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Research Article

RESEARCH ARTICLE

Bacterial community composition and predicted functional ecology of sponges, sediment and seawater from the thousand islands reef complex, West Java, Indonesia

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One sentence summary: The composition of Bacteria in four biotopes namely sediment, seawater and two sponge species were investigated in a coral reef ecosystem in West Java, Indonesia.

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ABSTRACT

In the present study, we assessed the composition of Bacteria in four biotopes namely sediment, seawater and two sponge species (*Stylissa massa* and *Xestospongia testudinaria*) at four different reef sites in a coral reef ecosystem in West Java, Indonesia. In addition to this, we used a predictive metagenomic approach to estimate to what extent nitrogen metabolic pathways differed among bacterial communities from different biotopes. We observed marked differences in bacterial composition of the most abundant bacterial phyla, classes and orders among sponge species, water and sediment. Proteobacteria were by far the most abundant phylum in terms of both sequences and Operational Taxonomic Units (OTUs). Predicted counts for genes associated with the nitrogen metabolism suggested that several genes involved in the nitrogen cycle were enriched in sponge samples, including *nosZ, nifD, nirK, norB and nrfA genes*. Our data show that a combined barcoded pyrosequencing and predictive metagenomic approach can provide novel insights into the potential ecological functions of the microbial communities. Not only is this approach useful for our understanding of the vast microbial diversity found in sponges but also to understand the potential response of microbial communities to environmental change.

Keywords: 16sRNA; coral reef; Jakarta; pyrosequencing; *Stylissa*; *Xestospongia*

INTRODUCTION

Sponges are abundant and conspicuous components of coral reef ecosystems. They play a key role in substrate and water column nutrient dynamics where their pumping capacity contributes to coupling productivity between the benthos and overlying water column (Southwell, Popp and Martens 2008; Fiore, Baker and Lesser 2013). Sponges host a diverse and

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abundant array of microbes including both Archaea and Bacteria and are sources of a wide range of bioactive compounds with significant pharmacological potential. The evolutionary and ecological success of sponges may in part be related to their intimate relationship with these microbial communities. (Sipkema *et al.* 2005). The chemical and microbial diversity and abundance found in sponges has led to them being model organisms for host–microbe interactions (Thacker and Freeman 2012; Hentschel *et al.* 2012). Up to 60% of the tissue volume of certain sponge species consists of microbes with a density exceeding 10^9 microbial cells per ml of sponge tissue, orders of magnitude greater than that found in seawater or sediment (Webster and Hill 2001). On the other hand, some sponge species harbor microbial concentrations similar to that found in seawater (Hentschel *et al.* 2012). Thus, major differences in microbial abundances among sponges exist, and this distinction has led to the terms HMA (high microbial abundance) versus LMA (low microbial abundance) sponges (Hentschel *et al.* 2003). In addition to differences in abundance, HMA sponges tend to have higher microbial diversity than LMA sponges, and the sponge metabolism seems to only be influenced by microorganisms in HMA sponges (Ribes *et al.* 2012). Consequently, many microbial studies have focused on HMA sponges. At present, very few bacteria found in sponges are culturable, but recent advances, such as pyrosequencing, now enable us to assess bacterial communities at an unprecedented level of detail. Up until now, 28 bacterial and 2 archaeal phyla have been recorded in sponge hosts (Hentschel *et al.* 2012). The exact roles of these microbes in sponges remain, however, largely unknown. It is generally accepted, that Bacteria play key roles in their host's health. Identifying the role and composition of Bacteria and other microbes in different biotopes is thus essential in order to gain a better understanding of the coral reef ecosystems and the role of Bacteria therein. Recently, it was suggested that sponge–microorganism symbioses are sensitive indicators of global environmental change (Hentschel *et al.* 2012). For example, the archaeal community associated with the pollutant-tolerant sponge species *Hymeniacidon heliophila*, *Paraleucilla magna* and *Petromica citrina* changed over a scale of a few kilometers, probably influenced by different eutrophication levels in the study region (Turque *et al.* 2010). Recently, it was discovered that nitrogen fixation in the marine environment has largely been underestimated and levels are very similar to terrestrial environments or even higher (Fiore *et al.* 2010). Nitrogen fixation not only occurs in free-living prokaryotes in the open sea and sediment, but also in marine microorganisms associated with invertebrate hosts. All nitrogen biogeochemical pathways have been reported in sponges including nitrogen fixation, nitrification, denitrification and anammox (Southwell, Popp and Martens 2008; Liu *et al.* 2012; Fiore, Baker and Lesser 2013). In the Florida Keys, Southwell, Popp and Martens (2008) suggested, given the magnitude of sponge nitrification rates, that the majority of benthic nitrification probably occurs in sponges. The composition and abundance of sponges and importantly their microbes can thus potentially exert a strong influence on the amount of dissolved inorganic nitrogen (DIN) in the water column with concomitant indirect effects on other marine benthic organisms. Thus, the release of excess DIN by sponges may adversely affect coral reef ecosystems by, among other things, promoting algal growth to the detriment of corals (Gonzalez-Rivero, Yakob and Mumby 2011; Fiore, Baker and Lesser 2013). However, nitrogen cycling is a very complex process and all the factors that govern the nutrient fluxes are not well understood. With the emergence of metagenomic methods, we can now predict and unravel the functional roles of microbial communities (Langille *et al.* 2013). Metagenomic data sets have provided novel insights into functions expressed by microorganisms in diverse microbial systems including the human microbiome, complex systems in waste water sludge and the open ocean (Liu *et al.* 2012).

In the present study, we assessed the composition of Bacteria in four biotopes, namely sediment, seawater and two sponge species at four different reef sites in a coral reef ecosystem in West Java, Indonesia. In addition to this, we used a predictive metagenomic analysis to estimate counts for genes involved in the nitrogen metabolism for bacterial communities in different biotopes.

MATERIAL AND METHODS

Sample collection and study area

The Jakarta Bay and Kepulauan Seribu coral reef system, also known as Thousand Islands (hereafter referred to as JBTI) consists of 105 islands or cay-crowned reefs, located to the northwest of Jakarta in the Java Sea (Cleary, Suharsono and Hoeksema 2006; Cleary *et al.* 2014). JBTI is subjected to strong precipitation rates and strong westerlies during the Northwest monsoon (wet season, from December to May) and strong easterlies during the Southeast monsoon (dry season, from June to November). The Jakarta conurbation has at present >12 million inhabitants and several rivers discharge storm water and sewage into the central sector of the bay (Rees *et al.* 1999).

Four sites were surveyed using SCUBA between 26 July and 10 August 2011 (Fig. 1) in three coastal zones, namely inshore (Bokor), midshore (Tidung Kecil) and offshore (Kelapa and Belanda). The inshore site is located about 20 km from the port of Jakarta and has poor water quality with high nutrient concentrations (Cleary *et al.* 2014). Severe contamination has been reported in the sediment and water of Jakarta Bay including heavy metals and pesticides (Williams, Rees and Setiapermana 2000). Bokor is the closest site to the city of Jakarta (20 km) and the furthest site (Belanda) is approximately 40 km from Bokor. Samples of sediment, seawater and tissue from the sponges *S. massa* and *X. testudinaria* were taken from each of the four sites. The two sponges studied are common reef sponges in the Indonesian archipelago although they inhabit different habitats.

Stylissa massa (LMA sponge) is a medium-sized orange-colored sponge that only occurs in very shallow water (0.5–3 m), whereas the giant barrel sponge *X. testudinaria* (HMA sponge) is a large sponge that grows mainly in deeper waters (3–50 m). Sediment samples were taken using mini cores; this consisted of sampling the top 5 cm of sediment with a plastic disposable syringe from which the end had been cut in order to facilitate sampling (Capone *et al.* 1992). Seawater samples were collected by filtering 1 L of seawater through a Millipore White Isopore Membrane Filter (GTTP04700, 47 mm diameter, 0.22 μm pore size). All samples were kept in absolute alcohol and in a cool box. After landing, tubes containing the samples were stored in a refrigerator at temperatures of about 0°C. In Portugal, the samples were stored at −20°C.

Total community-DNA extraction and 16S rRNA gene barcoded pyrosequencing

We isolated PCR-ready total community DNA from sediment, seawater and sponge samples using the FastDNA SPIN Kit (MP Biomedicals) following the manufacturer's instructions. Briefly, we prepared sediment samples by centrifuging each one for 30 min at 4400 rpm and 4°C; the membrane filter (seawater

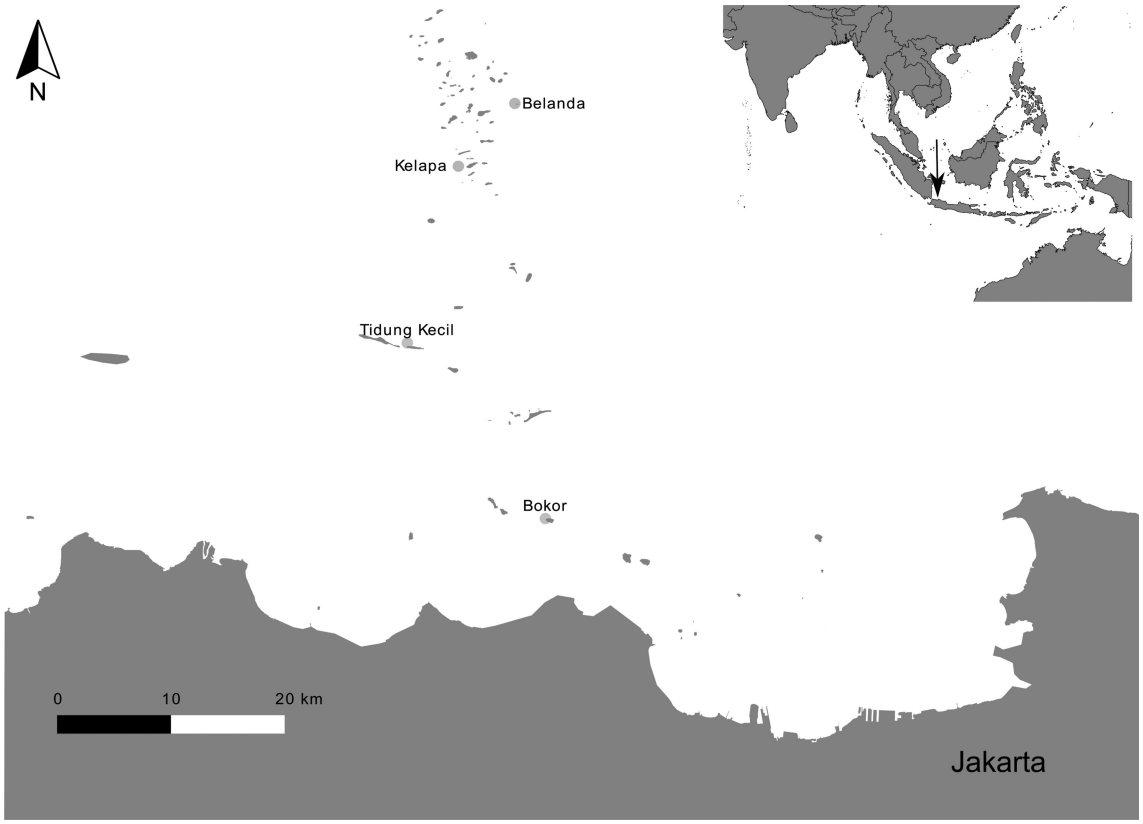

Figure 1. Map of the Jakarta Bay—Pulau Seribu reef system showing the location of the sample sites. The inset in the upper-right corner shows the location of the Jakarta Bay—Pulau Seribu reef system in southeast Asia.

sample) and sponge samples were each cut into small pieces. The whole membrane filter and 500 mg of sediment and sponge were transferred to Lysing Matrix E tubes containing a mixture of ceramic and silica particles. The microbial cell lysis was performed in the FastPrep Instrument (Q Biogene) for 80 s at the speed of 6.0 rpm. Extracted DNA was eluted into DNase/Pyrogen-Free Water to a final volume of 50 μl and stored at -20°C until use.

Prior to pyrosequencing, the amplicons of the bacterial 16S rRNA gene were obtained using bacterial specific primers 27F and 1494R (Gomes *et al.* 2001). After a denaturation step at 94°C for 5 min, 25 thermal cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 68°C were carried out followed by an extension step at 68°C for 10 min. Using the amplicons of the bacterial 16S rRNA gene as template, the V3V4 region was amplified, using barcoded fusion primers with the Roche-454 A Titanium sequencing adapters, a six-base barcode sequence, forward V3 primer 5′-ACTCCTACGGGAGGCAG-3′ (Yu *et al.* 2005) and V4 reverse degenerate primer 5′-TACNVRRGTHTCTAATYC-3′ (Vaz-Moreira *et al.* 2011).

Pyrosequencing and sequence analyses were performed using previously described methods (Pires *et al.* 2012; Cleary *et al.* 2013). Briefly, in QIIME, fasta and qual files were used as input for the split_libraries.py script. OTUs were selected using UPARSE with usearch7 (Edgar 2013). Chimera checking was performed using the UCHIME algorithm, which is the fastest and most sensitive chimera checking algorithm currently available (Edgar *et al.* 2011). OTU clustering was performed using the -cluster_otus command (cut-off threshold at 97%) (see Online Resource 1 for a detailed description). Closely related organisms of numerically abundant OTUs (≥100 sequences) were identified using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'blastn' tool with the -db argument set to nt (Zhang *et al.* 2000).

Higher taxon abundance

We tested for significant differences in the relative abundance of selected higher taxon groups (classes and orders) among biotopes with an analysis of deviance using the glm() function in R. Because the data were proportional, we first applied a glm with the family argument set to binomial. The ratio, however, of residual deviance to residual d.f. in the models substantially exceeded 1 so we set family to 'quasibinomial'. In the 'quasibinomial' family, the dispersion parameter is not fixed at 1 so that it can model overdispersion. Using the glm model, we tested for significant variation among biotopes using the anova() function in R with the F test, which is most appropriate when dispersion is estimated by moments as is the case with quasibinomial fits. Detailed descriptions of the functions used here can be found in R (when using R, typing ?glm in the terminal will give a help page for the glm function) and online in reference manuals (http://cran.r-project.org/web/packages/vegan/index.html; Accessed 05-12-2014).

Statistical analysis

A square matrix containing the presence and raw abundance of all OTUs per sample was imported into R (R Core Team 2013) using the read.table() function. Sequences not classified as bacteria or classified as chloroplasts or mitochondria were removed

prior to statistical analysis. The OTU abundance matrix was $\log_{10}(x + 1)$ transformed and a distance matrix constructed using the Bray–Curtis index with the vegdist() function in the vegan package in R (Oksanen *et al.* 2009). The Bray–Curtis index is one of the most frequently applied (dis)similarity indices used in ecology (Cleary 2003; de Voogd *et al.* 2006). Variation in OTU composition among biotopes (*S. massa* and *X. testudinaria*, sediment and seawater) was assessed with principal coordinates analysis (PCO) using the cmdscale() function in R with the Bray–Curtis distance matrix as input. We tested for significant variation in composition among biotopes using the adonis() function in vegan. In the adonis analysis, the Bray–Curtis distance matrix of species composition was the response variable with biotope as independent variable. The number of permutations was set at 999; all other arguments used the default values set in the function. Weighted averages scores were computed for OTUs on the first two PCO axes using the wascores() function in the vegan package.

BLAST, phylogenetic and predictive metagenome analysis

We used the NCBI BLAST command line 'blastn' tool with the -db argument set to nt to identify closely related organisms to numerically dominant OTUs (≥100 sequences) (Zhang *et al.* 2000). A phylogenetic tree including all dominant OTUs (≥100 sequences) and selected cultured organisms was constructed using the Mega5 program (http://www.megasoftware.net/; last checked 2014/07/02; Tamura *et al.* 2011) with the nearest-neighbor-interchange and generalized time-reversible model (Tavaré 1986) with gamma distributed and invariant sites. In the results, we present a bootstrap consensus tree based on 100 replicates (Felsenstein 1985). We used PICRUSt (Langille *et al.* 2013) to predict the metagenome of each sample. PICRUSt is a bioinformatics tool that uses marker genes, such as 16S rRNA, to predict metagenome gene functional content. These predictions are pre-calculated for genes in databases including KEGG (Kyoto Encyclopedia of Genes and Genomes) and COG (Clusters of Orthologous Groups of proteins). In the present study, we used the KEGG database. Output of PICRUST consists of a table of functional gene counts known as KEGG Orthologs (KOs). In the KEGG database, KOs are sets of homologous sequences, from a large array of organisms, that have been assigned a specific molecular function. KOs are, in turn, arranged hierarchically and grouped into biological pathways. Note that because of functional overlap, some KOs can be represented in more than one pathway. In the present study, we used the KEGG database and focused on selected KOs in the nitrogen metabolism pathway (K00368, K00376, KO2586, KO3385, KO04561 and KO10535). In addition to metagenomic data, we also used the -a option in the predict˙metagenomes.py script to obtain weighted nearest sequenced taxon index (NSTI) scores for each sample. NSTI scores are a means of quality control, which provide a summary of the extent to which OTUs in a given sample are related to reference OTUs. NSTI scores represent the average branch length separating an OTU from a reference OTU. A detailed description of these methods has been published previously (Cleary *et al.* 2013; Langille *et al.* 2013; Polónia *et al.* 2014) and can be found in the supplementary methods (Data S1, Supporting Information). We used R to generate bargraphs showing the percentage of total genes for each sample and the contribution of selected orders (obtained with the *metagenome˙contributions.py* script in PICRUSt) to the total gene count in each sample.

RESULTS

In the present study, sequencing yielded 36848 sequences, assigned to 3561 OTUs after quality control, OTU picking and removal of chimera, chloroplasts and mitochondria. A total of 325 OTUs remained unidentified at the level of domain and were not included in the statistical analyses, and 446 OTUs remained unclassified at the phylum level. Of those OTUs assigned to a phylum, these were assigned to 42 phyla, of which the Proteobacteria (1935 OTUs) was by far the most abundant in terms of both sequences and OTUs. Other phyla (>10 OTUs) included Bacteroidetes (386), Acidobacteria (160), Cyanobacteria (95), Chloroflexi (90), Actinobacteria (82), Gemmatimonadetes (68), Spirochaetes (43) Firmicutes (38), Verrucomicrobia (31), Nitrospirae (28), WS3 (28), GN02 (23), Planctomycetes (21), Tenericutes (17), Fibrobacteres (18), Sar406 (18) and Caldithrix (11). In addition to this, OTUs were assigned to 94 classes and 139 orders. Of the most abundant OTUs (≥100 sequences), 32 were identified from the sponges *X. testudinaria* and 19 from *S. massa*, 14 from seawater and 9 from sediment. Of these OTUs, none were most abundant in sediment, 8 were most abundant in seawater, 11 were most abundant in *S. massa* and 30 were most abundant in *X. testudinaria*. Only the sponges *S. massa* (5 OTUs) and *X. testudinaria* (27 OTUs) had abundant OTUs that were restricted to those particular biotopes.

BLAST identified closely related organisms to all 49 abundant (≥100 sequences) OTUs (Table S1, Supporting Information). The most abundant OTU overall was OTU 1, assigned to the order Thiohalorhabdales and restricted to *S. massa* hosts and represented by 4306 sequences. The three most abundant OTUs overall (OTUs 1, 2 and 4) were found exclusively or predominantly in *S. massa*. OTUs 2 and 4 were closely related to organisms isolated from the sponge *S. carteri* in Saudi Arabia (Table S1, Supporting Information). All of the OTUs found predominantly in *S. massa* were closely related to organisms isolated from sponges with the exception of OTU 27, which was closely related to an organism isolated from crustose coralline algae and OTU 14, which only had a maximum identity of 91.4 with an organism isolated from restored grassland. Of the abundant OTUs only one (OTU-103), assigned to the order Nitrospirales was found in all sponge hosts, but was absent from seawater and sediment. Only 14.9% of the OTUs (136 of 910) restricted to the non-host biotopes were shared between seawater and sediment and the majority (123 of 136) were not present in sponge hosts.

In seawater samples, the most abundant OTU (OTU 3), assigned to the order Oceanospirillales, was closely related to an organism isolated from a coral collected in Australia.

Higher taxon abundance

The abundance of all higher taxa shown in Fig. 2 differed significantly among biotopes with the exception of the class Synechococcophycideae. Some taxa were found predominantly or exclusively in a single biotope including the Anaerolineae and Syntrophobacterales in *X. testudinaria*, the Oceanospirillales in seawater and the Thiohalorhabdales in *S. massa*. The relative abundance of the most abundant OTU (Fig. 2; dominance) in each biotope was highest in the sponge *S. massa*, where more than 40% of sequences on average belonged to a single OTU. Dominance was lowest in the sediment biotope where just over 2% of sequences belonged to a single OTU on average.

Specific differences in relative abundance within individual sponges, seawater and sediment were the most obvious for *X. testudinaria* which generally harbored more cyanobacteria

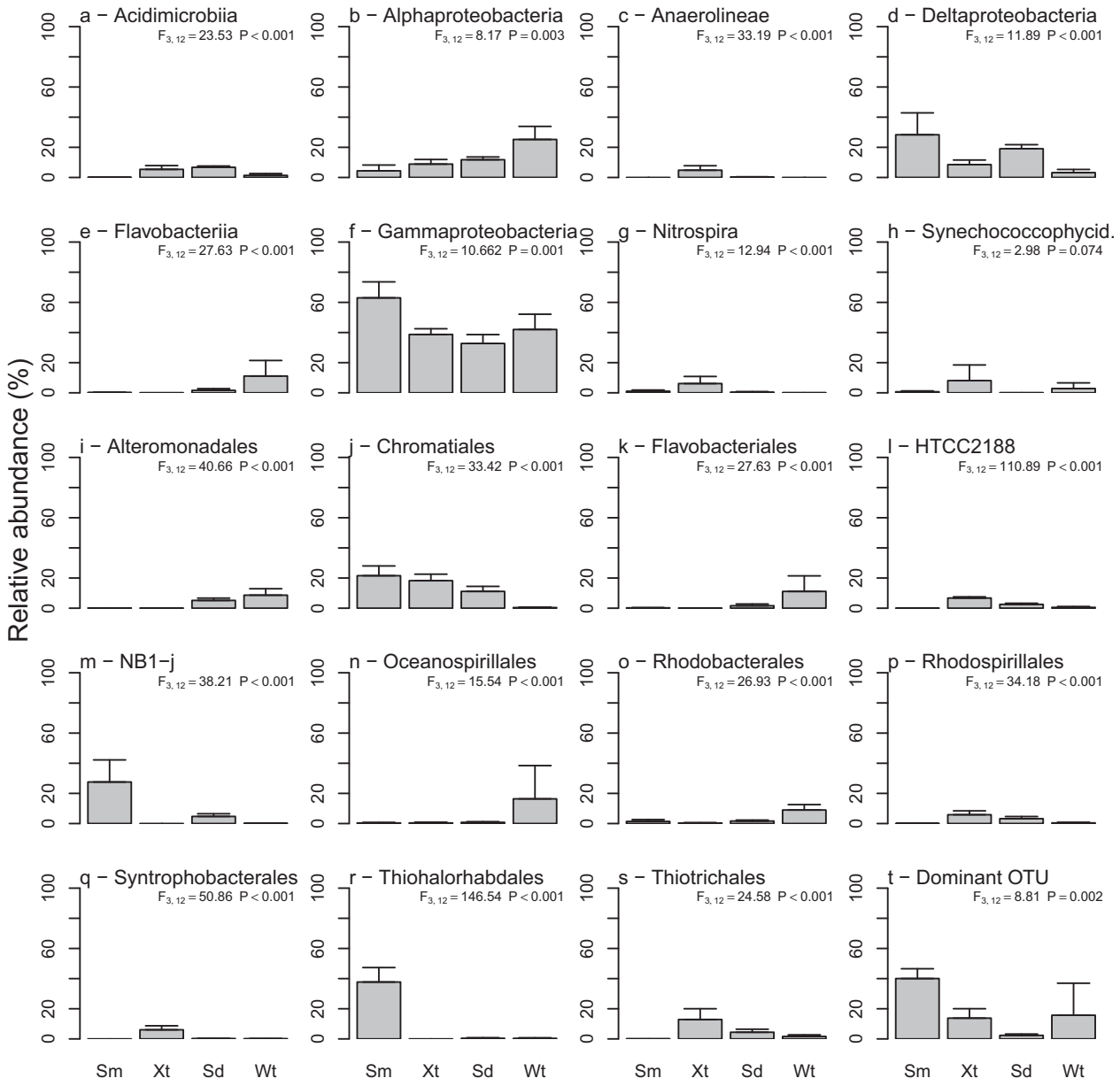

Figure 2. Mean relative abundance of the most abundant bacterial classes (a–h), orders (i–s) and the most abundant OTU (t) for samples from *S. massa* (Sm), *X. testudinaria* (Xt), sediment (Sd) and seawater (Wt). Error bars represent a single standard deviation. The dominant OTU represents the mean abundance for the single most abundant OTU in each sample, thus not necessarily the same OTU. Results of the GLM are shown in the top-right corner of each subfigure.

further away from Jakarta (sample X388 and X142) (Fig. 3). Also noteworthy is that the seawater sample of the reef of Kelapa harbored more Bacteroidetes.

Importance of biotope in structuring composition

There was a highly significant difference in composition among biotopes ($F_{3,12} = 6.83$, $P < 0.001$, $R2 = 0.630$). Variation among biotopes thus explained 63% of the variation in composition. A PCO (Fig. 4) of the first two axes shows four distinct clusters representing samples from the four biotopes. Although forming distinct clusters, samples from sediment and seawater were closer to one another in the ordination than either to the sponge samples. Axis 1 of the PCO separated samples from *X. testudinaria* and the other biotopes. Axis 2 separated samples from *S. massa* and the sediment and seawater biotopes.

Phylogenetic analysis

A phylogenetic analysis of the most abundant OTUs and various cultured organisms is presented in Fig. 5. *Xestospongia testudinaria* hosted three abundant closely related OTUs (OTUs 17, 24 and 3672) all assigned to the order HTCC2188. This clade was closely related to three OTUs (10, 43 and 47) assigned to the order Chromatiales. The closest cultured representative of these two clades was *Thioalkalivibrio paradoxus* (219857426). OTUs assigned to the class Nitrospira formed a well-supported clade (bootstrap value = 100) with a cultured Nitrospira (323573883). Within the

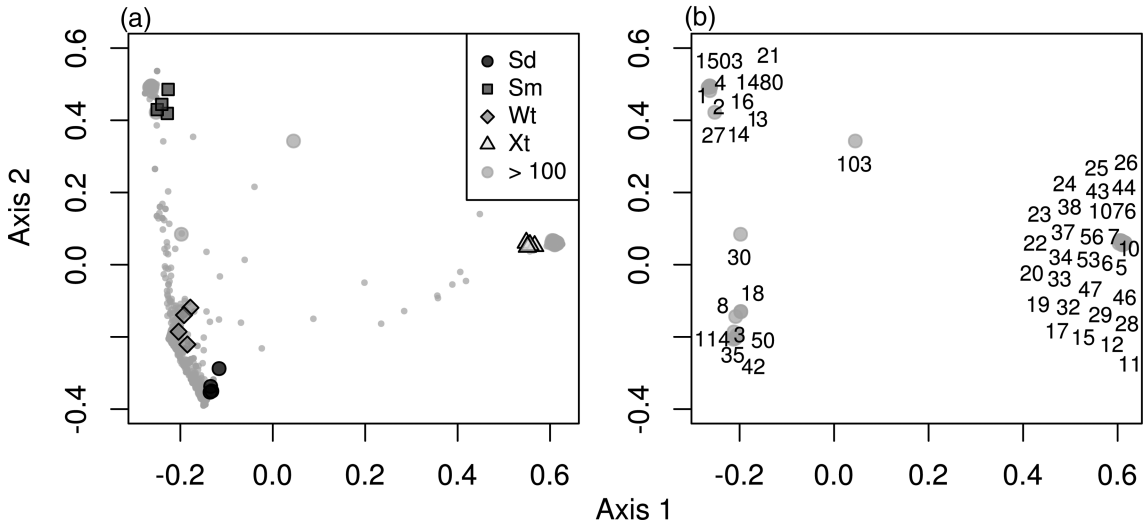

Figure 3. Ordination showing the first two axes of the PCO analysis. (**a**) Symbols represent samples from sediment (Sd), *S. massa* (Sm), seawater (Wt) and *X. testudinaria* (Xt). Very small light grey circles represent OTUs < 100 sequence reads; large light grey circles represent OTUs with >100 sequence reads. (**b**) Numbers represent abundant (≥100 sequence reads) OTUs referred to in Table S1 (Supporting Information).

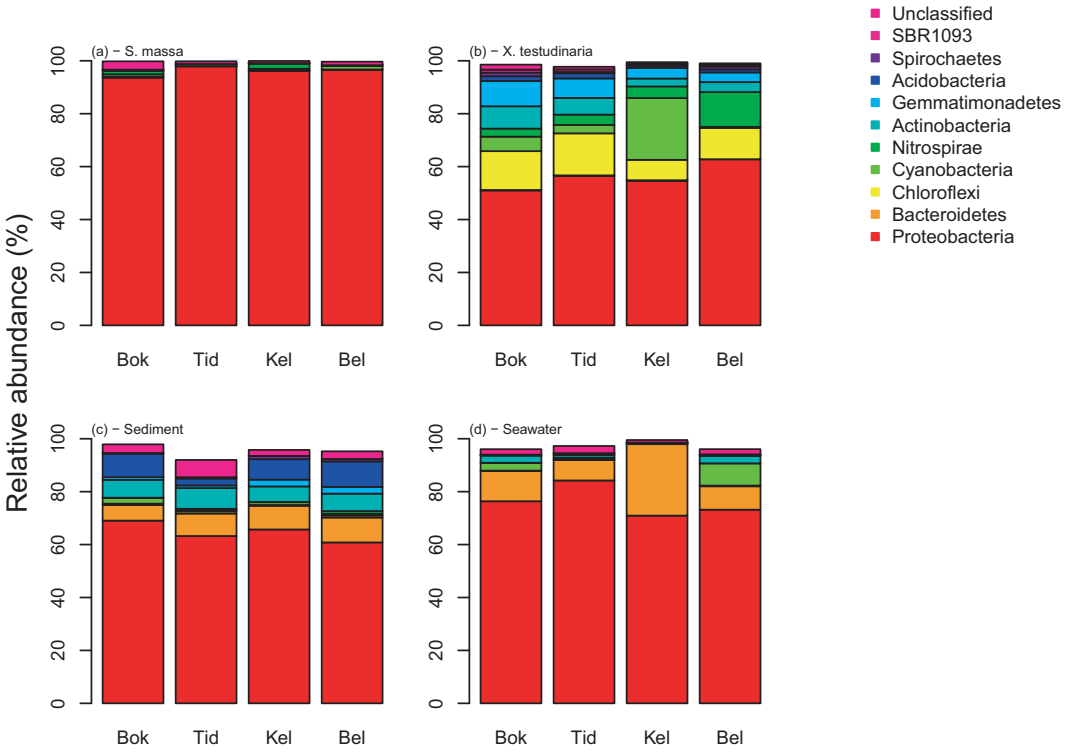

Figure 4. Stacked barplots showing the relative abundance of the 10 most abundant phyla sampled from the four biotopes, (**a**) *S. massa*, (**b**) *X. testudinaria*, (**c**) sediment and (**d**) seawater. The site codes (*x*-axis) are Bok: Bokor, Tid: Tidung Kecil, Kel: Kelapa and Bel: Belanda.

class Alphaproteobacteria, there was a strongly supported clade (bootstrap value = 100) consisting of OTU (–018) isolated from seawater and OTU (–027) isolated from *S. massa* and a cultured organism identified as *Roseovarius* sp. (327387758).

metagenome analysis

Mean (and standard deviation) NSTI values for the biotopes sampled in Jakarta were 0.22 (0.02) for *S. massa*, 0.21 (0.04) for *X. testudinaria*, 0.16 (0.01) for sediment and 0.12 (0.03) for seawater. NSTI values were thus relatively high for the sponge biotopes. The lowest NSTI values for sponge biotopes were 0.19 for sample SM-56 (*S. massa*) and 0.15 for sample XT-388 (*X. testudinaria*). There were pronounced predicted differences in enrichment among biotopes for a number of KOs belonging to the nitrogen metabolism pathway (Fig. 6). Nitrous oxide reductase *NosZ* (K00376) was predicted to be enriched in *X. testudinaria*, but showed very few counts in *S. massa*. This enzyme is important in the denitrification of nitrate to nitrogen. Enrichment for this enzyme was predicted to be primarily due to Chromatiales. For

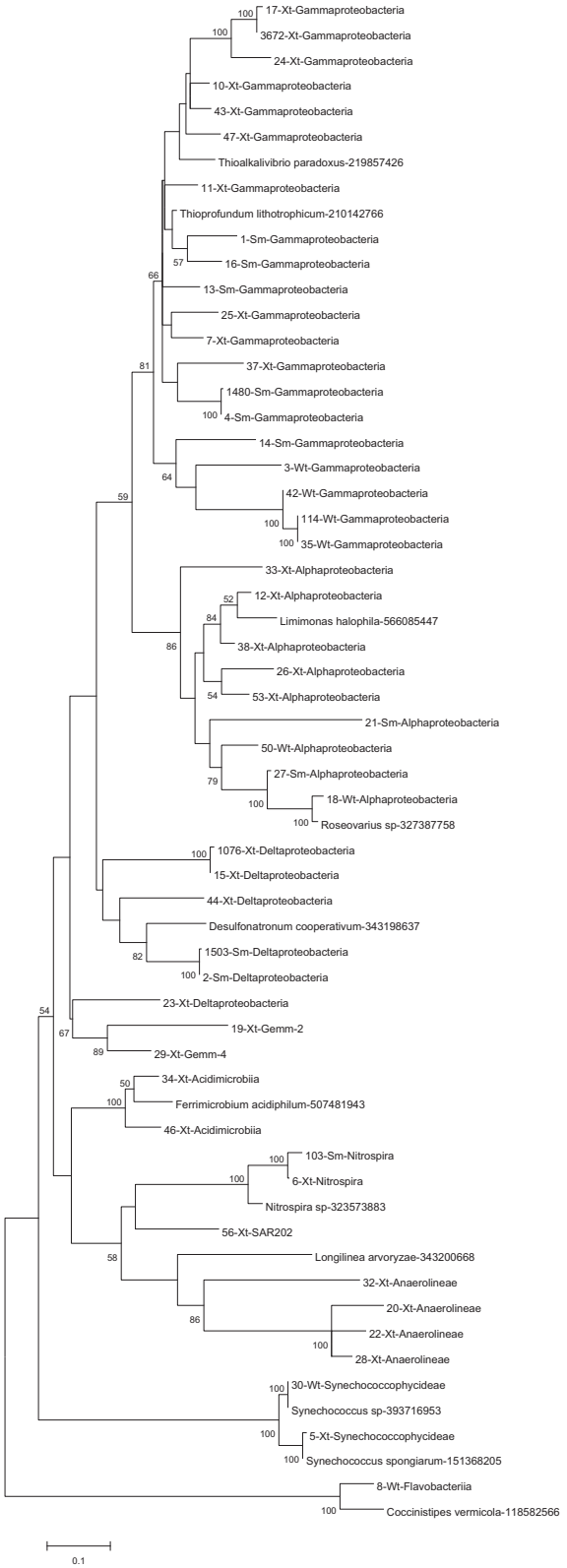

Figure 5. Phylogenetic tree of the bacterial 16S rRNA gene sequences recovered from *S. massa* (Sm), *X. testudinaria* (Xt), seawater (Wt) and sediment (Sd); bootstrap values lower than 50% were omitted. The number of each OTU is indicated as are GenBank GenInfo sequence identifiers of cultured bacterial sequences. Classes of Bacteria are indicated. OTUs are assigned to the following clusters Sm-found in *S. massa*, Xt-found in *X. testudinaria* and Wt-found in seawater.

water, the Oceanospirillales contributed most to counts for this KO. Nitrate reductase (K00370, K0371 and K00372) was predicted to be particularly enriched in *S. massa* and largely due to Chromatiales and Thiohalorhabdales and Thiotrichales in *X. testudinaria*. Interestingly, gene counts for the different sponge individuals enriched for various KOs varied considerably. For instance, sample XT-142 (*X. testudinaria*) was collected furthest away from Jakarta and had the highest gene counts for various KOs. Nitrite reductase *nirK* gene (K00368) and nitrite oxide reductase *norB* gene (K04561) were both enriched in *X. testudinaria*. Enrichment of K00368 was mainly predicted to be due to the order Nitrospirales, whereas enrichment of K04561 was mainly predicted to be due to Syntrophobacterales and Thiotrichales. The nitrifying gene hydroxylamine reductase (K010535) was particularly enriched in *S. massa* and this was mainly predicted to be due to the uncultivated order NB1-J. Also the gene *nifD* (KO2586) was enriched in both sponges and this was mainly due to NB1-J for *S. massa* and to Synthrophobacterales and Nitrospirales for *X. testudinaria*.

DISCUSSION

We recorded highly significant differences in the relative abundances of a number of classes and orders. The Oceanospirillales, for example, were largely restricted to seawater; the Anaerolineae and Syntropbacterales to the sponge *X. testudinaria* and the Thiohalorhabdales to *S. massa*.

The Gammaproteobacteria was by far the most abundant class. The relative abundance of the most abundant OTU in each biotope was highest in *S. massa*, where more than 40% of sequences on average belonged to a single OTU (OTU 1). This OTU was closely related to an organism previously isolated from the sponge *Phakellia fusca* from the South China Sea (Han *et al.* 2012). The second and third most abundant OTUs (orders NB1-J and Chromatiales, respectively) were closely related to organisms isolated from the LMA sponge *S. carteri* in Saudi Arabia (Lee *et al.* 2011). The most dominant bacteria of *S. carteri* from the Red Sea were assigned to Gammaproteobacteria, Oceanospirillales and Sphingobacteria. The sponge species *S. carteri* and *S. massa* are morphologically very similar to each other and often live sympatrically (Alvarez and Hooper 2010). Although we did not examine the tissue of *S. massa* by means of TEM, this sponge is considered to be an LMA sponge (Moitinho-Silva *et al.* 2014). The presence of a dominant OTU in an LMA sponge was observed in the Mediterranean Poecilosclerid sponge *Crambe Crambe* (Croué *et al.* 2013). This dominant bacterial OTU belonged to the Betaproteobacteria, and it was suggested that it had adapted to the presence of antibiotics produced by the sponge host. Moreover, the dominance of a single OTU would seem to imply that the symbiont is responsible for the biosynthesis of a less diverse range of bioactive compounds in this sponge species. Furthermore, it was recently suggested that LMA sponges typically have a low phylum-level diversity with dominance of proteobacterial phylotypes (Kamke, Taylor and Schmitt 2010; Giles *et al.* 2013; Gloeckner *et al.* 2013). Sponges of the genus *Stylissa* are the focus of scientific interest because they have been the source of highly interesting bioactive compounds, which include dimeric alkaloids such as dibromophakellin and sceptrin in addition to a series of brominated pyrrole alkaloids, and other brominated alkaloids. These compounds have attracted particular attention because of their ability to inhibit protein kinases (Ebada *et al.* 2015). In addition to this, *S. massa* produces a wide variety of antimicrobial compounds and their antibacterial activity is known

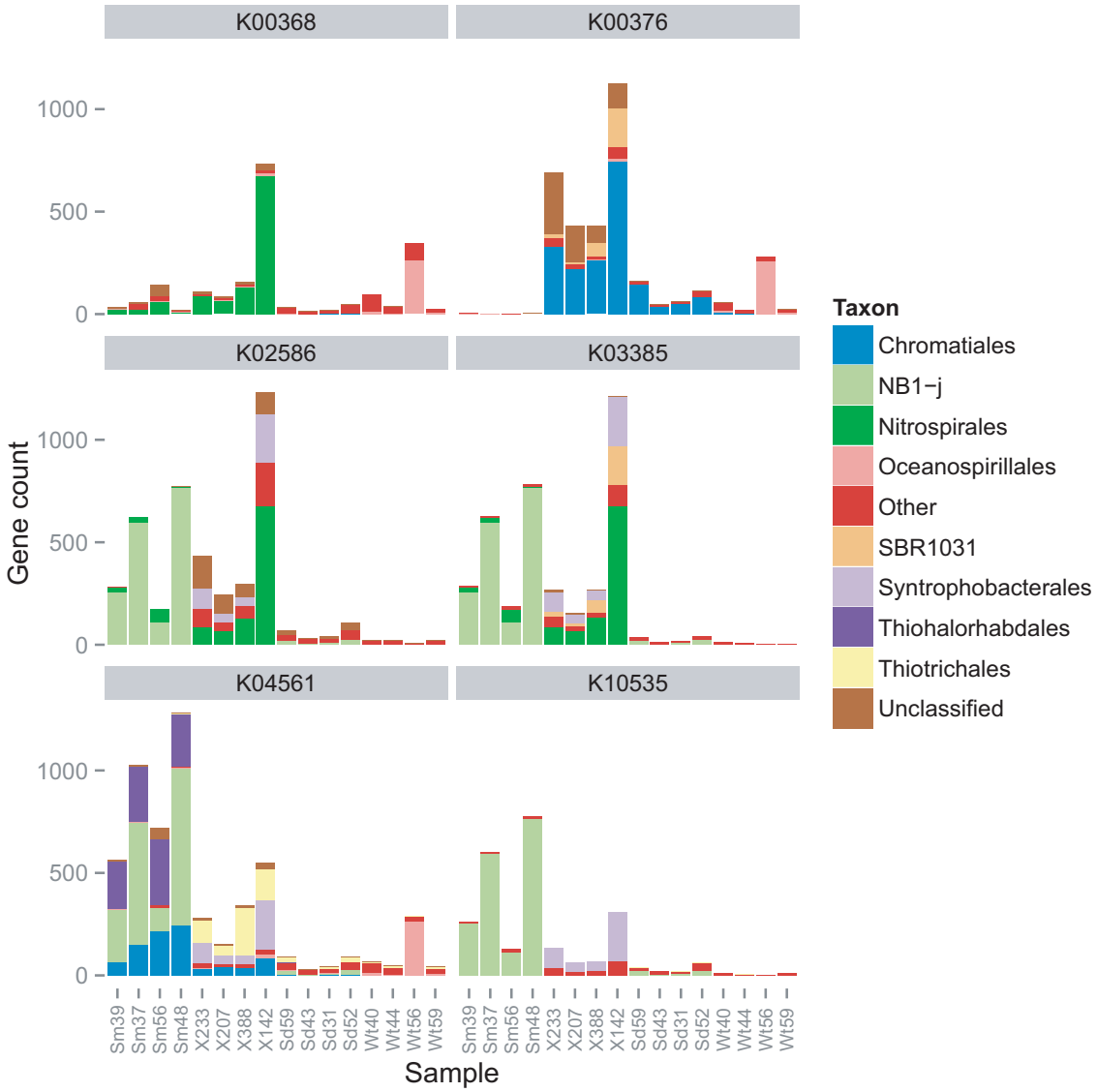

Figure 6. Stacked barplots showing the estimated gene count contributions (*y*-axis) of the 8 most abundant orders for K00368 (*nirK*, nitrite reductase), K00376 (*nosZ*, nitrous-oxide reductase), K02586 (*nifD*, nitrogenase molybdenum-iron protein alpha chain), K03385 (*nrfA*, nitrite reductase (cytochrome c-552), K04561 (*norB*, nitric oxide reductase subunit B) and K10535 (hao, hydroxylamine dehydrogenase)

to be highly selective against different bacterial strains. It has been hypothesized that this high selectivity may serve to establish natural sponge–microbial associations, while inhibiting settlement or growth of potential pathogens (Rohde *et al.* 2012).

In the present study, the most abundant OTUs of *X. testudinaria* belonged to the phyla Proteobacteria, Nitrospirae, Chloroflexi and Cyanobacteria. The microbial community of this sponge species has been assessed before from the Red Sea, North Sulawesi (Indonesia) and the Great Barrier Reef (Lee *et al.* 2011; Montalvo and Hill 2011; Montalvo *et al.* 2014). In these studies, the most abundant OTUs belonged to the Chloroflexi, Acidobacteria, Deltaproteobacteria and Actinobacteria in North Sulawesi, Chloroflexi, Proteobacteria and Firmicutes in samples from the Red Sea and Poribacteria in samples from the Great Barrier Reef. The pronounced differences in the bacterial communities from different regions may reflect geographical differences but probably also are due to methodological differences including sample preservation, tissue sampled, primer selection and the use of lysozymes. A recurring phenomenon in sponge microbiology is the high similarity among sponge-associated bacterial communities. Studies of *X. testudinaria* including the present, contrast with this general assumption, namely, the high degree of similarity among sponge-associated bacterial communities regardless of their host and geographical origin, but as mentioned, this may be due to sampling and laboratory artifacts (Hentschel *et al.* 2002).

In the present study, there were marked differences in the abundance of bacterial classes and orders among sponge species and non-host biotopes. The PCO ordination revealed significant compositional differences among the four biotopes. These results are in line with Lee *et al.* (2011), Cleary *et al.* (2013), Alex *et al.* (2013) and Webster *et al.* (2013). These data also confirm that the LMA sponge *S. massa* has a microbial community that is distinct from the surrounding seawater. Also, Moitinho-Silva *et al.* (2014) already remarked that its sister species, *S. carteri*, in the Red Sea harbored a distinct microbiota and that the common notion that LMA sponges only contain seawater bacteria is not correct.

The phylogenetic tree shows the relationship between the most abundant bacterial species (OTUs) and their closest cultured representatives. Within the clade Gammaproteobacteria, the closest cultured representative of the most abundant OTUs of *X. testudinaria* was *T. paradoxus*. This alkaliphilic obligatory autotrophic sulfur-oxidizing bacteria was recently described from soda lakes in Kenya and Egypt and is able to grow on thiocyanate as the sole energy, nitrogen and sulfur source (Sorokin *et al.* 2002). Interestingly, thiocyanate can act as a pollutant and is leached from concrete mixtures from construction sites. Many of the reefs in the JBTI have been used for coral mining, dredging and the construction of resorts and the rapidly increasing population of Jakarta has resulted in a continuous demand for construction.

The closest cultured representative of the most abundant OTU from *S. massa* was the lithotrophic *Thioprofundum lithotrophicum*. This species relies on an inorganic substrate via aerobic or anaerobic respiration and was described from a deep-sea hydrothermal vent chimney (Takai *et al.* 2009). Of the Alphaproteobacteria, the closest cultured representative of OTU 12 restricted to *X. testudinaria* was the extremely halophilic *Limimonas halophila*. This bacteria was only very recently described as a new genus and a new species from the hypersaline lake Aran-Bidgol in Iran (Amoozegar *et al.* 2013). *Synechococcus* is a widespread cyanobacterial genus and was abundant in *X. testudinaria*. Its closest relative was isolated from *X. muta* in the Caribbean. *Synechococcus spongiarum* is a cyanobacterial symbiont isolated from various sponge species around the world. Both abundant OTUs assigned to *Nitrospira* and isolated from *S. massa* and *X. testudinaria* were closely related to a cultured *Nitrospira*. *Nitrospira* are poorly studied and mainly consist of uncultured nitrite-oxidizing bacteria that are important in marine habitats; they are able to oxidize both ammonia and nitrite to nitrate (Lücker *et al.* 2010). However, it is known that water that is too rich in ammonia or has a low pH will inhibit *Nitrospira*'s nitrifying activity. The waters of the Bay of Jakarta are severely polluted and ammonia levels are probably much higher inshore than offshore as untreated sewage water from several rivers discharges directly run into the Bay. Overall, it is interesting to notice that most of the cultured representatives shown in the phylogenetic tree play a role in the nitrogen cycle.

Although nitrogen-fixing prokaryotes are known from the open ocean, they have also been isolated from a variety of hosts including sponges and corals. Recently, it was shown that the metabolism of the giant barrel sponges (*X. muta*) in the Caribbean might have a significant impact on the nitrogen biochemistry of reefs by releasing large amounts of DIN (Fiore, Baker and Lesser 2013). In the present paper, we used the recently developed bioinformatic tool PICRUSt to predict gene enrichment in our samples and the taxonomic affiliations of bacteria responsible for this enrichment. Due to previous studies addressing the importance of sponges in the nitrogen cycle, we chose to concentrate our efforts on genes involved in the nitrogen metabolism pathway. The nitrogen metabolism of bacterial and archaeal symbionts is closely linked to the sponge host, which secretes and accumulates ammonium. Several genes involved in the nitrogen cycle were predicted to be enriched in our sponge samples. The *nosZ* gene (K00376) was predicted to be particularly enriched in the sponge *X. testudinaria* and the order Chromatiales was mainly responsible for this enrichment. Chromatiales or purple sulfur bacteria are photosynthetic sulfur-oxidizing Gammaproteobacteria that fix carbon dioxide without evolving oxygen and are also known to be active nitrogen fixers (Proctor 1997). Nitrous oxide reduction (*nosZ*) is the final step in the denitrification pathway and represents the loss of biologically available nitrogen. Denitrification is the reductive respiration of nitrate or nitrite to N_2 or N_2O, and is carried out by a phylogenetically diverse group of bacteria, generally under anaerobic conditions. Beyond its importance in the oceanic nitrogen cycle, denitrification produces nitrous oxide, a gas implicated in both ozone destruction and global warming (Wang *et al.* 1976). The *nosZ* gene is mostly unique to denitrifying bacteria, although a few non-denitrifier species capable of reducing nitrous oxide have been identified as well (Zumft 1992).

Nitrite reductase [the gene *nirK* (K00368)] and nitrite oxide reductase [the gene *norB* (K04561)] were predicted to be enriched in *X. testudinaria* and the order Nitrospirales was mainly responsible for the enrichment of *nirK* whereas the Syntrophobacterales and Thiotrichales were mainly responsible for enrichment of *norB*. These orders were chiefly restricted to sponges in our study. In *S. massa*, the predicted gene count of *norB* was mainly due to the orders NB1-J and Thiohalorhabdales, the latter of which was only observed in this sponge. Nitrite reductase reduces nitrite to NO (nitrification) and is encoded by two structurally different genes: *nirK* and *nirS*; these genes are functionally and physiologically equivalent and appear to be mutually exclusive (Braker, Fesefeldt and Witzel 1998). Nitric oxide reductase is encoded by the *norB* gene and catalyzes the reduction of NO to N_2O. The nitrifying gene, hydroxylamine reductase (K10535), was predicted to be particularly enriched in the sponge *S. massa* and this was mainly due to the uncultivated order NB1-J.

The gene *nifD* (K02586) was predicted to be enriched in both sponges and this was mainly due to order NB1-J in *S. massa* and Synthrophobacterales and Nitrospirales in *X. testudinaria*. The *nifD* gene is one of the genes involved in the encoding of the enzyme nitrogenase that is responsible for the biological conversion of atmospheric nitrogen to ammonia or nitrogen fixation (Fani, Gallo and Liò 2000). The predicted variation in gene counts between the samples was quite large, and nitrogen fixation tended to be higher in the samples further away from Jakarta Bay, which may be attributed to suppression of nitrogen fixation due to elevated NH_3 concentrations inshore. In addition to this, gene counts for the gene *nrfA* (K03385) for the sample furthest away from the city of Jakarta was predicted to be much higher for the sponge samples. This gene is involved in the expression of the enzyme nitrite reductase that is responsible for dissimilatory nitrate reduction from nitrite to ammonia. Our data predict that all major transformations of nitrogen that occur in the nitrogen cycle are represented in our two sponges and that levels of enrichment in sponges differ from non-host biotopes. Hoffmann *et al.* (2009) already showed that anammox and nitrification can occur in the same sponge host and that sponges may function as unrecognized nitrogen sinks in the ocean. Interestingly, although it has been hypothesized that LMA sponges tend to depend on nutrient uptake from the water column, we show here that, although *S. massa* had a low diversity of bacteria, the bacteria present in this sponge appear to be distinct and abundant and, the predicted functional roles they play appear to be similar to those of the HMA sponge *X. testudinaria*, at least as far as the nitrogen energy metabolism is concerned.

PICRUSt provides a prediction of microbiome function based on marker genes, in this case 16S rRNA, but is not an actual measurement of such function. The reliability of PICRUSt, however, can be tested using various quality control methods including weighted NSTI scores. NSTI calculates dissimilarity between reference genomes and the metagenome under study and

was developed to evaluate the prediction accuracy of PICRUSt. Poorly characterized environments have relatively few reference genome sequences available thus lowering the accuracy of PICRUSt predictions for these genomes. In the present study, NSTI scores were relatively low for sediment and seawater and relatively high for both sponge species, a reflection of the relative novelty of the bacterial microbiomes of the coral reef sponges studied here. This can be seen by the relatively large contribution of OTUs belonging to unclassified orders in *X. testudinaria* with respect to their contribution in the gene count of K00376.

The large contribution of OTUs (particularly OTU 2) belonging to the deltaproteobacterial order NB1-J also partially explains the relatively high NSTI scores for *S. massa* given that this order is still poorly known within the Deltaproteobacteria. Mean scores for both sponge species were >0.20. The accuracy of PICRUSt decreased with increasing NSTI scores, but still produced reliable results for a dataset of soil samples with a mean NSTI score of 0.17 (Langille *et al.* 2013). Accuracy was markedly lower for a dataset from the Guerrero Negro microbial mat with a mean NSTI score of 0.23. This was, however, related to shallow sequencing at a depth that was insufficient to fully sample the community's genomic composition (Langille *et al.* 2013). The relatively high NSTI scores obtained for both sponge species here indicate that the PICRUSt predictions should be treated with caution. Results for the sponge samples though with the lowest NSTI scores (Sm-56 and XT-388) were similar to the other samples and also showed pronounced enrichment for the selected KOs shown in comparison to seawater and/or sediment. The results thus still provide some interesting insights into the potential function of the sponge microbiomes with respect to the nitrogen cycle that warrant future testing with studies that measure actual gene presence and/or expression.

Although we are beginning to understand the importance of sponges in regulating nutrient cycling in the Caribbean (Southwell, Popp and Martens 2008; de Goeij *et al.* 2013; Fiore, Baker and Lesser 2013), much less is known about the role sponges play in regulating nutrients in the Indo-Pacific, particularly in high-diversity coral reef habitats in the coral triangle, an area of the highest known coral reef diversity in the world (McLeod *et al.* 2010). This is a topic of particular importance because sponges are becoming increasingly important components of coral reefs and may even replace corals as the dominant reef component (McMurray, Henkel and Pawlik 2010; Bell *et al.* 2013). Moreover, many coral reefs in the Indo-Pacific are located near large urban areas, where marine assemblages in general, are structured by strong on-to-offshore gradients (de Voogd and Cleary 2008). In the JBTI, sponge diversity is impoverished near shore, but much more diverse offshore and environmental conditions are better (de Voogd and Cleary 2008). Although our samples were taken from different reefs in near and offshore locations, there were relatively little compositional differences between the samples, but the gene counts for the different KOs varied considerably.

To conclude, our data show that a combined barcoded pyrosequencing and predictive metagenomic approach can provide information on the functional ecology of the microbial communities that can be used to generate hypotheses for future studies.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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REFERENCES

Alex A, Silva V, Vasconcelos V, *et al.* Evidence of unique and generalist microbes in distantly related sympatric intertidal marine sponges (Porifera: Demospongiae). *PLoS One* 2013;**8**:e80653.

Alvarez B, Hooper JNA. Taxonomic revision of the order Halichondrida (Porifera: Demospongiae) of northern Australia. Family Dictyonellidae. *Beagle Rec N Terrritory Arts Sci* 2010;**26**:113–26.

Amoozegar MA, Makhdoumi-Kakhki A, Ramezani M, *et al. Limimonas halophila* gen. nov., sp nova, an extremely halophilic bacterium in the family Rhodospirillaceae. *Int J Syst Evol Micr* 2013;**63**:1562–7.

Bell JJ, Davy SK, Jones T, *et al.* Could some coral reefs become sponge reefs as our climate changes? *Glob Change Biol* 2013;**19**:2613–24.

Braker G, Fesefeldt A, Witzel KP. Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. *Appl Environ Microb* 1998;**64**:3769–75.

Capone DG, Dunham SE, Horrigan SG, *et al.* Microbial nitrogen transformations in unconsolidated coral reef sediments. *Mar Ecol-Prog Ser* 1992;**80**:75–88.

Cleary DFR. An examination of schale of assessment, logging and ENSO-induced fires on butterfly diversity in Borneo. *Oecologia* 2003;**135**:313–21.

Cleary DFR, Becking LE, Voogd NJ, *et al.* Habitat-and host-related variation in sponge bacterial symbiont communities in Indonesian waters. *FEMS Microbiol Ecol* 2013;**85**:465–82.

Cleary DFR, Polonia ARM, Renema W, *et al.* Coral reefs next to a major conurbation: a study of temporal change (1985–2011) in coral cover and composition in the reefs of Jakarta, Indonesia. *Mar Ecol-Prog Ser* 2014;**501**:89–98.

Cleary DFR, Suharsono, Hoeksema BW. Coral diversity across a disturbance gradient in the Pulau Seribu reef complex off Jakarta, Indonesia. *Biodivers Conserv* 2006;**15**:3653–74.

Croué J, West NJ, Escande ML, *et al.* A single proteobacterium dominates the microbial community of the crambescidine-containing sponge *Crambe crambe*. *Sci Rep* 2013;**3**:2583.

de Goeij JM, van Oevelen D, Vermeij MJA, *et al.* Surviving in a marine desert: the sponge loop retains resources within coral reefs. *Science* 2013;**342**:108–10.

de Voogd NJ, Cleary DFR. An analysis of sponge diversity and distribution at three taxonomic levels in the thousand

islands, Jakarta Bay reef complex, West-Java, Indonesia. *Mar Ecol* 2008;**29**:205–15.
de Voogd NJ, Cleary DFR, Hoeksema BW, Noor A, *et al.* Sponge beta diversity in the Spermonde Archipelago, Indonesia. *Mar Ecol-Prog Ser* 2006;**309**:131–42.
Ebada SS, Linh MH, Longeon A, *et al.* Dispacamide E and other bioactive bromopyrrole alkaloids from two Indonesian marine sponges of the genus *Stylissa*. *Nat Prod Res* 2015;**29**: 231–8.
Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 2013;**10**:996–8.
Edgar RC, Haas B, Clemente J, *et al.* UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 2011;**27**:2194–2200.
Fani R, Gallo R, Liò P. Molecular evolution of nitrogen fixation: the evolutionary history of the *nifD, nifK, nifE,* and *nifN* genes. *J Mol Ecol* 2000;**51**:1–11.
Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;**39**:783–91.
Fiore CL, Baker DM, Lesser MP. Nitrogen biogeochemistry in the Caribbean sponge *Xestospongia muta*: a source or sink of dissolved inorganic nitrogen. *PLoS One* 2013;**8**:e72961.
Fiore CL, Jarett JK, Olson ND, *et al.* Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends Microbiol* 2010;**18**:455–63.
Freeman CJ, Thacker RW. Complex interactions between marine sponges and their symbiotic microbial communities. *Limnol Oceanogr* 2011;**56**:1577–86.
Giles EC, Kamke J, Moitinho-Silva L, *et al.* Bacterial community profiles in low microbial abundance sponges. *FEMS Microbiol Ecol* 2013;**83**:232–41.
Gloeckner V, Hentschel U, Ereskovsky AV, *et al.* Unique and species-specific microbial communities in *Oscarella lobularis* and other Mediterranean *Oscarella* species (Porifera: Homoscleromorpha). *Mar Biol* 2013;**160**:781–91.
Gomes NCM, Heuer H, Schönfeld J, *et al.* Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant Soil* 2001;**232**:167–180.
Gonzalez-Rivero M, Yakob L, Mumby PJ. The role of sponge competition on coral reef alternative steady states. *Ecol Model* 2011;**222**:1847–53.
Han M, Liu F, Zhang F, *et al.* Bacterial and Archaeal symbionts in the South China Sea sponge *Phakellia fusca*: community structure, relative abundance, and ammonia-oxidizing populations. *Mar Biotechnol* 2012;**14**:701–13.
Hentschel U, Fieseler L, Wehrl M, Microbial diversity of marine sponges. In: Mueller W, *et al.* (ed.). *Molecular Marine Biology of Sponges*. Springer Verlag Heidelberg, 2003, 60–88.
Hentschel U, Hopke J, Horn M, *et al.* Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microb* 2002;**68**:4431–40.
Hentschel U, Piel J, Degnan SM, *et al.* Genomic insights into the marine sponge microbiome. *Nat Rev Microbiol* 2012;**10**: 641-54.
Hoffmann F, Radax R, Woebken D, *et al.* Complex nitrogen cycling in the sponge Geodia barretti. *Environ Microbiol* 2009;**11**:2228–43.
Kamke J, Taylor MW, Schmitt S. Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *ISME J* 2010;**4**:498–508.
Langille MGI, Zaneveld J, Caporaso JG, *et al.* Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 2013;**31**:814.
Lee OO, Wang Y, Yang J, *et al.* Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *ISME J* 2011;**5**:650–64.
Liu M, Fan L, Zhong L, *et al.* Metaproteogenomic analysis of a community of sponge symbionts. *ISME J* 2012;**6**: 1515–25.
Lücker S, Wagner M, Maixner F, *et al.* A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *P Natl Acad Sci USA* 2010;**107**:13479–84.
McLeod E, Moffitt R, Timmermann A, *et al.* Warming seas in the coral triangle: coral reef vulnerability and management implications. *Coast Manage* 2010;**38**:518–39.
McMurray SE, Henkel TP, Pawlik JR. Demographics of increasing populations of the giant barrel sponge Xestospongia muta in the Florida Keys. *Ecology* 2010;**91**:560–70.
Moitinho-Silva L, Bayer K, Cannistraci CV, *et al.* Specificity and transcriptional activity of microbiota associated with low and high microbial abundance sponges from the Red Sea. *Mol Ecol* 2014;**23**:1348–63.
Montalvo NF, Davis J, Vicente J, *et al.* Integration of culture-based and molecular analysis of a complex sponge-associated bacterial community. *PLoS One* 2014;**9**:e90517.
Montalvo NF, Hill RT. Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. *Appl Environ Microb* 2011;**77**:7207–16.
Oksanen J, Kindt R, Legendre P, *et al. vegan: Community Ecology Package. R Package Version 1.15-2*, 2009.
Pires ACC, Cleary DFR, Almeida A, *et al.* Denaturing gradient gel electrophoresis and barcoded pyrosequencing reveal unprecedented archaeal diversity in mangrove sediment and rhizosphere samples. *App Environ Microb* 2012;**78**: 5520–8.
Polónia ARM, Cleary DFR, Duarte LN, *et al.* Composition of archaea, sediment and sponges in the Kepulauan Seribu reef system, Indonesia. *Microb Ecol* 2014;**67**:553–67.
Proctor LM. Nitrogen-fixing, photosynthetic, anaerobic bacteria associated with pelagic copepods. *Aquat Microb Ecol* 1997;**12**:105–13.
R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2013. http://www.R-project.org/ (23 March 2015, date last accessed).
Rees JG, Setiapermana D, Sharp VA, *et al.* Evaluation of the impacts of land-based contaminants on the benthic faunas of Jakarta Bay, Indonesia. *Oceanol Acta* 1999;**22**:627–40.
Ribes M, Jimenez E, Yahel G, *et al.* Functional convergence of microbes associated with temperate marine sponges. *Environ Microbiol* 2012;**14**:1224–39.
Rohde S, Gochfeld D, Ankisetty S, *et al.* Spatial variability in secondary metabolites of the Indo-Pacific sponge *Stylissa massa*. *J Chem Ecol* 2012;**38**:463–75.
Sipkema D, Franssen MCR, Osinga R, *et al.* Marine sponges as pharmacy. *Mar Biotechnol* 2005;**7**:142–62.
Sorokin DY, Tourova TP, Lysenko AM, *et al.* Thioalkalivibrio thiocyanoxidans sp nov and *Thioalkalivibrio paradoxus* sp nov., novel alkaliphilic, obligately autotrophic, sulfur-oxidizing bacteria capable of growth on thiocyanate, from soda lakes. *Int J Syst Evol Micr* 2002;**52**:657–64.
Southwell MW, Popp BN, Martens CS. Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges. *Mar Chem* 2008;**108**:96–108.
Takai K, Miyazaki M, Hirayama H, *et al.* Isolation and physiological characterization of two novel, piezophilic, thermophilic

chemolithoautotrophs from a deep-sea hydrothermal vent chimney. *Environ Microbiol* 2009;**11**:1983–97.

Tamura K, Peterson D, Peterson N, *et al*. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731–2739.

Tavaré S. Some Probabilistic and statistical problems in the analysis of DNA sequences. *Lect Math Life Sci* 1986;**17**: 57–86.

Thacker RW, Freeman CJ. Sponge-microbe symbioses: recent advances and new directions. In: Becerro MA, Uriz MJ, Maldonado M, *et al*. (eds). *Advances in Sponge Science: Physiology, Chemical and Microbial Diversity, Biotechnology*, Vol. **62**. San Diego: Elsevier Academic Press Inc., 2012, 57–111.

Turque AS, Batista D, Silveira CB, *et al*. Environmental shaping of sponge associated archaeal communities. *PLoS One* 2010;**5**:e15774.

Vaz-Moreira I, Egas C, Nunes OC, *et al*. Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. *Antonie Van Leeuwenhoek* 2011;**100**:245–257.

Wang WC, Yung YL, Lacis AA, *et al*. Greenhouse effects due to man-made perturbations of trace gases. *Science* 1976;**194**:685–90.

Webster NS, Hill RT. The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-proteobacterium. *Mar Biol* 2001;**138**:843–51.

Webster NS, Luter HM, Soo RM, *et al*. Same, same but different: symbiotic bacterial associations in GBR sponges. *Front Microbiol* 2013;**3**:444.

Williams TM, Rees JG, Setiapermana D. Metals and trace organic compounds in sediments and waters of Jakarta Bay and the Pulau Seribu complex, Indonesia. *Mar Pollut Bull* 2000;**40**:277–85.

Yu Y, Lee C, Kim J, *et al*. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol Bioeng* 2005;**89**:670–679.

Zhang Z, Schwartz S, Wagner L, *et al*. A greedy algorithm for aligning DNA sequences. *J Comput Biol* 2000;**7**:203–14.

Zumft WG. The denitrifying prokaryotes. In: Balows A, Truper HG, Dworkin M, *et al*. (eds). *The Prokaryotes*, 2nd edn. New York: Springer-Verlag, 1992, 554–82.