottles and acidified using 100μL of a 35% HCl solution (pH below 2). Acidified samples were stored at −20 °C in the dark until
processing. DOC concentrations were measured by the high-temperature combustion method [148] using the TOC-VCMPH TOC analyzer (Shimadzu, Mandel, Canada). 10 ppm glutamic acid and artificial seawater Hansell standards (Hansell laboratory RS-MA University of Miami) were run in the analysis for calibration and quality control purposes.

### 3.6.3 Water column sampling and nucleic acid extraction

Water volumes of 400 mL were filtrated onto 47 mm 0.2-μm-pore size Supor filters (Pall Corporation, Ann Arbor, MI) and stored at −20 °C until further processing. DNA extraction was done following the protocol published by Bostrm and coworkers [217] for marine bacterioplankton communities with minor modifications. The protocol consists of a lysis step by adding 525μL of lysis buffer (0.4 M NaCl, 0.75 M sucrose, 20 mM EDTA and 50 mM Tris-HCl pH 9), 11μL of lysozyme (1mg mL$^{-1}$) and incubated 30 minutes at 37 °C. An additional incubation step of 12 hours at 55 °C with 60μL of 1% SDS and 3μL of proteinase K (100μg mL$^{-1}$). Precipitation of DNA was carried out with 0.1 volume of 3M NaAc and 0.6 volumes of isopropanol at −20 °C for 1 hour. Pellets were washed with 70% ethanol and eluted in 50μL of 10 mM Tris-HCL, pH 8.5. DNA was spectrophotometrically quantified using the BioPhotometer Plus (Eppendorf, Hamburg, Germany).

### 3.6.4 Sediment sampling and nucleic acid extraction

Sediment volumes of 5 mL were collected using sterile Falcon tubes and stored at −20 °C until further processing. DNA extraction was done using the PowerSoil™DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) using aliquots of 0.25 g of sediment samples. The obtained DNA was eluted in 50μL of 10 mM Tris-HCL, pH 8.5 and quantified using the BioPhotometer Plus (Eppendorf, Hamburg, Germany).
3.6.5 Sequence data and statistical analysis

A total of 124 samples were sequenced using a Genome Sequencer FLX system with Titanium chemistry (Roche) at LGC Genomics (Berlin, Germany). Roche/454-tagging and equimolar mixing of the 16S rRNA gene amplicons on 1/4 of the Pico Titer Plate were done by the company. To amplify the V3-V6 hypervariable regions of the 16S rRNA, the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 1061R (5'-CRRCACGAGCTGACGAC-3') were chosen as reported before (Ong et al., 2013). Data was processed using Mothur [218] and sequencing errors were removed by using PyroNoise [219]. Primer and barcodes were trimmed and sequences shorter than 500 nucleotides were excluded. In addition, reads with more than six homopolymers or with ambiguous bases were removed. Remaining sequences were used to produce multiple alignments against the 16S Silva database (release 123) [220]. At this stage, potential chimeras were removed using de novo and reference-based chimera detection using UCHIME [221]. The chimera-free generated files were used to taxonomically assign reads according to Greengenes database (released in May 2013) [222]. Sequences assigned as eukaryote, chloroplast or mitochondria were removed. OTUs were created at the 0.03 sequence difference level to calculate richness (Chao1) and Ace diversity index. Data were square-root transformed and normalized to relative abundances to satisfy assumptions of homogeneity of variance and to account for libraries of differing sequencing depths. Bray–Curtis dissimilarity distances were used to calculate successional trajectories and represented in a principal coordinates analysis (PCoA) using the R package “vegan” [223] for R. A three-way PERMANOVA using Adonis function was done to test the contribution of various factors to microbial community variations using the Bray–Curtis method. Significance tests among treatments and controls were analyzed by ANOSIM pairwise comparisons and SIMPER analysis was performed to identify contribution of individual OTUs to the variation...
between the treatments. Functional predictions on the bacterial community composition were made using Pareto scaling normalized OTU abundances and compared against the METAGENassist database to link taxonomy and phenotype [224]. Abundances per phenotype were compared between samples using ANOVA one factor and p-values were adjusted using Bonferroni correction. Figures were done in R using “Phyloseq” [225] and “ggplot2” [226] packages and FastTree [227].

3.6.6 Sequence Data deposition

The 454 pyrosequencing reads (raw data) are available from the NCBI Sequence Read Archive under the BioProject accession number PRJNA352106.
3.7 Supplementary information

Figure 3.6: DOC concentrations in coral reef mesocosms. Error bars denote ± SD. Asterisks indicate significantly different means compared to control at $p < 0.05$ (Holm–Sidak method).
Figure 3.7: Relative abundance of abundant phyla in coral and algae mesocosms. Only phyla with relative abundances higher than 2% are shown.
Figure 3.8: Relative abundance of genera at time points where significant different bacterial compositions were found.
Figure 3.9: Phylogenetic diversity of the OTUs with significant contribution to the enriched DIC effect. The 100 most significant (out of 332) OTUs were chosen to build the tree. Maximum-likelihood inference was done from a Jukes–Cantor matrix based on partial 16S rRNA gene sequence alignments.
Figure 3.10: Phylogenetic diversity of the OTUs with significant contribution to the DOC enrichment effect. The 100 out of 332 most significant OTUs were chosen to build the tree. Maximum-likelihood inference was done from a Jukes–Cantor matrix based on partial SSU rDNA sequence alignments.
Figure 3.11: Phylogenetic diversity of the OTUs with significant contribution to the combined effect. The 100 out of 332 most significant OTUs were chosen to build the tree. Maximum-likelihood inference was done from a Jukes–Cantor matrix based on partial SSU rDNA sequence alignments.
Table 3.1: Mesocosm water chemistry after 42 days of incubation. Average values (± SD) are given for each treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Salinity (NBS)</th>
<th>pH (μmol kg⁻¹)</th>
<th>TA (μmol kg⁻¹)</th>
<th>DIC (μatm)</th>
<th>HCO₃⁻ (μmol kg⁻¹)</th>
<th>Ω Aragonite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Algae mesocosms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.8 ± 0.4</td>
<td>35.6 ± 0.3</td>
<td>8.1 ± 0.024</td>
<td>2143 ± 72</td>
<td>456 ± 56</td>
<td>1732 ± 140</td>
<td>2.8 ± 0.02</td>
</tr>
<tr>
<td>DIC</td>
<td>26.0 ± 0.1</td>
<td>35.7 ± 0.3</td>
<td>8.0 ± 0.062</td>
<td>2206 ± 114</td>
<td>854 ± 115</td>
<td>2348 ± 41</td>
<td>2.3 ± 0.26</td>
</tr>
<tr>
<td>DOC</td>
<td>25.9 ± 0.2</td>
<td>35.6 ± 0.5</td>
<td>8.1 ± 0.046</td>
<td>2320 ± 86</td>
<td>604 ± 82</td>
<td>2130 ± 196</td>
<td>2.8 ± 0.26</td>
</tr>
<tr>
<td>Combined</td>
<td>26.6 ± 0.3</td>
<td>35.8 ± 0.6</td>
<td>7.9 ± 0.035</td>
<td>2195 ± 158</td>
<td>1170 ± 120</td>
<td>2823 ± 156</td>
<td>2.0 ± 0.14</td>
</tr>
<tr>
<td><strong>Coral mesocosms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>25.6 ± 0.1</td>
<td>36.2 ± 0.4</td>
<td>8.2 ± 0.016</td>
<td>2401 ± 89</td>
<td>423 ± 33</td>
<td>1833 ± 107</td>
<td>3.7 ± 0.24</td>
</tr>
<tr>
<td>DIC</td>
<td>25.2 ± 0.1</td>
<td>36.1 ± 0.3</td>
<td>8.1 ± 0.022</td>
<td>2333 ± 47</td>
<td>581 ± 45</td>
<td>1896 ± 55</td>
<td>2.8 ± 0.11</td>
</tr>
<tr>
<td>DOC</td>
<td>25.5 ± 0.4</td>
<td>36.1 ± 0.5</td>
<td>8.1 ± 0.029</td>
<td>2430 ± 111</td>
<td>520 ± 18</td>
<td>1924 ± 65</td>
<td>3.3 ± 0.33</td>
</tr>
<tr>
<td>Combined</td>
<td>25.3 ± 0.6</td>
<td>36.2 ± 0.2</td>
<td>8.1 ± 0.048</td>
<td>2310 ± 87</td>
<td>553 ± 64</td>
<td>1861 ± 61</td>
<td>2.9 ± 0.29</td>
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</tbody>
</table>
Table 3.2: Number of samples and total reads obtained before and after quality check.

<table>
<thead>
<tr>
<th>Sample pool</th>
<th>Number of samples</th>
<th>Average total reads obtained per sample</th>
<th>Final reads after quality check</th>
<th>Average sequence length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae sediments (AS)</td>
<td>28</td>
<td>1061</td>
<td>29703</td>
<td>639</td>
</tr>
<tr>
<td>Algae water column (AW)</td>
<td>30</td>
<td>1654</td>
<td>49619</td>
<td>619</td>
</tr>
<tr>
<td>Coral Sediments (CS)</td>
<td>31</td>
<td>782</td>
<td>24235</td>
<td>654</td>
</tr>
<tr>
<td>Coral water column (CW)</td>
<td>32</td>
<td>1577</td>
<td>52028</td>
<td>603</td>
</tr>
</tbody>
</table>

Table 3.3: Adonis analysis of the Bray–Curtis dissimilarities for bacterial OTU community structure in relation to habitat, time and treatment effects. Df = degrees of freedom; SS = sum of squares; F = F value. Bold numbers indicate statistical significance (P < 0.05); P-values are based on 1000 permutations.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>SS</th>
<th>F</th>
<th>$R^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>Treatment</td>
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<td>1.86140</td>
<td>0.0300</td>
<td><strong>0.0030</strong></td>
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<tr>
<td>Habitat</td>
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<td>12.9970</td>
<td>17.2018</td>
<td>0.2773</td>
<td><strong>0.0010</strong></td>
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<tr>
<td>Time</td>
<td>1</td>
<td>0.5080</td>
<td>2.0186</td>
<td>0.0109</td>
<td></td>
</tr>
<tr>
<td>Treatment * Habitat</td>
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<td>3.7350</td>
<td>1.6479</td>
<td>0.0797</td>
<td><strong>0.0220</strong></td>
</tr>
<tr>
<td>Treatment * Time</td>
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<td>0.9710</td>
<td>1.2846</td>
<td>0.0207</td>
<td><strong>0.0969</strong></td>
</tr>
<tr>
<td>Habitat * Time</td>
<td>3</td>
<td>1.2680</td>
<td>1.6776</td>
<td>0.0270</td>
<td><strong>0.0060</strong></td>
</tr>
<tr>
<td>Treatment * Habitat * Time</td>
<td>9</td>
<td>2.8130</td>
<td>1.2410</td>
<td>0.0600</td>
<td><strong>0.0250</strong></td>
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<tr>
<td>Residuals</td>
<td>92</td>
<td>23.1710</td>
<td>0.4944</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>123</td>
<td>46.8700</td>
<td>1.0000</td>
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Table 3.4: Pairwise ANOSIM between group vs. control treatments. AW = algal water, AS = algal sediments, CW = coral water, CS = coral sediments. Bold numbers indicate statistical significance (P < 0.05).

<table>
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<tr>
<th></th>
<th>AW</th>
<th>R²</th>
<th>P value</th>
<th>AS</th>
<th>R²</th>
<th>P value</th>
<th>CW</th>
<th>R²</th>
<th>P value</th>
<th>CS</th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC</td>
<td>0.044</td>
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<td>0.193</td>
<td>0.017</td>
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<td>0.043</td>
<td>0.204</td>
<td></td>
<td>0.084</td>
<td>0.111</td>
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<tr>
<td>DOC</td>
<td>0.074</td>
<td>0.125</td>
<td></td>
<td>0.381</td>
<td>0.003</td>
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<td>0.325</td>
<td>0.009</td>
<td></td>
<td>0.040</td>
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<tr>
<td>combined</td>
<td><strong>0.239</strong></td>
<td><strong>0.009</strong></td>
<td></td>
<td><strong>0.273</strong></td>
<td><strong>0.003</strong></td>
<td></td>
<td>−0.007</td>
<td>0.426</td>
<td></td>
<td>−0.032</td>
<td>0.644</td>
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</table>
Chapter 4
Chapter 4

Excess labile carbon promotes the expression of virulence factors in coral reef microbial populations


4.1 Abstract

Coastal pollution and algal cover are increasing in many coral reefs, resulting in higher dissolved organic carbon (DOC) concentrations. High DOC concentrations strongly affect microbial activity in reef waters and select for copiotrophic, often potentially virulent microbial populations. High DOC concentrations in coral reefs are also hypothesized to be a determinant for switching microbial lifestyles from commensals to pathogens, contributing to coral reef degradation. However, evidence correlating environmental conditions triggering this switch has yet to be provided. In this study,
we investigated gene expression of planktonic coral reef microbial populations under elevated concentrations of naturally abundant monosaccharides (glucose, galactose, mannose, and xylose) in algal exudates and sewage inflows. We assembled 27 near-complete (> 70%) microbial genomes through metagenomic sequencing, and determined expression patterns in those microbial populations through metatranscriptomic sequencing. Differential gene expression analysis revealed a shift in the central carbohydrate metabolism and the induction of metalloproteases, siderophores, and toxins among Alteromonas, Erythrobacter, Oceanicola and Alcanivorax populations. Sugar-dependent induction of virulence factors suggests a mechanistic link for the switch from commensal to a pathogenic microbial lifestyle, particularly relevant during increasing algal cover and human-derived pollution in coral reefs.

4.2 Introduction

Over the past decades, coral reefs have been rapidly degrading due to a combination of natural and anthropogenic factors that include over-fishing [17], pollution [228], and climate change [14, 229] leading to an increase in coral bleaching and diseases [111, 230]. These diseases are still poorly understood, although they represent an important cause of deterioration in these productive tropical marine communities [230, 231, 232]. Paleontological and ecological monitoring suggest an increase in the prevalence of reef diseases over the last decades [233, 234, 235], partially as a consequence of nutrient enrichment [114, 236, 237] and particularly to increases in dissolved organic carbon (DOC) [66, 67, 68]. DOC can enter coral reefs by coral and algal exudation (up to 40% of algal carbon produced in photosynthesis) in the form of a heterogeneous mixture of saccharides, proteins, and lipids [60, 61]. Exudates released by scleractinian corals are more similar in their sugar composition to reef waters and offshore DOC pools, whereas turf and fleshy macroalgal-derived DOC is enriched in the labile sugars
glucose, mannose, galactose, and xylose [61]. Allochthonous sources of DOC on coral reefs include sewage discharge [50], with a similar composition of dominant sugars compared to algal exudates [110].

The “Disease, Dissolved organic carbon, Algae and Microbe” (DDAM) model explains the impact of increased organic carbon in coral reefs [70]. DDAM proposes a positive feedback loop in which human-derived stressors such as overfishing and eutrophication, trigger macroalgae growth and promote the release of DOC-enriched exudates [57]. The mostly labile DOC fosters bacterial growth and oxygen removal, selecting for copiotrophs and opportunistic pathogens [52, 56, 61]. This, in turn completes the loop as it promotes coral bleaching and disease progression, providing space for algae to grow and become dominant on the reef. The etiology of many coral diseases, however, is poorly understood. Although numerous coral diseases have been described, causative agents have been identified only in a few cases with the incomplete satisfaction of Koch’s postulates [238, 239, 199]. Efforts towards the characterization of the whole microbial community rather than looking for single agents have made significant advances in the field. For instance, the appearance of signs of coral disease often correlates with shifts in coral-associated bacterial community composition [192, 240, 241], suggesting a more complex pathogenesis nature. To date, these diseases are believed to be opportunistic infections triggered by the exposure to environmental stressors (e.g., elevated temperature or nutrient enrichment), reducing host resistance and promoting the uncontrolled growth of opportunistic pathogens [115, 242]. These opportunistic pathogens can originate either from the coral holobiont (commensal symbionts) or the water column (environmentally acquired) and are also associated with healthy reefs, where they fulfill key functions to support the ecosystem, e.g. antibiotic production and nitrogen fixation [243, 244, 245].

Virulence can be understood as harm or morbidity caused by a microbe to its host.
Thus, identifying virulence determinants or factors (VF) can provide insights on the mechanistic basis of such harm [246]. VFs are molecules produced by pathogens that contribute to pathogenicity and typically include toxins, exoenzymes, adhesins, and secretion systems [246]. The expression of VFs is commonly correlated with the ability of a pathogen to invade and exploit host tissues. Nelson et al. [61] showed that algal exudates support the growth of bacterial populations among the families Vibrionaceae and Pseudoaltermonadaceae harboring high numbers of VFs. Temperature-dependent expression of virulence factors has been reported in several Vibrio species [247, 248]. Despite these studies, there has been no compelling evidence correlating other environmental factors with the activation of virulence factors in opportunistic coral reef pathogens.

Expression of VFs could provide a selective advantage for opportunistic microbes to gain access to nutrients. Therefore, these genes are often linked to general pathways of nutrient uptake [93]. As an example, so-called moonlighting proteins are involved in major metabolic processes such as the glycolytic pathway and stress responses, and they also have unexpected functions which contribute to microbial virulence [249]. Carbon catabolism is linked to bacterial virulence by the sensing of sugars through phosphotransferase system (PTS) transporters, which in turn activate carbon catabolite repression (CCR) [93, 250]. When different carbon sources are available, CCR allows bacteria to control the uptake of the preferred carbon substrate by disabling genes involved in the use of secondary carbon sources [251]. CCR is observed in most free-living heterotrophic bacteria and acts as a global regulator, controlling up to 10% of all bacterial genes, including several virulence factors [93, 252, 253]. The presence of CCR together with multiple transcriptional regulation networks allow copiotrophic bacteria to succeed in high-carbon concentrations [254].

In the present work, we characterized the microbial diversity of water samples before and after the addition of the most dominant monosaccharides (glucose, galactose,
mannose, xylose) previously reported for algal exudates and sewage discharge via 16S rRNA gene amplicon sequencing. Next, we recovered microbial population genomes via metagenomic binning, and subsequently mapped microbial mRNA reads to these population genomes to assess activity of distinct microbial community members under sugar enrichment. This allowed us to elucidate potential mechanisms associated with a shift from commensal to pathogenic microbial lifestyles driven by high sugar concentrations.

4.3 Materials and methods

4.3.1 Water sampling and incubations

Incubations consisted of 3 liters of 0.22 μm-filtered seawater inoculated with 1 liter of unfiltered reef seawater in a 4-liter polycarbonate bottle (sulfuric acid-cleaned and seawater-leached). Unfiltered seawater was collected at 10 m depth from Al-Fahal reef located 13 km offshore in the central Red Sea of Saudi Arabia at 9:30 am on 20/10/2014 and 03/11/2014, for the first and second set of experiments respectively. Every experiment consisted of 4 triplicated treatments, 2 sugar-amended and 2 unamended controls. Experiment 1 consisted of (B1) unamended control before incubation, (C1) unamended control after incubation, (Glu) after glucose incubation and (Gal) after galactose incubation. Experiment 2 consisted of (B2) unamended control before incubation, (C2) unamended control after incubation, (Man) after mannose incubation and (Xyl) after xylose incubation. Sugars were added to a final concentration of 500μM. The incubation time was 48 hours at 27 °C in the dark to avoid phytoplankton growth. Supplementary Fig. 4.6 shows a schematic representation of the different experiments and enrichments.
4.3.2 Cell density, DOC concentrations and efficiency calculations

Changes in cell abundance and DOC concentrations were measured over time during sugar incubations. Cell abundance was determined by flow cytometry using the protocol established by Nelson [255]. Briefly, unfiltered samples were fixed with paraformaldehyde to a final concentration of 0.4% and frozen (−80 °C) within 30 min of fixation. Fixed samples were thawed, mixed and stained with 1X SYBR Green for 1 hour at room temperature in the dark. Cell counts were done using the Guava® easyCyte flow cytometer (Millipore, Billerica, MA, USA). To measure DOC concentrations, triplicates of 20 mL samples were filtered through 0.45μm pore size Minisart-GF filters (Sartorius, Gottingen, Germany). The filtrate was collected in 25 mL pre-rinsed polyethylene HDPE bottles. 100μL of a 35% H₃PO₄ solution was added to acidify the samples (pH below 2) and subsequently stored at −20 °C in the dark until processing. DOC concentrations were measured in triplicates using the Apollo 9000 Total Organic Carbon (TOC) and Total Nitrogen Analyzer (TN) (Teledyne Tekmar, United States) at the Analytical Core Lab (ACL) at KAUST, Saudi Arabia. bacterial carbon change was determined as the difference in cell carbon (μmol C L⁻¹) before and after the incubation using a previously reported factor for coastal bacteria assemblages of 30 fg C cell⁻¹ [256]. Bacteria growth efficiency was calculated as the ratio of bacterial carbon production (rate of increase in bacterial carbon) to the rate of DOC removal [257].

4.3.3 Nucleic acid extraction and isolation

Triplicate water samples were pre-filtered subsequently through 10 and 3μm polycarbonate filters and collected onto 0.22μm polycarbonate filters. Filters were immediately frozen at −80 °C until nucleic acid extraction. Replicate filters were pooled for
nucleic acid extractions using the AllPrep DNA/RNA kit (Qiagen). Purified DNA was used for rRNA gene amplification and preparation of metagenomic sequencing libraries (see below).

About 200 ng of total RNA were amplified using the MessageAmp II-Bacteria kit (Ambion) as described previously [258, 259]. Prokaryotic mRNA was purified according to Stewart et al. [260] with slight modifications [261]. Briefly, rRNA was subtracted from the total RNA using anti-sense probes created by the in-vitro transcription of ribosomal genes (bacterial and archaeal 16S/23S and eukaryotic 18S/28S) at a final template-to-probe ratio of 1 : 2 (mass, per probe). Subsequently, mRNA was subjected to a polyA separation to exclude polyA+ eukaryotic mRNA from the polyA- prokaryotic mRNA. Metatranscriptomic TrueSeq libraries were sequenced using the Illumina MiSeq V3 system (Illumina, San Diego, CA, USA) with paired-end reads (2×300 bp). Metagenomic TrueSeq libraries were sequenced using the Illumina HiSeq 2000 system (Illumina, San Diego, CA, USA) with paired-end reads (2×150 bp) according to the manufacturer’s specifications.

4.3.4 16S-based diversity analysis

Microbial community composition was assessed via 16S rRNA sequencing before and after sugar enrichments. For this, 16S rRNA V5-V6 hypervariable regions were PCR amplified for 25 cycles using 16S primers 784F (5-AGGATTAGATACCCTGGTA -3) and 1061R (5- CRRACGAGCTGACGAC -3) designed by Andersson et al. [262] and have been shown to work well with marine environments [263]. PCR products were sequenced using Illumina MiSeq V3 (2×300 bp) according to the manufacturers specifications at the Bioscience Core Lab (KAUST, Saudi Arabia). Primer trimming, quality control, clustering and taxonomic classification were done in mothur v1.36.1 [218]. After filtering, quality control consisted of removing sequences shorter than 300 bp, with quality scores lower than 25 and those that occurred only once (sin-
Sequences were aligned to SILVA reference, release 119 [264], and clustered into operational taxonomic units (OTUs), defined at 97% similarity. Representative sequences from each cluster were taxonomically assigned using Greengenes reference taxonomy [222]. Chimeric sequences were identified with the de novo implementation of UCHIME in the mothur interface [221] and subsequently removed. Principal Coordinate Analysis (PCoA) using Bray–Curtis distance and diversity analyses (Shannon-Weaver and Chao1 indexes) were performed on OTU abundance data using the Phyloseq [265] R package, using output tables generated in Mothur.

4.3.5 Metagenome binning

Sequence reads were quality checked and trimmed for low-quality regions, adapter sequences and a minimum length of 75 bp using Trimmomatic v0.36 [266]. MEGAHIT v1.0.2 [267] was used to produce one assembly per experiment using the “metasensitive” option and kmer length of 81. Metagenome binning was done based on differential coverage and tetrancleotide signatures for each assembly separately using the binning tools GroopM v0.3.4 [268] and Metabat v0.25.4 [269]. Genome contamination and completeness were assessed using CheckM v1.0.3 [270]. To increase genome completeness, the “merge” option from CheckM was used and population genomes with a delta contamination lower than 3 and a merged contamination lower than 5 were merged. CheckM “unique” and “join” commands were used to compare and combine genomes generated from the different binning tools. The metagenomic binning procedure resulted in population genomes representing putative microbial taxa. A first taxonomic approximation was done based on universal, single-copy phylogenetic marker genes using specI [271], marker lineages from CheckM and sequence composition-based classifier [272]. Whole-genome based Average Nucleotide Identity (gANI) and Amino Acid Identities (AAI) between the query genomes and NCBI refer-
ence genomes were used to validate closest related species using JSpecies v1.2.1. [273] and the enveomics tools [274]. Open reading frames (ORFs) were predicted using Prodigal [275]. Predicted protein encoding genes were functionally annotated using Gene Ontology (GO) and myRAST server [276] according to SEED subsystems [277]. Specific processes were inferred from GO annotation as listed in the supplement (Table 4.3). For instance, microbial interaction genes were identified based on the Plant-Associated Microbe Gene Ontology (PAMGO) Consortium [278]. Virulence genes were assigned by a BLASTn v2.2.28 search against MvirDB [279] and VFDB [280] databases (both downloaded on 25/03/16), supplemented with GO terms related to pathogenesis (Table 4.3). Sugar transporters were identified using the Carbohydrate Active Enzymes database (http://www.cazy.org/) [281]. Genes involved in antibiotic resistance were deducted from the BLAST search against the Antibiotic Resistance Genes Database (ARDB) database (downloaded on 07/07/16) [282].

4.3.6 RNA mapping and gene expression analysis

Remaining rRNA were removed in-silico using SortMeRNA v2.0 [283]. Cleaned mRNA fragments were mapped to a multifasta file containing all annotated genomes using the Bowtie aligner v1.0.0 using the “best” option allowing a single hit [284]. Transcript-level estimates were obtained as mRNA read counts using the transcript abundance estimation tool eXpress v1.5.1 [285] and normalized by the size of the RNA library (sequencing depth) and the coverage of the population genome in the metagenome (population genome abundance). Differential expression, expressed by Log2 fold change (FC), was calculated for mRNA reads in each treatment compared to the control (no sugar amend after 48 h). A two-fold FC cut-off was used to determine up- and down-regulated genes.
4.3.7 Data deposition


4.4 Results and discussion

4.4.1 Microbial diversity and DOC consumption

To identify copiotrophic microbial taxa, we compared 16S rRNA gene-based microbial community diversity before and after addition of different sugars, previously reported to be among the most dominant in algal exudates and sewage discharge [61, 110]. We supplemented with glucose and galactose in the first experiment and with mannose and xylose in the second experiment (see section 4.3). The main differences in microbial composition between both controls were a higher relative abundance of Alteromonadaceae and lower Oceanospirillaceae of experiments 1 compared to experiment 2. These differences were likely due to small changes in inorganic nutrient concentrations driven by seasonality as previously reported [286] as well as by differences in DOC concentrations that we observed in reef water before the start of experiments 1 and 2 (Table 4.1).
Table 4.1: Microbial planktonic growth efficiencies after 48h sugar incubations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reef Natural DOC (μmol C L$^{-1}$)</th>
<th>DOC change (μmol C L$^{-1}$)</th>
<th>Bacterial C change (μmol C L$^{-1}$)</th>
<th>Bacterial specific growth rate (per day)</th>
<th>Bacterial growth efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (C1)</td>
<td>121.55 ± 28.23</td>
<td>$-14.25 \pm 5.96$</td>
<td>1.92 ± 0.44</td>
<td>0.3 ± 0.03</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>Glucose (Glu)</td>
<td>121.55 ± 28.23</td>
<td>$-362.91 \pm 77.5$</td>
<td>11.4 ± 1.72</td>
<td>0.66 ± 0.03</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Galactose (Gal)</td>
<td>$-383.34 \pm 124.09$</td>
<td>14.2 ± 2.39</td>
<td>0.69 ± 0.02</td>
<td></td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Control (C2)</td>
<td>$-98.98 \pm 93.2$</td>
<td>1.82 ± 0.65</td>
<td>0.45 ± 0.15</td>
<td></td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Mannose (Man)</td>
<td>69.57 ± 13.02</td>
<td>$-481.71 \pm 258.39$</td>
<td>1.69 ± 0.55</td>
<td>0.39 ± 0.09</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Xylose (Xyl)</td>
<td>$-516.19 \pm 171.24$</td>
<td>2.47 ± 0.84</td>
<td>0.5 ± 0.12</td>
<td></td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
Sugar enriched samples deviated from the controls towards less rich and less diverse bacterial communities (Fig. 4.1A-B). Microbial community shifts were characterized by increases in Rhodobacteraceae (Alphaproteobacteria) in galactose, mannose and xylose, Vibrionaceae (Gammaproteobacteria) in glucose, and Alteromonadaceae (Gammaproteobacteria) in both glucose and mannose enriched samples (see Fig. 4.1C). A recent study supporting the microbialization concept showed that oligotrophic coral-dominated waters are characterized by high abundance of Alphaproteobacteria, in contrast to enriched algal-dominated waters where Gammaproteobacteria are increased [52]. Linking our study to Haas et al. [52], our results suggest glucose and mannose are major contributors to reported bacterial shifts in impacted reefs. Furthermore, microbial communities had a significantly faster and more efficient growth in galactose and glucose enrichments (Table 4.1) indicating the fundamental role these sugars play at supporting an increase in microbial biomass in coral reefs.
Figure 4.1: Impact of high sugar concentrations on coral reef planktonic microbial diversity. (A) Alpha diversity indices show a decrease in OTU richness and diversity after sugar additions. (B) Principal coordinates analysis plot based on Bray–Curtis distances between planktonic microbial reef communities. Sugar supplemented samples (magenta) have a different planktonic microbial community composition compared to untreated samples (grey), evidenced by a shift from left to right along PC1. (C) Bar charts show the relative abundance of OTUs in water reef samples before and after addition of different sugars. Pronounced shifts of Alteromonadaceae, Rhodobacteraceae, and Vibrionaceae are evident. Outer and inner rings show taxonomical affiliation of these OTUs at genus and species level respectively. Gal = Galactose, Glu = Glucose, Man = Mannose, Xyl = Xylose.
4.4.2 Metagenomic binning and microbial population genomes

We assembled metagenomic reads from each experiment and calculated the abundance profile and tetranucleotide frequencies of each contig between treatments. This allowed us to assign co-abundant contigs with similar tetranucleotide frequencies into groups that represent the gene content of distinct microbial population genomes representing putative microbial taxa. We recovered more than 400 draft population genomes and only used those with total completeness above 70% for further analyses (Table 4.2).
Table 4.2: Microbial population genomes retrieved and assembled in this study.

<table>
<thead>
<tr>
<th>Population genome (experiment)</th>
<th>Completeness (%)</th>
<th>Contamination (%)</th>
<th>Bin size (Mbp)</th>
<th>GC (%)</th>
<th>Number of PEGs</th>
<th>Suggested taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>GML331 (E1)</td>
<td>98.76</td>
<td>2.36</td>
<td>3.35</td>
<td>62.2</td>
<td>3221</td>
<td>Hyphomonas sp.*</td>
</tr>
<tr>
<td>GM189 (E1)</td>
<td>98.09</td>
<td>2.71</td>
<td>3.3</td>
<td>45.4</td>
<td>3207</td>
<td>Alteromonas sp.*</td>
</tr>
<tr>
<td>GM_23 (E1)</td>
<td>97.66</td>
<td>0</td>
<td>2.92</td>
<td>55.1</td>
<td>2982</td>
<td>Rhodobacteraceae</td>
</tr>
<tr>
<td>MB_1 (E2)</td>
<td>95.12</td>
<td>1.8</td>
<td>3.59</td>
<td>63</td>
<td>3408</td>
<td>Oceanicola sp.</td>
</tr>
<tr>
<td>MB_3 (E1)</td>
<td>92.2</td>
<td>0.56</td>
<td>2.07</td>
<td>63</td>
<td>1747</td>
<td>Flavobacteriaceae</td>
</tr>
<tr>
<td>GM_439 (E1)</td>
<td>90.79</td>
<td>3.35</td>
<td>4.72</td>
<td>45.8</td>
<td>3736</td>
<td>Alteromonas sp.</td>
</tr>
<tr>
<td>MB_4a (E1)</td>
<td>90.69</td>
<td>1.64</td>
<td>2.85</td>
<td>64.3</td>
<td>2724</td>
<td>Erythrobacter sp.</td>
</tr>
<tr>
<td>GM_293 (E2)</td>
<td>88.2</td>
<td>5</td>
<td>2.15</td>
<td>53.8</td>
<td>2094</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>GM_554 (E2)</td>
<td>87.65</td>
<td>5.44</td>
<td>2.76</td>
<td>59.8</td>
<td>2451</td>
<td>Alcainivora sp.*</td>
</tr>
<tr>
<td>merged_357,358 (E1)</td>
<td>83.36</td>
<td>8.21</td>
<td>2.8</td>
<td>66.6</td>
<td>2619</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>GM_66 (E2)</td>
<td>81.61</td>
<td>6.68</td>
<td>2.37</td>
<td>64.3</td>
<td>2257</td>
<td>Erythrobacter sp.</td>
</tr>
<tr>
<td>GM_20 (E1)</td>
<td>81.4</td>
<td>1.91</td>
<td>2.64</td>
<td>43.8</td>
<td>2283</td>
<td>Pseudoalteromonas</td>
</tr>
<tr>
<td>GM_34 (E2)</td>
<td>80.4</td>
<td>3.2</td>
<td>1.54</td>
<td>55.6</td>
<td>1379</td>
<td>Euryarchaeota</td>
</tr>
<tr>
<td>merged_809,810 (E1)</td>
<td>80.36</td>
<td>8.27</td>
<td>3.38</td>
<td>48.7</td>
<td>2993</td>
<td>Alteromonas sp.</td>
</tr>
<tr>
<td>MB_2 (E2)</td>
<td>80.24</td>
<td>0</td>
<td>1.46</td>
<td>47.3</td>
<td>1292</td>
<td>Flavobacteriaceae</td>
</tr>
<tr>
<td>MB_5a (E1)</td>
<td>79.34</td>
<td>2.42</td>
<td>1.68</td>
<td>59.3</td>
<td>1625</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>GM_808 (E1)</td>
<td>77.7</td>
<td>2.83</td>
<td>5.17</td>
<td>59.8</td>
<td>4654</td>
<td>Labrenzia sp.</td>
</tr>
<tr>
<td>MB_4b (E2)</td>
<td>76.2</td>
<td>0.01</td>
<td>1.76</td>
<td>58.8</td>
<td>1706</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>MB_5b (E2)</td>
<td>75.63</td>
<td>1.54</td>
<td>1.7</td>
<td>57.6</td>
<td>1463</td>
<td>Flavobacteriaceae</td>
</tr>
<tr>
<td>GM_50 (E2)</td>
<td>73.74</td>
<td>7.13</td>
<td>3.06</td>
<td>45.1</td>
<td>2625</td>
<td>Alteromonas macleodii</td>
</tr>
<tr>
<td>GM_88 (E2)</td>
<td>73.42</td>
<td>4.5</td>
<td>2.34</td>
<td>44.5</td>
<td>2112</td>
<td>Gammaproteobacteria</td>
</tr>
<tr>
<td>MB_7 (E1)</td>
<td>72.08</td>
<td>0.43</td>
<td>1.31</td>
<td>57.4</td>
<td>1358</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>merged_515,516 (E1)</td>
<td>72.08</td>
<td>5.22</td>
<td>2.25</td>
<td>61.5</td>
<td>2018</td>
<td>Rhodobacteriaceae</td>
</tr>
<tr>
<td>merged_287,630 (E2)</td>
<td>71.62</td>
<td>8.61</td>
<td>2.03</td>
<td>63</td>
<td>1907</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>MB_8 (E1)</td>
<td>71.33</td>
<td>0</td>
<td>1.09</td>
<td>50.3</td>
<td>998</td>
<td>Euryarchaeota</td>
</tr>
<tr>
<td>MB_9 (E1)</td>
<td>71.2</td>
<td>0.27</td>
<td>1.61</td>
<td>60.6</td>
<td>1419</td>
<td>Euryarchaeota</td>
</tr>
</tbody>
</table>

PEGs: Protein encoding genes
(*) population genome contains 16S rRNA sequence
Annotation and gene expression analyses were conducted for the selected 27 population genomes, after determining nucleic acid (ANI) and amino acid identities (AAI) of complete reference genomes to infer closest related organisms. Cutoffs of ANI > 94% and AAI > 90% are commonly accepted as bacterial species [287] and in spite of an absence of a specific genus cutoff for ANI or AAI, intragenus and interspecies nucleotide identities often vary between ~ 62% to 95% for ANI [288, 289]. Two population genomes, GM−44 (Ruegeria mobilis) and GM−50 (Alteromonas macleodii) could be assigned to the species level based on ANI and AAI comparisons (Table 4.4). For the majority of the remaining population genomes, which likely represent undescribed species of known genera, we used taxonomic assignment based on a polyphasic approach that combines ANI, AAI, genome clustering using marker genes, and 16S rRNA where possible. Population genomes comprised a wide diversity of taxa including different populations of the copiotrophic Alteromonas and several members of the Rhodobacteraceae, such as Oceanicola, Ruegeria, and Labrenzia (Table 4.2).

Active populations were identified using RAST annotation [276] of induced biological processes (Figs. 4.1B and 4.7). Most active population genomes included members of the Gammaproteobacteria genera Alteromonas, Pseudoalteromonas, Alcanivorax, Hahella, and Alphaproteobacteria genera, Oceanicola and Erythrobacter. Most of these comprise microbes typically found in coral reefs [290, 291, 292], but are often linked to nutrient-enriched and degraded reefs including fish farm effluents [48], oil-contaminated reefs [293], and coral diseases [192, 240, 241].

4.4.3 Sugar transporter diversity and microbial metabolism

The recovery of near-complete population genomes allowed us to investigate mechanisms triggered by the sensing and uptake of dissolved monosaccharides. The first step in understanding sugar uptake was to evaluate the diversity of sugar transporters. The most common transporters among the population genomes were galac-
tose ABC transporters, mannose PTS, and glucose/mannose MFS porters. The man-
nose PTS is known to be not only an important uptake system for mannose and
glucose but it has a major role in the transcriptional regulation and the preferential
utilization of monosaccharides [294, 295]. PTS was mostly expressed in active pop-
ulation genomes and was also tightly linked with the activation of virulence factors
(Fig. 4.2A-B) (discussed below). Copiotrophs were expected to possess a wide array
of low-affinity and highly specific transporters, contrary to oligothrophs with a rel-
atively smaller number of broad-specificity and sufficiently high-affinity transporters
as previously suggested [296]. This was confirmed in the most abundant population
genomes (Fig. 4.2C), such as GM−23 (Rhodobacteraceae), which possessed 5 differ-
ent transporters for sugar uptake (Fig. 4.2A). Most of these transporters belonged to
the low-affinity ABC transporters and the multi-sugar Major Facilitator Superfamily
(MFS) in comparison to the low presence of high-affinity phosphotransferase system
(PTS) transporters. The presence of several glucose uptake systems is suggested to
serve as backup transporters that can be used under different environmental condi-
tions [297]. For instance low-affinity systems are expected to be induced in sugar-rich
environments and high-affinity transporters when low amounts of glucose are avail-
able.
Figure 4.2: Sugar transporter diversity and expression in microbial population genomes. (A) Diversity of sugar transporters across microbial population genomes. Each row represents the presence of ABC, PTS, or MFS transporters involved in the uptake of galactose (blue), glucose (magenta), mannose (yellow), and xylose (green) in microbial population genomes. (B) Heat map depicting number of induced genes, across microbial population genomes across SEED subsystems under different sugars. (C) Relative abundance of each microbial population genome in the metagenome.
Using metagenomics, Haas et al. [52] found that coral-dominated reefs waters were enriched for genes involved in the more energy efficient Embden–Meyerhof–Parnas (EMP) pathway, while algal-dominated reefs waters were enriched with genes involved in the less efficient Entner–Doudoroff (ED) and pentose phosphate pathways (PP). To test whether we see a similar pattern, we evaluated the expression of genes involved in the EMP, ED, and PP pathways in the population genomes, and could corroborate previous findings in some of the copiotrophic populations (Fig. 4.3). We found evidence for a shift from EMP to ED and PP in the population genomes of Alteromonas and Erythrobacter species, suggesting a lower cost, faster and less efficient organic carbon remineralization under high carbon availability as previously argued [298, 52].
Figure 4.3: Expression of genes involved in different glycolytic pathways. (A) Entner–Doudoroff (ED) in green, Embden–Meyerhof–Parnas (EMP) in red and pentose phosphate pathways (PP) in blue. (B) Each row represents a population genome and each column represents a distinct gene. Expression levels are colored white for low intensities and red for high log2 fold changes comparing control and treatment of sequencing depth- and bin coverage-normalized mRNA counts.
4.4.4 Expression of microbial interaction genes

In general, microbial symbioses can be beneficial, neutral, or pathogenic [299]. Microbial interaction genes comprise several mechanisms by which microbes colonize, grow in, and occasionally cause detrimental effects to hosts and host tissues [300]. The expression of interaction genes in the population genomes suggest that these microbial planktonic communities have the potential of establishing symbiosis with other organisms. We classified interaction genes into distinct categories including biofilm formation, adhesion, secretion systems, and genes involved in pathogenesis among others. Numerous interaction genes identified in the population genomes belonged to antibiotic transport and production (Fig. 4.8). Antibiotic-producing bacteria are often reported in association with numerous marine organisms [301, 302, 303] and consequently, coral mucus-associated bacteria have been considered the first line of coral defense [243, 304]. Contrastingly, free-living bacteria are shown to be irregular antibiotic producers [305, 306]. Therefore, exhibiting this trait could represent an advantageous strategy to colonize and effectively outcompete native benthic bacteria in new habitats. Another important group of interaction genes, “viral processes”, included viral-host interactions (Fig. 4.8). The latter suggests that viral infections are playing major roles driving bacterioplankton community diversity also in this study, as has been shown repeatedly in different marine ecosystems [307, 308, 309].

A large number of interaction genes were assigned as virulence factors due to their high homology with previously reported virulence factors in other microbial species. We characterized the population genomes based on their metagenome abundance and the number of induced interaction genes and virulence factors into the following microbial lifestyles: 1) the larger the increase in abundance after sugar enrichment the more likely to have a copiotrophic lifestyle (Fig. 4.4A), 2) the larger the number of interaction genes, the more likely this population genome represents an opportunistic
symbiont (Fig. 4.4A and 4.4B), 3) the larger the number of virulence factors, the more likely this population genome represents an opportunistic pathogen (Fig. 4.4B). Based on these criteria, we chose the following six opportunistic pathogens with copiotrophic lifestyles and defined them as Potential Opportunistic Pathogens (POPs): MB_1 (Ruegeria), GM_189 (Alteromonas), GM_439 (Alteromonas), GM_554 (Alcanivorax), MB_4a (Erythrobacter) and GM_66 (Erythrobacter).

4.4.5 Virulence Potential in Opportunistic Pathogens

Much has been discussed regarding opportunistic pathogens in the field of coral diseases, but little is known in regard to the responsible microbes and their underlying mechanisms. The POPs we describe above represent candidate bacterial taxa, as they are found commonly associated with healthy organisms and ecosystems as well as disturbed ecosystems and diseased marine organisms. For instance, Erythrobacter species and members of the Rhodobacteracea family have been reported in association with healthy coral tissue [310, 311], but their participation in plague-like diseases in corals is strongly suggested [192, 312, 313]. Alteromonas species are commonly found in several marine pelagic and benthic ecosystems [314, 315, 316] and in association with various marine organisms, including numerous coral species [317, 318, 319, 320]. However, Alteromonas species have also been previously related to marine diseases in shrimps [321], oyster [322], corals [240], and fish-farm impacted coastal waters [48]. In this matter, we highlight the importance of not only relating the presence of certain bacterial genera and/or taxa with a disease, but rather looking at possible biotic and abiotic factors turning commensal bacteria into pathogens. In Fig. 4.5, we show genes previously linked with pathogenesis in each POP. We additionally present major antibiotic resistance mechanisms and environmental sensing mechanisms that can contribute to the regulation of such virulence factors.
Figure 4.4: Interaction and virulence factor genes in microbial population genomes. (A) Change in population abundance in the metagenome in relation to the differentially expressed bacterial interaction genes. Increasing numbers on the y-axis represent organisms with rapid growth under sugar enrichments, while increasing numbers on the x-axis denote bacterial species with a high number of expressed interaction genes. Population genomes that are more likely to initiate host interactions (i.e., bacteria that grow rapidly under sugar enrichment, expressing a high number of interaction genes) are highlighted in a gray area. (B) Black bars represent the total number of interaction genes (left) and virulence factors (right) present in each population genome. Colored bars represent the number of differentially expressed genes under either galactose (blue), glucose (magenta), mannose (yellow), or xylose (green) enrichments. Stars denote population genomes with the largest number of virulence factors and correspond to the selected population genomes from (A).
Figure 4.5: Expression of genes involved in pathogenesis in potential opportunistic pathogens. Each diagram represents a bacterial cell and each side of the cell membrane represents a group of cellular processes. Antibiotic resistance on the upper side of the membrane, environmental sensing on the right side of the membrane, bacterial secretion systems and adhesion on the lower part, and extracellular proteins and toxic compounds on the left part.
4.4.6 Expression of aggregation genes to promote virulence potential

A common feature among many pathogens is their ability to adhere to surfaces, colonize them and survive adverse conditions in the form of biofilms [323, 324]. As evidence of these processes, genes involved in adhesion such as the Type IV secretion system and the translocation and assembly module (TAM) were induced under sugar enrichment. Membrane proteins tamA/B, part of TAM, are involved in the assembly of surface structures essential to host-pathogen interactions including adhesion and host invasion [325], and deletion of these elements diminishes the virulence of several pathogens [326, 327, 328]. Furthermore, POPs strongly induced eps and ams genes, which code for exopolysaccharide (EPS) production and export proteins, important for biofilm formation under sugar enrichment. Biofilms not only constitute a protected lifestyle for bacteria and promote pathogenesis but also cause impacts from an ecological perspective in coral reef ecosystems. For instance, marine biofilms containing Alteromonas species can influence benthic community succession by inducing the settlement and metamorphosis of cnidarian larvae and algal zoospores [329, 330] while inhibiting the settlement of polychaetes [331]. Some pathogens overcome host microbiota defenses through the production of antibiotics. For instance, biofilm formation in Ruegeria is tightly linked to antibiotic production and motility [332].

4.4.7 Expression of genes related to iron uptake to promote virulence potential

Another group of virulence factors induced after sugar enrichment is related to iron uptake. Iron is limited in the ocean and at the same time an essential micronutrient. Iron transporters and siderophores (iron chelating compounds) are common strategies among bacteria to acquire iron. Extracellular siderophores are rarely pro-
duced by pelagic bacteria, but are more common on particle-attached and benthic marine microorganisms [333, 334]. Some pathogens can produce siderophores to sequestrate iron from the host or other living organisms and use it to support their own growth [335]. The most common genes related to iron uptake processes found in the POPs were the TonB-dependent transporters and the outer protein IrgA. TonB-dependent iron acquisition is one of the main mechanisms permitting bacterial growth in a wide range of iron-limited environments, including host colonization in several pathogens [336, 337, 338]. IrgA is the outer membrane receptor of vibriobactin, a siderophore produced by *Vibrio* species, and extracellularly transported by TonB [339, 340]. The induction of multiple TonB elements and siderophore receptors strongly suggests iron scavenging as an important mechanism used by POPs for successful growth. However little is known about marine siderophore substrates and mechanisms by which they contribute to virulence in marine pathogens.

### 4.4.8 Expression of toxins and proteases to promote virulence potential

The third group of sugar-induced virulence factors found in the POPs was “toxins and proteases”. An example of a non-proteinaceous endotoxin was lipopolysaccharide (LPS), evidenced in the population genomes GM.189 (*Alteromonas* sp.) and GM.554 (*Alcanivorax* sp.) by the induction of the LPS biosynthetic genes *galU, algG, algA*. LPS is involved in the modulation of marine symbiosis [341] but also has an important role in pathogenesis of several bacterial species, including *Vibrio* [342], by eliciting strong immune responses in host cells. Among induced extracellular toxins, membrane-disrupting and proteolytic toxins were prevalent (Table 4.5). Two extracellular zinc metalloproteases were induced by the POP GM.189 (*Alteromonas* sp.) and MB.1 (*Oceanicola* sp.). Similar extracellular zinc metalloproteases are widely distributed among several marine bacteria and largely in *Vibrio* species [343, 344, 345].
Only few mechanisms of these metalloproteases are well described, as in the case of the hemagglutinin produced by *V. cholerae* to degrade intestinal protective mucus in humans [346]. Additionally, we provide evidence for the induction of subtilisin-like serine proteases in GM−554 (*Alcanivorax* sp.), known to have antifouling activity via degradation of EPS biofilms [347]. We hypothesize that the expression of this protease is related to either functioning as a negative regulator of their own biofilm formation or as a competitive mechanism against other bacteria. Among membrane-disrupting toxins, we found the induction of genes encoding for homologs of the pore-forming cytotoxins TlyA and TlyC in the metagenome POPs GM−66 (*Erythrobacter*) and MB−1 (*Oceanicola* sp.). Homologs of these toxins from a wide range of bacteria have shown the ability to lyse erythrocytes, leukocytes, phagosomal vacuoles and gastric epithelium cells [348, 349]. Another group of membrane-disrupting toxins induced by sugar enrichment were the calcium-dependent pore-forming cytotoxins (RTX toxins). Three RTX homologs were expressed in the POP MB−1 and two elements involved in the RTX toxin translocation in the POPs GM−189 and GM−439 (*Alteromonas* sp.). RTX proteins are potent virulent factors with cytotoxic and hemolytic activities toward a broad range of animal cells [350], however, there is little evidence to date about the role of RTX toxins in marine diseases. Among marine bacterial species, RTX toxins have been found in several *Vibrio* species [351, 352], *Pseudoalteromonas agarivorans* [353] and in members of the *Roseobacter* clade including *Phaeobacter* [354] and *Ruegeria pomeroyi* [355]. These proteins are characterized by tandemly repeated nonapeptides with the consensus sequence GGXGDX[X/L/I/V/W/Y/F]X (where X is any amino acid) in the C-terminal half of the protein, as observed in RTX homologs expressed by MB−1 (*Oceanicola* sp.) (Fig. 4.9). Expression of toxin-related proteins is consistent with expression of genes of the type I to IV secretion systems, known to be involved in the transport of several virulence factors including effector proteins and toxins [356]. For instance, one main feature of all RTX toxins is their conserved
mechanisms of transport mediated by the type 1 secretion system [357, 358]. Elements of the type 1 secretion system were induced in POPs expressing RTX-related homologs, suggesting the export of these protein homologs during sugar incubations. The production of marine toxins from bacteria has gained considerable attention in the search for biologically active compounds for industrial and medical applications [359, 360, 361]. However, the ecological role of these toxins has been poorly studied in the field of marine diseases.

4.4.9 Energy-dependent regulation of virulence factor gene expression

Based on expression data from this study, we suggest two possible mechanisms of virulence factor regulation. First, the activation of global regulators linked to sugar catabolism and second by activation of environmental sensing responses. Catabolism-dependent regulation relies on the PTS-dependent modulation of intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP), via the activation of carbon catabolite repression (CCR) [362]. As a consequence, high glucose levels are accompanied by low intracellular cAMP levels. The concentration of cAMP in the cell is a key determinant for the expression of a large number of genes, including catabolism of alternative sources of carbon and virulence. Virulence factors can be expressed under low [363, 364, 365] and high [108] cAMP intracellular levels. One example of such a sugar-dependent gene regulation in marine pathogens is the natural competence in *Vibrio cholera* induced by chitin and repressed by any preferred PTS-dependent sugars [366]. Levels of *cyt* and *crp* genes encoding the adenylate cyclase and the cyclic AMP (cAMP) receptor protein (CRP), vary among the POPs with the different sugars (Table 4.6). Based on our results we speculate that GM_189 (Altoromonas sp.) and GM_554 (Alcanivorax sp.) possibly express virulence factors under low cAMP concentrations. We also found high induction of second messengers con-
nected to carbon catabolism like bis-(3'5')-cyclic dimeric guanosinmonophosphate (c-di-GMP). Diguanylate cyclase activity (required for c-di-GMP synthesis) was induced under galactose in POPs GM\_189 and MB\_4 and under mannose in POPs GM\_554 (Alcanivorax sp.), GM\_66 and MB\_1 (Oceanicola sp.). The switch between planktonic and attached lifestyles in several bacteria including Roseobacter clade members is balanced by this second messenger [332, 367, 368, 369]. This suggests sugar catabolism likely was an important regulator promoting settlement and virulence factor expression in POPs.

\subsection*{4.4.10 Environmental sensing-dependent regulation of virulence factor gene expression}

The second mechanism suggested to be involved in virulence factor regulation is the activation of environmental sensing responses. An early environmental cue detected by bacteria after sugar enrichment may involve changes in the surrounding osmotic pressure. We found the prevalent induction of the two component system for osmotic upshift sensing, EnvZOmpR, in the POPs. Under high solute concentrations, EnvZOmpR trigger water efflux and compatible solute accumulation [370, 371], but also act as global regulator involved in the transcription of several proteins involved in pathogenesis, such as bacterial secretion system type III in Salmonella typhimurium [372]. Osmoregulatory mechanisms are also linked with the activation of bacterial motility and chemotaxis in a way that cells can be repelled under low osmolarity, attracted under optimal osmolarity and repelled under high osmolarity causing tumbling motility [373, 374]. In the same way, induction of chemotaxis was evidenced by the expression of several genes encoding for proteins involved in chemotaxis in all POPs (Table 4.6). It remains to be determined to what extent the pathways, identified here, are upregulated at sugar concentrations that are typically found in coral reefs exposed to wastewater outfalls [375].
Another environmental factor commonly determining the regulation of virulence is low dissolved oxygen concentrations. Low dissolved oxygen concentrations can promote coral reef degradation by the increase of toxic substances, such as sulfide, a product of bacterial sulfate reduction and desulfuration in black band disease in corals [376]. We found an induction of genes involved in nitrate and sulfate reduction in the POPs (Table 4.6). These processes are prevalent in benthic and particle-attached bacteria, but not common in bacterioplankton communities. However, the increase in labile organic carbon results in increased bacterial respiration, creating microaerophilic environments suited for chemoheterothrophic activity, as previously reported on impacted reefs [21, 377]. Furthermore, we provided evidence for the induction of the sensor kinase MprB, a hypoxia-responsive regulator, linked to virulence expression in *Mycobacterium tuberculosis* [378]. MprAB directly participates in the expression of sigma factors including extracytoplasmic function (ECF) sigma factors SigE and SigB that in turn regulate the expression of numerous stress-responsive genes [378, 379].

We additionally found the induction of a few other regulators previously linked with virulence, such as PhoP-PhoQ, stimulated by Mg$^{2+}$/Ca$^{2+}$ [380], the RegX3-SenX3 with unknown environmental stimulus [381], and elements of quorum sensing (Table 4.6) that might be involved in the expression of VF.

### 4.5 Conclusions

Anthropogenic pollution and algal cover have continuously increased in coral reefs worldwide, leading to an increase of dissolved organic carbon (DOC) concentrations. These high DOC concentrations have been shown to influence microbial diversity and activity and are hypothesized to switch microbial lifestyles from commensal to pathogenic. The underlying mechanisms are however largely unknown. By assaying
bacterial community composition and gene expression under experimental enrichment of sugars abundant in algal exudates, we propose opportunistic bacteria in the families Alteromonadaceae, Vibrionaceae, and Rhodobacteracea to be candidate taxa for this switch, based on their copiotrophic behavior and their potential abilities to become harmful to other marine organisms under high sugar concentrations. These opportunistic pathogens displayed a shift in their metabolic capabilities and expression of a wide set of virulence factors (VF) including metalloproteases, siderophores, and toxins as well as numerous mechanisms of antibiotic resistance. For the first time, this study shows DOC-dependent expression of VFs and shifts in metabolic capacities of coral reef microbial populations within the context of reef-health. These results corroborate the “Disease, Dissolved organic carbon, Algae and Microbe” (DDAM) hypothesis, providing new insights to the mechanistic link between elevated DOC concentrations and the switch from free-living to attached stages, as well as from commensal to pathogen.
4.6 Supplementary information

![Experimental setup schematic](image)

Figure 4.6: Schematic representation of the experimental setup: coral reef water amended with monosaccharides.

Table 4.3: Gene ontology (GO) terms used for functional annotation of microbial population genomes. Functional annotation included all child terms of the parental terms indicated in the table.

<table>
<thead>
<tr>
<th>GO term</th>
<th>Name</th>
<th>Used to infer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0004016</td>
<td>Adenylate cyclase activity</td>
<td>cAMP synthesis</td>
</tr>
<tr>
<td>GO:0030552</td>
<td>cAMP binding</td>
<td>cAMP receptor protein (CRP)</td>
</tr>
<tr>
<td>GO:0052621</td>
<td>Diguanylate cyclase activity</td>
<td>Diguanylate cyclase synthesis</td>
</tr>
<tr>
<td>GO:0019419</td>
<td>Sulfate reduction</td>
<td>Sulfate reduction</td>
</tr>
<tr>
<td>GO:0008940</td>
<td>Nitrate reductase activity</td>
<td>Nitrate reduction</td>
</tr>
<tr>
<td>GO:0009372</td>
<td>Quorum sensing</td>
<td>Quorum Sensing</td>
</tr>
<tr>
<td>GO:0044403</td>
<td>Symbiosis, encompassing mutualism through parasitism</td>
<td>Bacterial interaction genes</td>
</tr>
<tr>
<td>GO:0006935</td>
<td>Chemotaxis</td>
<td>Chemotaxis</td>
</tr>
<tr>
<td>GO:0009405</td>
<td>Pathogenesis</td>
<td>Virulence factors</td>
</tr>
</tbody>
</table>
Figure 4.7: Gene expression patterns in microbial population genomes. Black bars denote bin abundance in the metagenome and dots represent log2 fold change comparing control and treatment of normalized mRNA counts. Log2 fold changes above 2 are colored in red and below $-2$ are colored in blue. Log2 fold change values between $-2$ and 2 are grey. Active populations are evidenced by a higher numbers of red dots.
Figure 4.8: Expression of bacterial interaction genes in selected microbial population genomes. Interaction genes were clustered at the biological process (BP) level according to gene ontology (GO) annotation. Size denotes the number of genes in each group of interaction genes. Expression is represented in log2 fold change after comparing control and experimental treatments of normalized mRNA counts. Shapes correspond to different sugars in experiment 1 (A) and 2 (B).
Figure 4.9: Calcium-binding motifs of RTX homologs found in the population genome MB_1 (*Oceanicola* sp.).
**Table 4.4: Taxonomic assignment of microbial population genomes using average nucleic acid identities (ANI), average amino acid identities (AAI), SpecI, PhyloPythiaS, and CheckM marker lineages.**

<table>
<thead>
<tr>
<th>Population genome</th>
<th>SpecI</th>
<th>CheckM marker lineage</th>
<th>PhyloPythiaS (assignments %)</th>
<th>Closest related species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GM 331</strong></td>
<td><em>Hyphomonas neptunium</em> (79.73)</td>
<td>c. <em>Alphaproteobacteria</em> (UID3422)</td>
<td></td>
<td><em>Hyphomonas neptunium</em> (71.58</td>
</tr>
<tr>
<td><strong>GM 189</strong></td>
<td><em>Alteromonas</em> sp. SN2 (77.11)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alteromonas mackoidii</em> (68.37</td>
</tr>
<tr>
<td><strong>GM 23</strong></td>
<td><em>Roseobacter</em> sp. SK209-2-6 (79.8)</td>
<td>f. <em>Rhodobacteraceae</em> (UID3360)</td>
<td></td>
<td><em>Rosoavarius nubinhibens</em> (69.36</td>
</tr>
<tr>
<td><strong>MB 1</strong></td>
<td><em>Oceancola butaniensis</em> (85)</td>
<td>f. <em>Rhodobacteraceae</em> (UID3361)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>MB 3</strong></td>
<td>Unassigned (n/a)</td>
<td>k. <em>Bacteria</em> (UID2569)</td>
<td>Actinobacteria (40.45)</td>
<td><em>Robignitales biformata</em> (65.39</td>
</tr>
<tr>
<td><strong>GM 439</strong></td>
<td><em>Alteromonas</em> sp. SN2 (76.46)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alteromonas mackoidii</em> (69.31</td>
</tr>
<tr>
<td><strong>MB 4</strong></td>
<td><em>Erythrobacter</em> sp. SD-21 (85.09)</td>
<td>o. <em>Sphingomonadales</em> (UID3310)</td>
<td></td>
<td><em>Erythrobacter citreus</em> (82.44</td>
</tr>
<tr>
<td><strong>GM 23</strong></td>
<td>Unassigned (n/a)</td>
<td>c. <em>Alphaproteobacteria</em> (UID3305)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 554</strong></td>
<td><em>Alcanivorax</em> sp. DG881 (88.07)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>MB 4a</strong></td>
<td><em>Oceancola batsensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 14</strong></td>
<td>Unassigned (n/a)</td>
<td>k. <em>Bacteria</em> (UID1453)</td>
<td>Actinobacteria (84.10)</td>
<td><em>Gordonia paraflavum</em> (65.58</td>
</tr>
<tr>
<td><strong>GM 66</strong></td>
<td><em>Erythrobacter</em> sp. SD-21 (86.56)</td>
<td>o. <em>Sphingomonadales</em> (UID3310)</td>
<td></td>
<td><em>Erythrobacter citreus</em> (83.32</td>
</tr>
<tr>
<td><strong>GM 50</strong></td>
<td><em>Pseudomonas marinomarinus</em> (74.57)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alcanivorax jadensis</em> (83.53</td>
</tr>
<tr>
<td><strong>GM 20</strong></td>
<td><em>Oceancola butaniensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
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<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 88</strong></td>
<td>Unassigned (n/a)</td>
<td>p. <em>Euryarchaeota</em> (UID3)</td>
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<td>35.27)</td>
</tr>
<tr>
<td><strong>MB 2</strong></td>
<td><em>Alcanivorax</em> sp. DG881 (88.07)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>MB 4a</strong></td>
<td><em>Oceancola batsensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 554</strong></td>
<td>Unassigned (n/a)</td>
<td>k. <em>Bacteria</em> (UID2569)</td>
<td>Actinobacteria (84.10)</td>
<td><em>Gordonia paraflavum</em> (65.58</td>
</tr>
<tr>
<td><strong>MB 4a</strong></td>
<td><em>Oceancola batsensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 50</strong></td>
<td><em>Pseudomonas marinomarinus</em> (74.57)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alcanivorax jadensis</em> (83.53</td>
</tr>
<tr>
<td><strong>MB 2</strong></td>
<td>Unassigned (n/a)</td>
<td>p. <em>Euryarchaeota</em> (UID3)</td>
<td>Thermophiles actinomycetes (61.78</td>
<td>35.27)</td>
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<tr>
<td><strong>MB 4a</strong></td>
<td><em>Oceancola batsensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
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<td><strong>GM 50</strong></td>
<td><em>Pseudomonas marinomarinus</em> (74.57)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alcanivorax jadensis</em> (83.53</td>
</tr>
<tr>
<td><strong>MB 2</strong></td>
<td>Unassigned (n/a)</td>
<td>p. <em>Euryarchaeota</em> (UID3)</td>
<td>Thermophiles actinomycetes (61.78</td>
<td>35.27)</td>
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<tr>
<td><strong>MB 4a</strong></td>
<td><em>Oceancola batsensis</em> (85)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4444)</td>
<td></td>
<td><em>Oceancola butaniensis</em> (77.80</td>
</tr>
<tr>
<td><strong>GM 50</strong></td>
<td><em>Pseudomonas marinomarinus</em> (74.57)</td>
<td>c. <em>Gammaproteobacteria</em> (UID4761)</td>
<td></td>
<td><em>Alcanivorax jadensis</em> (83.53</td>
</tr>
<tr>
<td><strong>MB 2</strong></td>
<td>Unassigned (n/a)</td>
<td>p. <em>Euryarchaeota</em> (UID3)</td>
<td>Thermophiles actinomycetes (61.78</td>
<td>35.27)</td>
</tr>
</tbody>
</table>
Table 4.5: Extracellular proteases and toxins expressed in potential opportunistic pathogens (POPs).

<table>
<thead>
<tr>
<th>Virulence</th>
<th>Gene name</th>
<th>Uniprot</th>
<th>Contig ID</th>
<th>% of identity</th>
<th>length</th>
<th>e-value</th>
<th>bit score</th>
<th>Log2 fold change Glucose</th>
<th>Log2 fold change Galactose</th>
<th>Log2 fold change Mannose</th>
<th>Log2 fold change Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTX toxin translocase</td>
<td>hlyB</td>
<td>P15492</td>
<td>GM_118_Lpeg_1875</td>
<td>41.11</td>
<td>443</td>
<td>8.00E-69</td>
<td>2.37</td>
<td>3.97</td>
<td>9.53</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>RTX toxin translocase</td>
<td>hlyB</td>
<td>P15492</td>
<td>GM_239_Lpeg_3127</td>
<td>41.59</td>
<td>315</td>
<td>4.00E-57</td>
<td>206</td>
<td>1.81</td>
<td>8.95</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Cytotoxin</td>
<td>tlyC</td>
<td>O05961</td>
<td>GM_26_Lpeg_296</td>
<td>40</td>
<td>170</td>
<td>3.00E-32</td>
<td>123</td>
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Chapter 5
Chapter 5

Discussion

5.1 Outline

DOC is one of the abiotic factors related to the promotion of opportunistic pathogens, largely due to its role in fueling the microbial loop and boosting heterotrophic microbial activity. This thesis assessed the effects of DOC concentrations on microbial diversity and activity in coral reef surrounding water. To achieve this, two manipulative experiments exhibiting elevated sugar concentrations were framed. Using a wide variety of methods ranging from the measurement of organic carbon pools to the use of “omics” techniques, the data produced in this work supports the negative impacts DOC enrichment has on coral reefs. Furthermore, the framework of this thesis is addressed from various perspectives such as organic carbon transformation, carbon metabolism, microbial diversity and the DOC-mediated switch between commensals to pathogens lifestyles in planktonic microbes.
5.2 Who are the unseen players fueling the positive coral degradation feedback on coral reefs?

In congruency with the results of this thesis, previous ex-situ and in-situ manipulative experiments, and field surveys have shown that increasing algal cover in coral reefs has led to the promotion of the families *Alteromonadaceae* [382], *Enterobacteraceae* [52] and *Rhodobacteraceae* [61]. In this work the addition of glucose caused increases of *Erythrobacteraceae* in algal- and coral-dominated mesocosms. However, members of the *Erythrobacteraceae* family have been related to exposition to coral exudates [61]. In the same manner, *Vibrionaceae*, *Alteromonadaceae* and *Rhodobacteraceae* rapidly respond to allochthonous glucose inputs as well, as is shown in sewage and fish farm enrichment gradients [48, 383, 384].

In spite of these groups showing consistent responses to DOC enrichments, bacterial taxa indicators of low-quality water are difficult to establish due to the variable DOC composition among the different benthic primary producers (turf, fleshy, calcifying and cyanobacterial mats) and anthropogenic-derived pollutants. Assessing the individual bacterial response to the main sugars found in algal exudates was therefore planned to overcome this limitation and elucidate the contribution of each sugar to the bacterial community responses observed. The results presented in chapter 4 showed sugar-dependent shifts in bacterial communities evidenced by increases in *Rhodobacteraceae* (Alphaproteobacteria) in galactose, mannose and xylose, *Vibrionaceae* (Gammaproteobacteria) in glucose, and *Alteromonadaceae* (Gammaproteobacteria) in both glucose and mannose enriched samples. Despite the fact that every bacterial assembly responds in a different manner which depends on taxonomical composition, correlating specific bacterial groups with DOC main components is a promising starting point to understand and predict the response of bacterial communities under DOC enrichment.
5.3 Bacterial metabolic shifts and their ecological implications

The success of copiotrophic bacteria in nutrient-rich environments can be partially explained by their inefficient growth [385]. The results of this thesis showed that copiotrophic populations (e.g. *Alcanivorax*, *Alteromonas*, and *Erythrobacter*) experienced trade-offs between the rate and yield of adenosine triphosphate (ATP) production (i.e. metabolic shift). These metabolic shifts correspond to the alternation of glycolytic pathways from the low but more energy efficient Embden–Meyerhof–Parnas (EMP) pathway to the fast but less efficient Entner–Doudoroff (ED) and pentose phosphate (PP) pathways. Similar patterns were previously observed at larger scales, comparing metagenomes from algal- versus coral dominated reefs [52]. When organisms compete for shared resources, fast and inefficient ATP production emerges as a selective advantage to out-compete low and efficient resource users [298, 386, 387]. One way to measure growth efficiency is by the amount of carbon incorporated into biomass per carbon resource consumed. Therefore, inefficient growth will result in higher bacterial biomass but lower energy entering higher trophic levels [388]. This reallocation of ecosystem trophic structure towards higher microbial biomass and energy use is referred to as microbialization [17]. Consequently, the microbialization score is a metric to assess the level of human impact on a reef system [389].

5.4 Excess of labile carbon mediates virulence factor expression

A larger number of virulence factors in metagenomes of algal- versus coral-dominated reefs suggest the increasing benthic algal cover as a factor to facilitate pathogen pro-
liferation in coral reef waters [52, 61, 382, 390]. The results of this thesis (chapter 4) contribute to the often proposed, but rarely tested, assumption that opportunistic pathogens benefit from DOC inputs on coral reefs waters. The results presented in this thesis suggest that DOC enrichment not only selects for opportunistic pathogens but also mediates the expression of virulence factors through direct (e.g. carbon catabolite repression pathway) and indirect (e.g. quorum sensing, osmotic stress, and hypoxia) mechanisms. Hence, excess labile DOC concentrations can be considered driving factors promoting the switch from commensal to pathogen (i.e. pathogenic switch) in some microbial planktonic populations. By assessing gene expression in individual microbial populations it was possible to pinpoint bacterial taxa involved in such pathogenic switches. Chapter 4 shows the activation of virulence factors in copiotrophic populations of the genera *Alcanivorax*, *Alteromonas*, *Erythrobacter* and *Oceanicola* under sugar enrichment.

The results shown in chapter 4, suggest two main strategies used by opportunistic pathogens to colonize and effectively outcompete native benthic bacteria on coral surfaces (Fig. 5.1). Both strategies involve the ability to move towards these sugar-rich exudates, evidenced by the upregulation of proteins involved in chemotaxis and flagellar systems in all opportunistic pathogens. The first strategy, possibly mediated by *Alteromonas* and *Alcanivorax* populations, proposes the settlement on coral surfaces, evidenced the activation of a large number of genes involved in adhesion and biofilm formation. The competition on the mucus is maintained by the large number of genes involved in antibiotic resistance. Negative interactions are mediated by iron chelation and coral immune response to lipopolysaccharide, as previously shown [391]. Iron transfer in anthozoans (Cnidaria) is done through vesicles containing iron-bound transferrins [392]. However, chelator-facilitated removal of iron from transferrin has not been addressed in coral-associated microbes. The second strategy, possibly mediated by *Oceanicola* and *Erythrobacter*, involves the production of
extracellular proteins with cytotoxic activity. The accumulation of such toxins in the diffusion-limited environments (e.g. diffusive boundary layer) in direct contact with benthic organisms may promote physical damage to corals or their symbiotic algae.

5.5 Two sides of the same coin

The need of untangling the numberless interactions between microbes and corals from the coral disease perspective, has resulted in two possible explanations. The first explanation involves putative pathogens and the second one opportunistic infections. Examples of proposed causative agents include *Thalassomonas loyana* [393] and *Aurantimonas coralicida* [394] for white plague-like diseases, *Vibrio carchariae* for white band disease [395], *Vibrio harveyi* [396] and *Vibrio coralliilyticus* [397] for white syndrome, *Serratia marcescens* for acroporid serratiosis [198], and the sulfide-oxidizing consortia for the black band disease [193], among many others. Assessing coral diseases is a challenging task and part of the success has much to do with an accurate diagnostic of the disease and the culturability of the etiological agent. Those limitations have largely resulted in the inability to fulfill Koch’s postulates or the establishment of multiple pathogens for one coral disease [398].

On the other hand, opportunistic infections are believed to be triggered by the exposure to environmental stressors (e.g., elevated temperature or nutrient enrichment) that can reduce host resistance and promote opportunistic microbes [115, 242]. This type of infections often involve imbalances in the coral microbiota (i.e. dysbiosis) [192, 399, 400]. However, the non-trivial question is whether this dysbiosis is the cause or consequence of disease. In spite of these restraints, which clearly need further examination, our results suggest that elevated DOC is not promoting primary infections caused by pathogen transmission, but rather promoting opportunistic infections, which are likely to be feeding the DDAM positive feedback. Opportunistic
Figure 5.1: Proposed strategies used by opportunistic pathogens. Sugar enrichment causes changes in bacterial communities favoring copiotrophic bacteria. These copiotrophs move towards sugar-rich exudates produced by algae. The first strategy involves the settlement on coral surfaces and the production of extracellular siderophores. Siderophores are proposed to chelate iron from soluble transferrins that carry Fe$^{3+}$, therefore depleting available iron to corals. Lipopolysaccharide (LPS) can activate coral immune response. The second strategy involves bacterial groups capable of producing diverse groups of proteins with cytotoxic activity. These toxins accumulate in the diffusive boundary layer and promote physical damage to corals or their symbiotic algae.
infections are represented in this context by mechanisms such as iron chelation, hypoxia and the accumulation of toxic substances that can contribute to coral stress and vulnerability. The occurrence of these opportunistic infections is expected to happen in impacted reefs with high microbialization scores. Furthermore, it does not deny the occurrence of putative and polymicrobial infectious in coral diseases.

### 5.6 The other face of DOC enrichment on coral reefs

Aggregate formation processes have been widely documented during phytoplankton blooms in temperate waters [401, 402], whereas these processes in coral reef ecosystems have been almost neglected. The results of this thesis show that coral and algal exudates either directly constitute a source of TEP or represent a source of precursors that are abiotically transformed into TEP. This suggests that the benthic community potentially impacts aggregate formation processes and therefore, has an influence in the carbon and energy budgets in coral reefs [81, 403] and sedimentation fluxes [84].

The results of this thesis show that elevated DOC concentrations promoted aggregate formation in coral reef mesocosms, presumably by a glucose-mediated TEP formation. Moreover, to have an approximation of global stressors interacting with high DOC concentrations in coastal coral reefs, the effect of ocean acidification was evaluated (measured as high DIC). The results presented in chapter 2 suggest a synergistic interaction between high DOC and high DIC concentrations, evidenced as five-time larger total aggregated volume than in the individual treatments. The coupling of excess carbon from sugar enrichment with the lowering of seawater pH by 0.2 units or more, can significantly increase TEP buoyancy [131], resulting in longer residence time in the water column that may further enhance aggregate formation. Floating rather than sinking aggregates will result in the retention of energy sources
coming from these aggregates to benthic feeders as well as hampering of light penetration [20].

5.7 Perspectives

The assessment of organic carbon transformations in the water column gave some interesting insights on this processes in coral reef waters. For future studies, one important aspect to consider is the contribution of the different carbon species found in DOC (e.g. benthic exudates) to the formation of TEP and consequently to the formation of particle aggregates. The results of this thesis show significant increases in total aggregated volume when combined simultaneously elevated DOC and DIC. In the same way, temperature and inorganic nutrient enrichment, common stressors in coastal areas, should be considered to have a better approximation of the mass transfer between organic carbon pools in coral reef systems. Furthermore, particle aggregates are known to be hot-spots of bacterial activity and are often considered vectors for transferring bacterial pathogens. Assessing the role of particle aggregates in transferring opportunistic pathogens from the water column to benthic organisms can enlighten some links into marine disease ecology and transmission.

The findings of this thesis also propose interesting bacterial-coral interactions that could potentially be feeding the positive loop of coral reef degradation. The existence of these opportunistic interactions encourages new efforts to move from the characterization of single pathogens towards a more holistic understanding of the interaction of the host with its microbiome and transient bacteria from the water column. Moreover, future studies should incorporate the mechanisms by which environmental factors are modulating these interactions and their consequences to coral health. The combination of metagenome binning and metatranscriptomics represents a powerful approach that can be extended to facilitate the characterization of uncultivable
etiological agents in coral diseases and investigate possible mechanisms involved in the disease development. Finally, the use of model host (e.g. *Aiptasia*) to investigate microbe-coral interactions, should overcome several of the limitations regarding metadata availability, as well as their ability to grow in aquaria and be experimentally manipulated.

5.8 Conclusions

This thesis has provided a brief overview of what is known about the impacts of DOC enrichment in coral reefs, as well as three studies characterizing organic carbon transformations, microbial diversity and activity of planktonic microbial communities under elevated sugar concentrations. Collectively, these three studies provide compelling evidence that high DOC concentrations impact negatively on coral reefs by promoting aggregate formation and microbial activity. The findings of this thesis further support the DDAM model and contribute to it by providing a mechanistic explanation whereby elevated sugar concentrations mediates the expression of virulence factors. The expression of virulence factors propose some mechanisms (e.g. invasion, iron chelation, accumulation of toxic substances), suggested to play an important role in feeding the positive feedback of coral reef degradation. Finally, this thesis provides a first assessment to gain a broader understanding of marine bacterioplankton life strategies. However, further functional analyses of bacterial communities associated with coral reef ecosystems are needed to corroborate the role of the suggested mechanisms in coral-bacteria interactions.
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134


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