Microbial Diversity in a Military Impacted Lagoon (Vieques, Puerto Rico) as Revealed by Metagenomics

Lizbeth Dávila-Santiago[a,b,c], Natasha DeLeón-Rodriguez[b*], Katia LaSanta-Pagán[a,*], Janet K. Hatt[b], Zohre Kurt[b*], Arturo Massol-Deyá[a#] and Konstantinos T. Konstantinidis[b,c#]

[a] Department of Biology, University of Puerto Rico, Mayagüez, PR

[b] School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA

[c] School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA

Running Head: Microbial Diversity in a Military Impacted Lagoon

[#]Address correspondence to Konstantinidis Konstantinos, kostas@gatech.edu and Arturo Massol-Deyá, arturo.massol@upr.edu

[*]Present address: Natasha DeLeón-Rodriguez, Puerto Rico Science, Technology, and Research Trust, San Juan, PR. Zohre Kurt, Department of Environmental Engineering, Middle East Technical University, Ankara, Turkey

Keywords: lagoons metagenomes, natural attenuation, biodegradation genes

Abstract

The Anones Lagoon, located in the island municipality of Vieques, Puerto Rico (PR), received extensive bombing during military practices by the US Navy for decades. After military activities ceased in 2003, the bombing range was designated as part of a larger Superfund site by US EPA. Here, we employed shotgun metagenomic sequencing to investigate how microbial communities responded to pollution by heavy metals and explosives at this lagoon. Sediment samples (0-5 cm) from Anones were collected in 2005 and 2014 and compared to samples from two reference lagoons, i.e., Guaniquilla, Cabo Rojo (a natural reserve) and Condado, San Juan (PR's capital city). Consistent with selection under low anthropogenic impacts, Guaniquilla exhibited the highest degree of diversity with lower frequency of genes related to xenobiotics metabolism among the three lagoons. Notably, a clear shift was observed in Anones, with *Euryarchaeota* becoming enriched (9% of total) and a concomitant increase in community diversity, by about one order of magnitude, after almost 10 years without bombing activities. In contrast, genes associated with explosives biodegradation and heavy metal transformation significantly decreased in abundance in Anones 2014 (by 91.5%). Five unique population genomes were recovered from the Anones 2005 sample that encoded genetic determinants implicated in biodegradation of contaminants. Collectively, these results provided new insights into the natural attenuation of explosive contaminants by the benthic microbial communities of the Anones lagoon and could serve as reference points to enhance bioremediation actions at this site and for assessing other similarly impacted sites.

Importance

This study represents the first assessment of the benthic microbial community

in the Anones Lagoon in Vieques, Puerto Rico after the impact of intense pollution by bombs and unconventional weapons during military training exercises. Evaluating the microbial diversity of Anones, represents an opportunity to assess the microbial succession patterns during the active process of natural attenuation of pollutants. The culture-independent techniques employed to study these environmental samples allowed the recovery of almost complete genomes of several abundant species that were likely involved in the biodegradation of pollutants and thus, represented species responding to the strong selection pressure posed by military activities. Further, our results showed that natural attenuation has proceeded to a great extend ten years after the cease of military activities.

Introduction

Military exercises have left a legacy of pollution worldwide and represent one of the most substantial anthropogenic disturbances of natural ecosystems. Activities such as naval maneuvers and arms testing have resulted in the contamination of air, water, and soil with heavy metals and explosive compounds (1, 2, 3, 4, 5). In cases where military bases or ranges have been established in natural areas, a wide range of environmental damages has been documented (2, 3, 6, 7). At the moment, little is known about the magnitude of contamination by military training activities since a full-disclosure of what type and quantity of contaminants were used in any given location is typically unavailable. This leads to a potentially serious environmental hazard since explosive compounds cause not only physical damage of the environment where they detonate but also may have long-term consequences due to trace contamination or bi-products that can be arrested in soil, sediments, water, and even leak in groundwater (3, 8). Two of the most commonly used nitramine

explosives in military activities since World War II is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). Both are categorized as carcinogenic by the Environmental Protection Agency (US EPA). Other explosives commonly used are octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 2,4-dinitrotoluene (2,4-DNT) (3, 8). In general, insufficient information is available for the current pollution of soil and sediments by these explosives.

In Puerto Rico (PR), significant areas of the Island municipality of Vieques were used by the US Navy in military training exercises for over 60 years. One of the most impacted areas is the Anones Lagoon located at the center of the former Atlantic Fleet Weapons Training Facilities (9). In 2004, a year after the military activities ceased, the US EPA designated the area as a Superfund Site. Only a few studies on the military impacts on Vieques have been conducted by the US EPA and local scientists (10, 11, 12, 13, 14). These studies highlighted the impacts to plants, algae, animals, and public health caused by the presence of metals, explosive compounds and bi-products. Unfortunately, the ecological impacts in the Anones Lagoon by the long-term bombing are still poorly understood.

The study of microorganisms in these atypical sites could reveal unique diversity associated with environmental pollution and could help estimate, better understand and model the biological attenuation processes associated with the area. For instance, certain microbial species can serve as indicators of pollution or an environmental disturbance (15). Microorganisms can also express specific genes to biodegrade or biotransform contaminants into less hazardous compounds. Specific metabolic pathways for degrading toxic compounds generated by military activities have been elucidated, and are often enriched in this type of environment that is hostile to life (16, 17, 18, 19). Genes, including nitroreductases, have been shown to be

involved in degrading different explosives (20, 21, 22, 23, 24, 25). Although, these approaches have advanced our understanding of the role of microbes in military impacted sites, all rely on culture-dependent techniques in the laboratory and thus, the relevance of the results obtained from *in-situ* activities and processes remain frequently inaccessible.

Various studies have been conducted using metagenomics analysis to better understand environments such as wastewater treatment plants (26), contaminated freshwater and marine sediments by pesticides (27, 28), and contaminated sediments by oil spills (15). Military sites remaining comparatively much less studied by culture-independent techniques, especially in terms of studying the corresponding microbial communities across time. To our knowledge, there has not been a metagenomics survey of the Vieques military-impacted site or similar tropical lagoon sites. Yet, the relative abundance of microorganisms over time and their gene complement at Vieques could provide new insights into the process of natural attenuation of explosive pollutants or enhanced restoration, and serve as biomarkers for predicting the fate of explosives.

We hypothesized that microbial communities in Anones were enriched in broad adaptive strategies, and perhaps unique biodegradation genes of explosives selected by the long-term history of diverse pollution while increase in microbial diversity could indicate progress of natural attenuation processes. We employed shotgun whole-community metagenomics to test this hypothesis and characterize the genetic diversity and biodegradation genes at the Anones Lagoon. Comparative metagenomics with microbial communities from undisturbed and human-impacted lagoons in Puerto Rico revealed that the Anones sediment communities harbor novel diversity that has apparently contributed to the natural restoration of the site.

Results

Site and sample description

The three lagoons used in this study were exposed to different natural or anthropogenic effects. In the case of Guaniquilla Lagoon, The Department of Natural and Environmental Resources of Puerto Rico has since 2002 designated the lagoon as a natural wildlife reserve for native and endemic species (29). On the other hand, Condado lagoon is located in San Juan, which is the capital city (~400,000 inhabitants) and urban area of Puerto Rico. Anthropogenic activities such as urban development with significant sewage discharges has been extensively reported at the site. However, in 2013 the lagoon was designated as an estuarine reservoir (30). Finally, Anones lagoon, the most severely impacted site, was exposed to military training exercises for over 60 years, since it was located in center of the former Atlantic Fleet Weapons Training Facilities (10). At the present time, the lagoon is a Superfund Site designated by US EPA since 2004 and been placed under the jurisdiction of US Fish and Wildlife Service (USFWS). The lagoon is connected to Bahía Salinas del Sur, the bay to the open sea, through a small channel and traces from bullets and bombs are easily spotted at the surface throughout.

Each sediment sample had similar physicochemical characteristics since all lagoons experience similar climatic and edaphic conditions. All samples originated from the surface sediment of each lagoon, as shown in Table 1. In general, pH measurements were nearly neutral, between 7.10 and 7.75, with Anones-2005 being the most alkaline. All samples exceeded the ocean's typical salinity (~3.5%), except Condado. Heavy metals measurements showed that Condado and Anones-2005 had the highest concentration of lead and cadmium, 15.8µg/g, 0.7µg/g and 34.9µg/g,

0.3µg/g, respectively. Anones-2014 exhibited the highest level for copper, 63.4µg/g, while Guaniquilla had the lowest concentration for all three elements (Table 1). According to the interim freshwater sediment quality guidelines (ISQG) of the Canadian Council of Ministers of the Environments (CCME) (31), Anones-2005 exceeded the cadmium guideline of 0.6 mg/kg, while Anones-2014 exceeded the copper guideline of 35.7 mg/kg. These reference thresholds indicate the possible adverse biological effects on aquatic systems (32)(CCME 1995). Interestingly, Anones-2005 was the only sample in which explosives were detected above the U.S EPA method 8330B limit.

Microbial community diversity patterns

DNA extraction for each of the four sediment samples (Anones-2005, Anones-2014, Condado and Guaniquilla) was performed in triplicate. The pool of the triplicate samples from each site was sequenced at about 5 Gbp/sample. As typical of metagenomics surveys of complex sediment samples, sequencing did not cover the total DNA diversity sampled (33). Nonpareil 3.0, a database-independent metric of microbial community complexity and alpha-diversity (33, 34), with default parameters showed that the coverage of each microbial community by sequencing was 28%, 33%, 67.5%, and 13% for Guaniquilla, Condado, Anones-2005, and Anones-2014, respectively. Coverage values and Nonpareil curve projections for complete coverage showed that Anones-2005 saturated faster and required the least sequencing effort for complete coverage, while Anones-2014 required the highest (Figure 1A), revealing that Anones-2005 possessed the least diverse microbial community. In particular, the Nonpareil index of sequence diversity (N_d), revealed that Guaniquilla had the highest value N_d = 21.22, closely followed by Anones-2014

176 at 20.81, whereas Anones-2005 the smallest, N_d =19.68 (note that N_d is a logarithmic
177 scale, thus a difference of 1 corresponds to 10 fold the absolute difference).
178 Comparing the metagenomes from this study to several reference metagenomes
179 determined previously (34), revealed that Guaniquilla and Anones-2014 metagenomes
180 were comparable to other typical sediment samples in terms of coverage and
181 microbial diversity, whereas Anones-2005 was similar to freshwater or heavily
182 impacted environments.
183
184 *Taxonomic composition and relatedness among the sites*
185 pH and salinity have been known to strongly influence the presence and/or
186 relative abundance of microbial populations (35, 36). In Anones-2005 and 2014, the
187 relative abundance of the two most dominant phyla, *Proteobacteria* and *Bacteroidetes*
188 was similar based on MyTaxa analysis of assembled the metagenomic contig
189 sequences (Figure 2A). In Anones-2005, *Firmicutes* was the third most abundant
190 phylum, making up ~12% of the total; with the remaining phyla collectively making
191 up only 9% of the population. In contrast, Anones-2014 was characterized by high
192 abundance of *Euryarchaeota* (9% of the total), followed by *Firmicutes* (5%),
193 revealing a clear broad taxonomical shift after 9 years, accompanied by an increase in
194 microbial diversity. Further, Anones-2005 was dominated by a few operational
195 taxonomic units (OTUs) based on 16S rRNA gene fragments recovered from the
196 metagenomes (Figure 2B), consistent with a low-diversity microbial community.
197 The constantly human-impacted lagoon at Condado was dominated by
198 *Bacteroidetes* (68%) and *Proteobacteria* (29%); and only 3% of the metagenomic
199 sequences were assignable to other phyla. Meanwhile, the Guaniquilla lagoon, the
200 least impacted ecosystem by human activity, did not appear to have a dominant

phylum with higher than ~30% overall relative abundance. Also, Guaniquilla had the highest abundance of unclassified OTUs (7% of the total) and OTUs assigned to *Ignavibacteriae* (7%), which contrasted with <0.5% *Ignavibacteriae* in the other metagenomes (Figure 2A). Consistent with these findings, the MASH-based distances among the metagenomes showed that Condado was the most distant from either of the Anones samples, reflecting presumably its constant human-induced impact, while Guaniquilla appeared to be more similar to Anones-2014 in complexity (Fig. S2).

To further investigate if the high abundance of the *Bacteroidetes* phylum in Condado lagoon reflected the presence of human-gut associated taxa (due to the location of this lagoon in the city of San Juan), the metagenomic reads were searched against the human gut microbiome IGC reference database (37) for high identity matches (>95% of nucleotide identity). Human gut-associated *Bacteroidetes* genera such as *Bacteroides, Prevotella* and *Porphyromonas* were present in Condado at very low relative abundances (< 0.001%), similar to the other lagoons (Fig. S3). Further, 40% of the detected *Bacteroidetes* belonged to the *Flavobacteria* 3% to *Cytophagia* and 3% to *Shingobacteria* classes, which are typically associated with natural settings such as fresh/saltwater, soil, activated sludge, and compost (38). Hence, it appears that environmentally-adapted populations made up most of the *Bacteroidetes* signal in Condado.

Biodegradation genes of explosives and heavy metals

In the Anones samples, a significant functional shift was observed between 2005 and 2014 samples (Figure 3). Anones-2014 showed an average decrease of 91.5% in reads mapping to several key genes encoding enzymes involved in the biodegradation of explosives and nitroreductases, including cytochrome P450-like

protein (*xplA)*, xenobiotic reductase B *(xenB)*, xenobiotic reductase A *(xenA)*, major oxygen-insensitive nitroreductase (*nfsA*), oxygen-insensitive nitroreductase B (*nfsB*), oxygen-insensitive nitroreductase *(nitA)*, oxygen-insensitive nitroreductase *(nitB)*, cadmium-transporting ATPase P-type (*cadA)*, zinc, cobalt and lead efflux system (*zntA)*, Pb(II) resistance ATPase (*pbrA)*, copper-exporting P-type ATPase A *(copA)*, copper-exporting P-type ATPase B *(copB)* genes compared to Anones-2005; while *xplA, xenB, nfsB, nitA* and *cadA* did not recruit any reads. In fact, Anones-2014 looked more similar to the reference, pristine lagoons in this respect, e.g., only 0.017%, 0.018% and 0.027% of the total reads mapped to the above mentioned genes for Anones-2014, Guaniquilla, and Condado, respectively, contrasting with 0.2% for Anones-2005, i.e., a ~10 fold higher abundance, on average (Table S2). Interestingly, *copA*, a copper-exporting P-type ATPase, appeared to be the gene with the highest number of matching reads in all metagenomes. This finding is consistent with *in-situ* copper concentrations in each sediment lagoon, which were higher than the other two heavy metals assessed.

Novel organisms in Anones 2005

Due to the low complexity of Anones-2005, we were able to recover the genome of five metagenome-assembled genomes (MAGs) representing relatively abundant populations, with high completeness (>83.2%) and low contamination (<10%) using binning techniques (Table 2). The estimated size of these genomes ranger between 2.6 and 4.5 Mbp. Their closest relative in NCBI's RefSeq prokaryotic genome database showed <50% genome-aggregate amino-acid identity (AAI) and was affiliated with different phyla, indicating that these genome bins (MAGs) represent diverse, novel genera, if not families (39). MAG 2 especially appeared to

represent a class-level novel taxon with a low GC (%) content of 31.7% in comparison with the other MAGs (Table S3). Interestingly, all MAGs recovered in Anones-2005 did not appear to be present 9 years later in Anones-2014 (Fig. S4). MAG 3 had a related but distinct microbial population in the Condado metagenome (~90% AAI; Fig. S4). Also, MAG 2 and MAG 7 appear to have a related microbial population in Guaniquilla (Fig. S4).

MAG 1 (*Rhodospirilaceae* sp., *Alphaproteobacteria*) had a relative abundance of 12% (of the total metagenome) and, together with MAG 3 (*Gammaproteobacteria* sp.) (6.5%), were assignable to the *Proteobacteria* phylum. MAG 2 (*Mollicutes* sp.) (4.8%) was assignable to the *Tenericutes* phylum. *Mollicutes* (MAG 2) and *Bacteroidetes* sp. (MAG 5) represented more deep-branching members of the *Tenericutes* and *Bacteroidetes* phyla, respectively, compared to MAG 1 and MAG 3, based on their best AAI values against RefSeq genomes, and phylogenetic relationships using 57 universal housekeeping genes (Figure 4). All five genomes together accounted for about ~29% of the total metagenome.

Functional description of MAGs

As expected, subsystem categories such as protein metabolism, cofactors, vitamins, prosthetic group, pigments, and amino acid and derivatives were the most abundant pathways in each MAG, followed by DNA and RNA metabolism (Table S4). In addition, each MAG encoded various specialized functions such as: (1) cellular response to DNA damage, (2) response to heat, (3) response to stress, (4) sodium ion transport, (5) SOS response, while no MAG represented photosynthetic bacteria (Figure 5). All MAGs also encoded various manually verified genes with significant homology (e.g., >30% amino acid identity across >70% of the gene length)

to genes previously shown to be involved in the transformation and biodegradation of explosives and heavy metals resistance. A brief description of the functional gene content of each MAG follows:

MAG 1 (*Rhodospirilaceae* sp.):

The most abundant MAG appears to belong to the *Proteobacteria* phylum, has a genome size of 3.1Mbp, a GC content of 62.6%, and coding density of ~90%. MAG 1 harbors genes for sulfate assimilation, including adenylyl-sulfate kinase (*cysC*) and phosphoadenosine phosphosulfate reductase (*cysH*) genes. Therefore, this genome is likely from a sulfate-reducing bacterium using sulfate as terminal electron acceptor. Interestingly, MAG 1 is unique among the other four MAGs in harboring the full gene complement (e.g., 25 genes) of a nitrogen fixer, including nitrogenase iron protein 1 (*nifH*). MAG 1 also has genes for chemotaxis, including flagellar motor switch proteins, such as *fliN* and *fliG*, indicating that it is potentially motile. Presence of efflux system proteins involved in response to antibiotics were also detected. In addition, MAG 1 harbors a (predicted) homologous gene to xenobiotic reductase (*xenA*) and copper exporting (*copA*) (Table S5).

MAG 2 (*Bacilli sp.*) and MAG 7 (*Spirochaetales sp.*):

MAG 2 was the most deep-branching of all MAGs with respect to the available genomes of isolates in NCBI's RefSeq prokaryotic database and did not appear to be motile, with a genome size of 2.3Mbp, GC content of 31.7%, and coding density of ~93%. MAG 2 has genes for resistance to antibiotics, reactive oxygen species, and ultraviolet radiation (UV) by homodimerization activity. Viral genome integration proteins, including *int* for integrase functions, appears to be present,

indicating vphage predation for this population. Using PHASTER with default parameters (40), only this MAG showed the presence of phage-like proteins, including transposase, portal protein, integrase, head protein and terminase in three different contigs. However, the phage genome was not intact/complete, but most likely represents a remnant prophage. For specific genes of interest, this MAG harbors (predicted) genes associated with cadmium transporter (*cadA*) and copper exporter (*copA),* xenobiotic reductase (*xenA*) and oxygen nitroreductase (*nitB*) (Table S5).

MAG 7 has a genome size of 2.7Mbp, GC content of 50.8%, and coding density of ~93.2%. This genome harbors sulfur utilization proteins such as L-cystine-binding protein (*fliY*) and antibiotic resistance genes (e.g., efflux pump systems). Genes encoding the homologous functions for oxygen nitroreductase (*nitA*), xenobiotic reductase (*xenA*), copper exporting (*copB*) were also observed (Table S5).

MAG 3 (*Gammaproteobacteria sp.*):

The second most abundant genome appears to belong to the *Proteobacteria* phylum, has a genome size of 3.2Mbp, GC content of 51.2%, and coding density of ~90%. The presence of nitrate and nitrate reductases indicated that MAG 3 was a denitrifying bacterium encoding primarily facultative anaerobic heterotrophic lifestyle. MAG 3 also harbors genes for chemotaxis (*fliN* and *fliG*) and thus, like MAG 1, appears to be motile. Also present were genes for antibiotic resistance (e.g., efflux pump system), and response to gamma and ultraviolet radiation. For specific genes of interest, MAG 3 appears to carry a gene encoding the XenB protein, which works under anaerobic conditions and less toxic compounds are produced during biodegradation, (50%, that has a high amino acid identity to a previously

325 characterized XenB (41)), oxygen nitroreductase (*nfsB)*, and copper exporting (*copA*)
326 genes (Table S5).
327
328 MAG 5 (*Bacteroidetes sp.*):
329 MAG 5 has a genome size of 3.2Mbp, GC content of 42.2%, and coding
330 density of ~92.5%. It harbors sulfate assimilation genes, including phosphoadenosine
331 phosphosulfate reductase (*cysH*); therefore could be a sulfate-reducing bacterium,
332 likely using sulfate as terminal electron acceptor. It also harbors nitrous-oxide
333 reductase (*nosZ*) gene, i.e., nitrous oxide reductase to atmospheric dinitrogen. MAG 5
334 also possesses genes for *fliN* and *fliG* genes involved in chemotaxis and thus, likely
335 represents a motile population. Also, present are genes involved in antibiotic
336 resistance (e.g., efflux system), response to radiation, including UV by
337 homodimerization activity, and presence of *int* gene, for viral/prophage integration.
338 Finally, this MAG possesses homologs of oxygen nitroreductase (*nfsA)*, copper
339 exporting (*copA*, *copB*), cadmium transporter (*cadA*), and lead resistance (*pbrA*)
340 genes (Table S5).
341
342 **Discussion**
343 The microbial community structure of surface sediment samples from three
344 different coastal lagoons in Puerto Rico was evaluated. Anones was impacted by a
345 major and toxic disturbance; the lagoon was sampled two years after continuous
346 pollution disturbance stopped and then 11 years later. Condado has continuous
347 anthropogenic disturbance due to its proximity to Puerto Rico's capital city. Finally,
348 Guaniquilla lagoon which is a proxy for a pristine lagoon, since it is a natural reserve.
349 Collectively, our results showed that microbial diversity of the Anones Lagoon during
350 2005 was negatively affected by military activities since microbial community was

ten times or more less complex than the average sediment from reference lagoons or Anones nine years later, and encoded at least 10 times more genes related to biotransformation of xenobiotics and heavy metals. These results indicated that continuously bombing for decades selected for a few populations well-adapted to an environment containing explosive chemicals. Nonetheless, about a decade later, conditions became favorable for bacterial diversity to recover, at least partially. Our findings are consistent with several studies that have documented reduction in microbial community diversity following a major environmental disturbance (42), but also enrichment of specific microbes after exposure to explosives (19, 43, 44, 45, 46, 47).

The substantial microbial community shifts from 2005 to 2014 in Anones also indicated a well-adapted/enriched community to the explosives and under transition in 2005. The major changes observed in microbial composition and diversity nine years after indicates that changes were likely occurring at the end of 2003, when bombing ceased, and the 2005 sample represented an early recovery stages of the microbial community from the use of explosives. Anones-2005 also had the most alkaline pH among the sampling sites, contrary to other military impacted zones with acidic pH (7), which is consistent with a transitional state for the 2005 community. Unfortunately, samples from 2003 or earlier were not available to further corroborate these interpretations. Further, enrichment of *Archaea* has previously been shown to be related to community recovery from an oil spill (15), and *Euryarcheaota* were collectively much more abundant in Anones 2014 vs. 2005. Therefore, it appears that such archaeal populations may represent good indicators of less-polluted ecosystems or recovered communities.

375 The Anones-2014 microbial community was characterized by a higher
376 number of microbial taxa and lower frequency of biodegradation process genes
377 compared to Anones-2005 or Condado lagoons. The decrease in relative abundance of
378 known genes related to the transformation/biodegradation of explosive compounds
379 between 2005 and 2014 samples strongly indicated that Anones Lagoon has been
380 undergoing natural attenuation. For instance, nitroreductases such as those detected in
381 the Anones-2005 metagenomes and MAGs are known to act by cleaving
382 nitroaromatics rings, including those found in explosives like TNT (22, 23, 24, 25).
383 These findings further confirmed that microbes are capable of faster recovery and
384 adaptation when compared to multicellular organisms (48, 49, 50), and can be used as
385 more sensitive biomarkers of the current state and future projection of ecosystem
386 recovery (51).

387 Bioinformatics functional prediction of the recovered MAGs from Anones-
388 2005 indicated that the organisms sampled were, at least partially, responsible for the
389 bioremediation enhancement in Anones. MAGs encoded genes related to explosives
390 and heavy metals (e.g., cadmium, copper, and lead resistance genes), including genes
391 for the biodegradation/transformation of nitroaromatics such as TNT and RDX (16,
392 17, 18), that were absent in other lagoons or Anones-2014. Also, based on AAI
393 values, these organisms represented at least novel families, revealing that disturbed
394 tropical sites by military activities may have selected for novel organisms that need to
395 be studied in more detail both taxonomically as well as functionally for their
396 biodegradation/transformation potential.

397 The differences in microbial community composition observed in the
398 Anones samples are unlikely to be attributed to seasonal effects or sample
399 heterogeneity. First, our samples represent composite samples of multiple DNA

extractions in order to reduce sample-specific patterns and were collected at the same time of the year (summer). Further, previous studies have shown that bacterial communities inhabiting sediments of tropical coastal lagoons do not show strong seasonal patterns (52, 53), consistent with relative small seasonal variations in temperature in the tropics.

In conclusion, this study has revealed the potential functions and organisms associated with transformation/biodegradation of explosives and resistance of heavy metals in a former bombing range. Even though our results indicated that natural attenuation has been occurring since 2005 in Anones, it is important to note that it only involved the surface of the lagoon's sediment. Thus, the structure of microbial communities residing at deeper sediments remains uncharacterized. Several questions also remain to be addressed in future studies; most notably, how much the identified microbial populations contributed to natural attenuation and if their activities are adequate for complete bioremediation of a site constantly disturbed for over 60 years by military activities. Tracking temporal shifts after the disturbance, coupled to *in-situ* rate measurements, could be a useful approach to better quantify the role of benthic microbes for natural attenuation of explosives and other environmental pollutants, and provide biomarkers for better modeling the attenuation process and predicting the toxic effects of specific chemical compounds. The genes and genome sequences recovered here can also provide reference points for future experiments related to the remediation of Anones or other contaminated sites, e.g., by providing sequences for qPCR assays.

Materials and Methods

Sampling

Soil samples from the sediment surface (0-5cm), where heavy metals and explosives residues were mainly deposited (6, 7), were collected in Corning® 50mL centrifuge tubes from Anones at two time points (October 2005 and June 2014). Similarly, sediment surface soil smaples from the pristine Guaniquilla and urban impacted and Condado lagoons (Fig. S1) were collected in June 2013. All samples were stored at 4°C until further analyzed. Temperature measurements were taken *in-situ* by immersion of a mercury thermometer in sediment; a salinity refractometer was used to measure salinity of water above the sampled sediment, and pH measurements were taken on the samples in the laboratory with a pH meter (ATI Orion Model 230A).

Heavy Metal and *Explosive Concentrations*

To measure heavy metals an acid extraction was done, approximately 3g of homogenous sediment sample was incinerated for 3hrs at 600°C in ceramic crisols. After incineration, samples were pulverized with approximately 1mL of concentrated HCl and transferred to a 250mL beaker by rinsing with 1mL of HCl for a total of three washes in order to transfer all the sample to the beaker. One mL of nitric acid was added to each sample while warming samples without drying, for a total of two washes and a last wash of 3mL concentrated HCl. By the end of the washes, the sediment was white in color. The acid extraction was then filtered through a Whatmann filter #40 and 5% HCl added for a total of 100mL of sample. Lead (Pb), cadmium (Cd), and copper (Cu) were measured by atomic absorption spectrophotometer (Perkin Elmer Model AA100). Concentrations for RDX and 2,6-DNT were measured by High Performance Liquid Chromatography (HPLC) according to U.S EPA method 8330B (54).

DNA extraction protocol

Metagenomic DNA extraction was performed with the modified DNA extraction protocol Method 2 using the QIAamp DNA Micro Kit from QIAGEN® (55). Briefly, approximately 0.5g of homogeneous sediment sample was subjected to a two-step cell lysis. First, a chemical lysis was performed by addition of an enzyme cocktail as follows: Mutanolysin (1,500 u/ml), Lysostaphin (510 u/ml), and Lysozyme (10mg for 2ml), plus a lysis buffer [0.5M EDTA and of 1M Tris (pH 8.3)] and incubation at 37°C for 1h with rotation to mix. Second, a physical lysis was performed with approximately 0.22g of each bead (0.1mm glass and 0.5mm ziconia/silica beads) and 600µl Phenol:Chloroform at 3,500rpm for 1:30 min. After centrifugation at 3,000rpm for 5min and transferring of the supernatant to a new microtube, AL Buffer (QIAGEN kit) and 100% EtOH were added to the supernatant. The rest of the protocol followed the QIAamp DNA Micro Kit manual from step 17 to protocol end.

High-throughput sequencing and sequences processing

Community DNA (libraries) was sequenced using an Illumina MiSeq reagent V2 kit for 500 cycles (2 x 250 bp paired end run) on an Illumina MiSeq instrument (located in the School of Biological Sciences, Georgia Institute of Technology). Prior to sequencing, DNA sequencing libraries were prepared using the Illumina Nextera XT DNA library prep kit according to manufacturer's instructions except the protocol was terminated after isolation of cleaned double stranded libraries. Library concentrations were determined by fluorescent quantification using a Qubit HS DNA kit and Qubit 2.0 fluorometer (ThermoFisher Scientific) and samples run on a High

Sensitivity DNA chip using the Bioanalyzer 2100 instrument (Agilent) to determine library insert sizes. Adapter trimming and de-multiplexing of sequenced samples was carried out on the MiSeq instrument. Raw metagenomic reads were trimmed using Solexa Q4 (56). Each resulting trimmed pair-end read was merged together with its sister read, when overlapping, using PEAR with default parameters (57) (Table S1).

Metagenome coverage and de-novo assembly

Nonpareil, which assesses coverage of extracted community DNA by sequencing based on the frequency of unmatched reads in the metagenomic datasets (35, 58), was used determine the level of microbial community coverage and sequence diversity. In the present study, Nonpareil 3.0 was used, which represents a faster, k-mer-based method than the original Nonpareil, and in addition, provides an estimation of the alpha-diversity of the sample (58). Assembly of the metagenomic reads was performed with IDBA 1.1.1 (59) with a minimum k-mer value of 35 and a maximum value of 75. The k-mer size that resulted in the highest number of assembled reads was selected for each metagenome.

Taxonomic classification of DNA sequences and estimation of in-situ relative abundance

Taxonomic classification was assessed in two ways: (1) assembled contigs or binned genomes were classified using the stand-alone MyTaxa analysis (60) and reported at the Phylum level; (2) 16S rRNA gene-encoding reads were identified using Parallel-META 2.0 (61) followed by QIIME 1.9.0 (62) for taxonomic classification and the top 75 most abundant genera reported.

Trimmed reads were mapped on predicted genes of contigs using BLAT in

order to assess relative abundance of the gene or population bin, based on a minimum cut-off for a match of 97% nucleotide identity. Finally, a distance matrix was developed to estimate sequence relatedness between metagenomes based on MASH distance analysis (63) at the whole metagenome level, and visualized using the PCA plot function of the QIIME principal_coordinates.py script (62).

Gene prediction and functional annotation of biodegradation genes

Gene prediction was performed with MetaGeneMark (64) using trimmed metagenomic reads or assembled contig sequences as input. Predicted genes were compared against a manually curated *in-house* database of biodegradation genes using BLAST (65) for complete alignment, conservation of functional domains and at least 30% amino acid identity (minimum bitscore cutoff of 60 for a match). The database included genes related to explosives (*xplA, xenB, xenA, nfsA, nfsB, nitA, nitB*) and heavy metals resistance for Pb, Cd and Cu (*bmtA, cadA, zntA, pbrA, cadD, copA, copB*).

Recovering population genomes by binning

Assembled contigs for each metagenome were binned into MAGs using MaxBin software (66) in order to obtain whole or partial genomes. For each MAG, coverage, genome size, completeness, and contamination were estimated using the HMM.essential.rb script as implemented in the Microbial Genomes Atlas (MiGA) webserver (67). Average Amino Acid identity (AAI) values (68) against the RefSeq prokaryotic genome database from NCBI (https://www.ncbi.nlm.nih.gov/refseq) were also computed by the MiGA webserver.

To further improve the quality of the recovered MAG sequences, the likely phylogenetic origin of the contigs was evaluated as follows: every gene encoded by the contigs of a MAG was searched against the RefSeq prokaryotic genome database for its best match. Contigs that provided matches to different taxonomic families or provided highly divergent AAI values were manually removed from the MAG. Phylogenetic relationships of the resulting MAGs and their best-three matching RefSeq genomes were determined based on their AAI values as well as sequence alignment of 57 essential genes shared by all genomes using RAxML (69) and were checked for consistency. Functional analysis for each MAG was performed with SEED and Swiss-Prot database for more specific functional prediction. Predicted genes were also compared to manually curated biodegradation genes related to explosives and heavy metals resistance as described above.

The raw sequences of each metagenome are available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/SRP156313) under Bioproject "Vieques metagenomes" (PRJNA483958) and accession numbers: SAMN09754619, SAMN09754620, SAMN09754621, SAMN09754622. MAG sequences are available through http://enve-omics.ce.gatech.edu/data/vieques

Acknowledgments:

Special thanks to Cacimar Zenón for his help during the sampling trips to Vieques, Elba Díaz for advice on chemical analysis, and Casa Pueblo in Adjuntas for support during the project. Thanks also to Juliana Soto, Luis Miguel Rodríguez-R and Carlos Rodríguez Minguela for helpful discussions related to the analysis of the

metagenomic datasets. This research was supported by U.S. National Science Foundation (award 1241046).

Conflict of interest

The authors declare no conflict of interest.

References

1. Lee IS, Kim OK, Chang YY, Bae B, Kim HH, Baek KH. 2002. Heavy Metal Concentrations and Enzyme Activities in Soil from a Contaminated Korean Shooting Range. J Biosci Bioeng 94:406–411.

2. Kim S, Baek K, Lee I. 2010. Phytoremediation and microbial community structure of soil from a metal-contaminated military shooting range: Comparisons of field and pot experiments. J Environ Sci Health A Tox Hazard Subst Environ Eng 45:389–394.

3. Pichtel J. 2012. Distribution and Fate of Military Explosives and Propellants in Soil: A Review. Appl Environ Soil Sci 2012: 617236.

4. Jung H, Yun S, Choi B, Kim H, Jung M, Kim S, Kim K. 2010. Geochemical studies on the contamination and dispersion of trace metals in intertidal sediments around a military air weapons shooting range. J Soils Sediments 10:1142–1158.

5. Etim EU, Onianwa PC. 2012. Lead contamination of soil in the vicinity of a military shooting range in Ibadan, Nigeria. Toxicol Environ Chem, 94:895–905.

574

575 6. Jenkins TF, Pennington JC, Ranney TA, Berry Jr TE, Miyares PH, Walsh ME,
576 Hewitt AD, Perron NM, Parker LV, Hayes CA, Wahlgren EG. 2001.
577 Characterization of Explosives Contamination at Military Firing Ranges. U.S.
578 Army Engineer Research and Development Center Cold Regions Research and
579 Engineering Laboratory.

580

581 7. Clausen J, Robb J, Curry D, Korte N. 2004. A case study of contaminants on
582 military ranges: Camp Edwards, Massachusetts, USA. Environ Pollut 129:13–21.

583

584 8. Rylott EL, Lorenz A, Bruce NC. 2011. Biodegradation and biotransformation of
585 explosives. Curr Opin Biotechnol 22:434–440.

586

587 9. Sasha Davis J, Hayes-Conroy JS, Jones VM. 2007. Military pollution and natural
588 purity: seeing nature and knowing contamination in Vieques, Puerto Rico.
589 GeoJournal 69: 165–179.

590

591 10. Díaz E, Massol-Deyá A. 2003. Trace Element Composition in Forage Samples
592 from a Military Target Range, Three Agricultural Areas, and One Natural Area in
593 Puerto Rico. Caribbean Journal of Science 39:215–220.

594

595 11. Ortiz-Roque C, López-Rivera Y. 2004. Mercury contamination in reproductive
596 age women in a Caribbean island: Vieques. J Epidemiol Community Health 58,
597 756–757.

598

12. Massol-Deyá A, Pérez D, Pérez E, Berrios M, Díaz E. 2005. Trace Elements Analysis in Forage Samples from a US Navy Bombing Range (Vieques, Puerto Rico). Int J Environ Res Public Health 2:263–266.

13. Hernández-Delgado EA, Montañez-Acuña A, Otaño-Cruz A, Suleimán-Ramos SE. 2014. Bomb-cratered coral reefs in Puerto Rico, the untold story about a novel habitat: from reef destruction to community-based ecological rehabilitation. Rev Biol Trop 62:183–200.

14. Díaz E, Pérez D, Pérez E, Delgado Acevedo Johanna, Massol-Deyá A. 2018. Longitudinal survey of lead, cadmium, and copper in seagrass Syringodium filiforme from a former bombing range (Vieques, Puerto Rico). Toxicol Rep 5:6-11.

15. Rodriguez-R, LM, Overholt WA, Hagan C, Huettel M, Kostka JE, Konstantinidis KT. 2015. Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. ISME J 9:1928-1940.

16. Thompson KT, Crocker FH, Fredrickson HL. 2005. Mineralization of the Cyclic Nitramine Explosive Hexahydro-1,3,5-Trinitro-1,3,5-Triazine by Gordonia and Williamsia spp. Appl Environ Microbiol 71:8265–8272.

17. Seth-Smith HMB, Edwards J, Rosser SJ, Rathbone DA, Bruce NC. 2008. The Explosive-Degrading Cytochrome P450 System Is Highly Conserved among Strains of Rhodococcus spp. Appl Environ Microbiol 74:4550–4552.

18. Indest KJ, Jung CM, Chen H, Hancock D, Florizone C, Eltis LD, Crocker FH. 2010. Functional Characterization of pGKT2 , a 182-Kilobase Plasmid Containing the xplAB Genes, Which Are Involved in the Degradation of Hexahydro-1,3,5-Trinitro-1,3,5- Triazine by Gordonia sp. Strain KTR9. Appl Environ Microbiol 76:6329–6337.

19. Jayamani I, Cupples AM. 2015. A comparative study of microbial communities in four soil slurries capable of RDX degradation using illumina sequencing. Biodegradation 26: 247–257.

20. Zenno S, Koike H, Tanokura M, Saigo K. 1996. Gene Cloning, Purification, and Characterization of NfsB, a Minor Nitroreductase from Escherichia coli, Similar Biochemical Properties to FRase I, the Major Flavin Reductase in Vibrio fischeri. J. Biochem, 120:736–744.

21. Blehert DS, Fox BG, Chambliss GH. 1999. Cloning and Sequence Analysis of Two Pseudomonas Flavoprotein Xenobiotic Reductases. J of Bacteriol 181:6254–6263.

22. Seth-Smith HM, Rosser SJ, Basran A, Travis ER, Dabbs ER, Nicklin S, Bruce NC. 2002. Cloning, Sequencing, and Characterization of the Hexahydro-1,3,5-Trinitro-1,3,5-Triazine Degradation Gene Cluster from Rhodococcus rhodochrous. Appl Environ Microbiol 68:4764–4771.

649 23. Kutty R, Bennett GN. 2005. Biochemical characterization of trinitrotoluene
650 transforming oxygen-insensitive nitroreductases from Clostridium acetobutylicum
651 ATCC 824. Arch Microbiol 184:158–167.
652
653 24. Roldán MD, Pérez-Reinado E, Castillo F, Moreno-Vivián C. 2008. Reduction of
654 polynitroaromatic compounds: the bacterial nitroreductases. FEMS Microbiol Rev
655 32:474–500.
656
657 25. Halasz A, Manno D, Perreault NN, Sabbadin F, Bruce NC, Hawari J. 2012.
658 Biodegradation of RDX Nitroso Products MNX and TNX by Cytochrome P450
659 XplA. Environ Sci Technol 46:7245–7251.
660
661 26. Fang H, Cai L, Yu Y, Zhang T. 2013. Metagenomic analysis reveals the
662 prevalence of biodegradation genes for organic pollutants in activated sludge.
663 Bioresour Technol 129:209–218.
664
665 27. Sangwan N, Lata P, Dwivedi V, Singh A, Niharika N, Kaur J, Anand S, Malhotra
666 J, Jindal S, Nigam A, Lal D, Dua A, Saxena A, Garg N, Verma M, Kaur J,
667 Mukherjee U, Gilbert JA, Dowd SE, Raman R, Khurana P, Khurana JP, Lal R.
668 2012. Comparative Metagenomic Analysis of Soil Microbial Communities across
669 Three Hexachlorocyclohexane Contamination Levels. PLoS One 7(9).
670
671 28. Fang H, Cai L, Yang Y, Ju F, Li X, Yu Y, Zhang T. 2014. Metagenomic analysis
672 reveals potential biodegradation pathways of persistent pesticides in freshwater
673 and marine sediments. Sci Total Environ 470-471:983–992.

29. Fuentes-Santiago, G., and Quevedo-Bonilla, V. 2002. Documento de Designación Reserva Natural Punta Guaniquilla. Departamento de Recursos Naturales y Ambientales. San Juan, Puerto Rico.

30. DRNA. (2016). Plan de Manejo para la Reserva Natural Estuarina de la Laguna del Condado. Departamento de Recursos Naturales y Ambientales. San Juan, Puerto Rico.

31. Canadian Council of Ministers of the Environment (CCME). 2001. Canadian sediment quality guidelines for the protection of aquatic life: Summary Tables. Updated. In Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, Winnipeg.

32. Canadian Council of Ministers of the Environment (CCME). (1995). Protocol for the derivation of Canadian sediment quality guidelines for the protection of aquatic life. CCME EPC-98E. Prepared byEnvironment Canada, Guidelines Division, Technical Secretariat of the CCME Task Group on Water Quality Guidelines, Ottawa.

33. Rodriguez-R, L.M., and Konstantinidis, K.T. 2014. Estimating coverage in metagenomic data sets and why it matters. ISME J 8:2349–2351.

34. Rodriguez-R LM, Konstantinidis KT. 2014. Nonpareil: a redundancy-based approach to assess the level of coverage in metagenomic datasets. Bioinformatics

699 30:629–635.

700

701 35. Canfora, L., Bacci, G., Pinzari, F., Lo Papa, G., Dazzi, C., and Benedetti, A. 2014.
702 Salinity and Bacterial Diversity: To What Extent Does the Concentration of Salt
703 Affect the Bacterial Community in a Saline Soil? PLoS One 9(9).

704

705 36. Yang, J., Ma, L., Jiang, H., Wu, G., and Dong, H. 2016. Salinity shapes microbial
706 diversity and community structure in surface sediments of the Qinghai-Tibetan
707 Lakes. Sci Rep 6:6–11.

708

709 37. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, Manimozhiyan Arumugam,
710 Kultima JR, Prifti E, Nielsen T, Juncker AS, Manichanh C, Chen B, Zhang W,
711 Levenez F, Wang J, Xu X, Xiao L, Liang S, Zhang D, Zhang Z, Chen W, Zhao H,
712 Yousuf Al-Aama J, Edris S, Yang H, Wang J, Hansen T, Nielsen HB, Brunak S,
713 Kristiansen K, Guarner F, Pedersen O, Doré J, Ehrlich SD, Consortium M, Bork
714 P, Wang J. 2014. An integrated catalog of reference genes in the human gut
715 microbiome. Nat Biotechnol 32:834–841.

716

717 38. Thomas F, Hehemann JH, Rebuffet E, Czjzek M, Michel G. 2011. Environmental
718 and gut Bacteroidetes: the food connection. Front Microbiol 2:93.

719

720 39. Konstantinidis KT, Tiedje JM. 2005. Towards a Genome-Based Taxonomy for
721 Prokaryotes. Journal of Bacteriology, 187: 6258-6264.

722

723 40. Arndt D, Grant J, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS. 2016.

724 PHASTER: a better, faster version of the PHAST phage search tool. Nucleic
725 Acids Res 44:W16–W21.
726
727 41. Fuller ME, McClay K, Hawari J, Paquet L, Malone TE, Fox BG, Steffan RJ.
728 2009. Transformation of RDX and other energetic compounds by xenobiotic
729 reductases XenA and XenB. Appl Microbiol Biotechnol 84:535–544.
730
731 42. Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, Huber DH,
732 Langenheder S, Lennon JT, Martiny JBH, Matulich KL, Schmidt TM,
733 Handelsman J. 2012. Fundamentals of microbial community resistance and
734 resilience. Front Microbiol 3:1–19.
735
736 43. Bhatt M, Zhao JS, Monteil-Rivera F, Hawari J. 2005. Biodegradation of cyclic
737 nitramines by tropical marine sediment bacteria. J Ind Microbiol Biotechnol
738 32:261–267.
739
740 44. Meyers SK, Deng S, Basta NT, Clarkson WW, Wilber GG. 2007. Long-Term
741 Explosive Contamination in Soil: Effects on Soil Microbial Community and
742 Bioremediation. Soil & Sediment Contam 16:61–77.
743
744 45. George I, Eyers L, Stenuit B, Agathos SN. 2008. Effect of 2,4,6-trinitrotoluene on
745 soil bacterial communities. J Ind Microbiol Biotechnol 35:225–236.
746
747 46. Travis ER, Bruce NC, Rosser SJ. 2008. Short term exposure to elevated
748 trinitrotoluene concentrations induced structural and functional changes in the soil

749 bacterial community. Environ Pollut 153:432–439.

750

751 47. George IF, Liles MR, Hartmann M, Ludwig W, Goodman RM, Agathos SN.
752 2009. Changes in soil Acidobacteria communities after 2 , 4 , 6-trinitrotoluene
753 contamination. FEMS Microbiol Lett 296:150–166.

754

755 48. Rocheleau S, Lachance B, Kuperman RG, Hawari J, Thiboutot S, Ampleman G,
756 Sunahara GI. 2008. Toxicity and uptake of cyclic nitramine explosives in ryegrass
757 Lolium perenne. Environ Pollut 156:199–206.

758

759 49. El-Serehy HA, Aboulela H, Al-Misned F, Kaiser M, Al-Rasheid K, El-Din HE.
760 2012. Heavy metals contamination of a Mediterranean Coastal Ecosystem,
761 Eastern Nile Delta, Egypt. Turk J Fish Aquat Sci 12:751–760.

762

763 50. Koide S, Silva JAK, Dupra V, Edwards M. 2016. Bioaccumulation of chemical
764 warfare agents, energetic materials, and metals in deep-sea shrimp from discarded
765 military munitions sites off Pearl Harbor. Deep-Sea Research II 128:53-62

766

767 51. Brooks AN, Turkarslan S, Beer KD, Yin Lo F, Baliga NS. 2012. Adaptation of
768 cells to new environments. Wiley Interdiscip Rev Syst Biol Med 3:544–561.

769

770 52. Pringault O, Duran R, Jacquet S, Torréton JP. 2008. Temporal Variations of
771 Microbial Activity and Diversity in Marine Tropical Sediments (New Caledonia
772 Lagoon). Microb Ecol 55:247–258.

773

774 53. Azandégbé A, Poly F, Andrieux-Loyer F, Kérouel R, Philippon X, Nicolas J.
775 2012. Influence of oyster culture on biogeochemistry and bacterial community
776 structure at the sediment – water interface. FEMS Microbiol Ecol 82:102–117.
777
778 54. US Environmental Protection Agency (EPA). 2006. Method 8330B (SW-846):
779 Nitroaromatics, Nitramines, and Nitrate Esters by High Performance Liquid
780 Chromatography (HPLC), Revision 2.
781
782 55. Yuan S, Cohen, D.B., Ravel, J., Abdo, Z., and Forney, L.J. 2012. Evaluation of
783 Methods for the Extraction and Purification of DNA from the Human
784 Microbiome. PLoS One 7(3).
785
786 56. Cox MP, Peterson DA, Biggs PJ. 2010. SolexaQA: At-a-glance quality
787 assessment of Illumina second-generation sequencing data. BMC Bioinformatics,
788 11:485.
789
790 57. Zhang J, Kobert K, Flouri T, Stamatakis A. 2014. PEAR: a fast and accurate
791 Illumina Paired-End reAd mergeR. Bioinformatics 30:614–620.
792
793 58. Rodriguez-R LM, Gunturu S, Tiedje JM, Cole JR, Konstantinidis KT. 2018.
794 Nonpareil 3: Fast Estimation of Metagenomic Coverage and Sequence Diversity.
795 mSystems 3:3.
796
797 59. Peng Y, Leung H, Yiu SM, Chin FYL. 2010. IDBA - A Practical Iterative de
798 Bruijn Graph De Novo Assembler. RECOMB 6044:426–440.

799

800 60. Luo C, Rodriguez-R LM, Konstantinidis KT. 2014. MyTaxa: an advanced
801 taxonomic classifier for genomic and metagenomic sequences. Nucleic Acids Res
802 42:1–12.

803

804 61. Su X, Pan W, Song B, Xu J, Ning K. 2014. Parallel-META 2.0: Enhanced
805 Metagenomic Data Analysis with Functional Annotation, High Performance
806 Computing and Advanced Visualization. PLoS One 9:3.

807

808 62. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Elizabeth K
809 Costello EK, Fierer N, Gonzalez Peña A, Goodrich JK, Gordon JI, Huttley GA,
810 Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge
811 BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J,
812 Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-
813 throughput community sequencing data. Nat Methods 7:336-336.

814

815 63. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S,
816 Phillippy AM. 2016. Mash: fast genome and metagenome distance estimation
817 using MinHash. Genome Biol 17:32

818

819 64. Zhu W, Lomsadze A, Borodovsky M. 2010. Ab initio gene identification in
820 metagenomic sequences. Nucleic Acids Res 38:1–15.

821

822 65. Altschul SF, Lipman DJ. 1990. Protein database searches for multiple alignments.
823 Proc Natl Acad Sci USA 87:5509–5513.

824

825 66. Wu Y, Tang Y, Tringe SG, Simmons BA, Singer SW. 2014. MaxBin: an
826 automated binning method to recover individual genomes from metagenomes
827 using an expectation-maximization algorithm. Microbiome 2:1-18.

828

829 67. Rodriguez-R L, Gunturu S, Harvey W, Roselló-Mora R, Tiedje James, Cole JR,
830 Konstantinidis K, NAR, Webserver issue, 2018, in press.

831

832 68. Rodriguez-R LM, Konstantinidis KT. 2014. Bypassing Cultivation To Identify
833 Bacterial Species. Microbe 9:111-118.

834

835 69. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-
836 analysis of large phylogenies. Bioinformatics 30:1312–1313.

837 **Table 1**. Physicochemical measurements of the sediment samples from each lagoon.

Lagoon (sample)	Date	pH	Temperature (°C)	Salinity (%)	Pb (µg/g)	Cd (µg/g)	Cu (µg/g)	RDX (µmoles/kg)	2,6-DNT (µmoles/kg)
Anones	2005	7.75	ND[a]	3.8	15.788	0.748	21.320	7.164	10.516
Anones	2014	7.10	35.7	7.1	17.664	0.585	63.382	< 0.2µM	< 0.1µM
Condado	2013	7.54	ND[a]	0	34.898	0.258	31.218	< 0.2µM	< 0.1µM
Guaniquilla	2013	7.12	ND[a]	7.0	9.321	0.263	17.714	< 0.2µM	< 0.1µM

[a] Not determined.

838

839

840 **Table 2**. Statistics of the MAGs recovered from the Anones-2005 Lagoon by

841 MaxBin.

MAG	Average Coverage	Genome Size (MB)	Completeness (%)	Contamination[a] (%)	Estimated Genome Size[b] (MB)	Estimated Relative Abundance [c] (%)	AAI[d] (%)	Anones-2014	Condado	Guaniquilla
MAG 1	37.6	3.1	100.0	1.9	3.1	12	*Magnetospirillum magneticum* AMB 1 (50.21%)	No matches	No matches	No matches
MAG 2	20.6	2.3	89.7	3.1	2.6	4.8	*Mollicutes* bacterium HR1 (45.82%)	No matches	No matches	Related population
MAG 3	20.1	3.2	99.1	0.9	3.3	6.5	*Thioalkalivibrio sulfidiphilus* HL EbGr7 (50.2%)	No matches	Related population	No matches
MAG 5	9.7	3.9	86.9	4.0	4.5	3.9	*Rhodothermus marinus* DSM 4252 (42.73%)	No matches	No matches	No matches
MAG 7	6.2	2.7	83.2	9.0	3.2	1.7	*Spirochaeta smaragdinae* DSM 11293 (50.01%)	No matches	No matches	Related population

842

843 [a] Posible chimeras.

844 [b] Represents genome size divided by completeness.

845 [c] Represents genome size times the coverage times read length divided by total of

846 reads of metagenome.

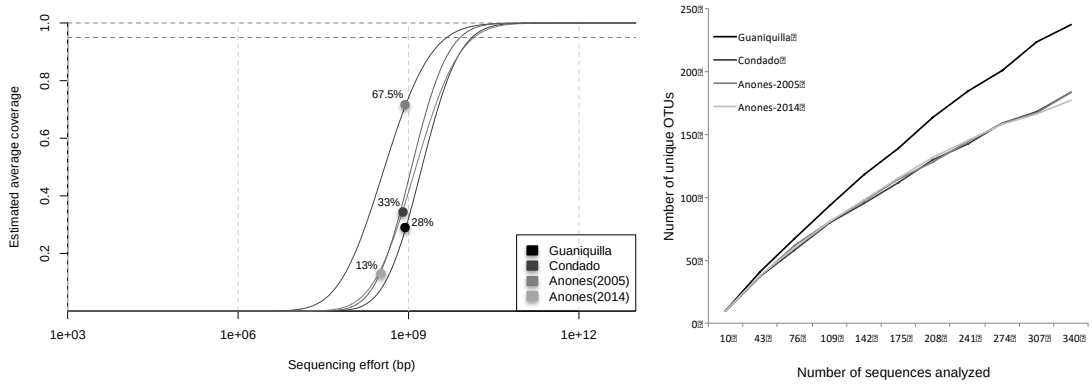
847 [d] Closest relative based on Average Amino Acid Identity.

848

849

850

851



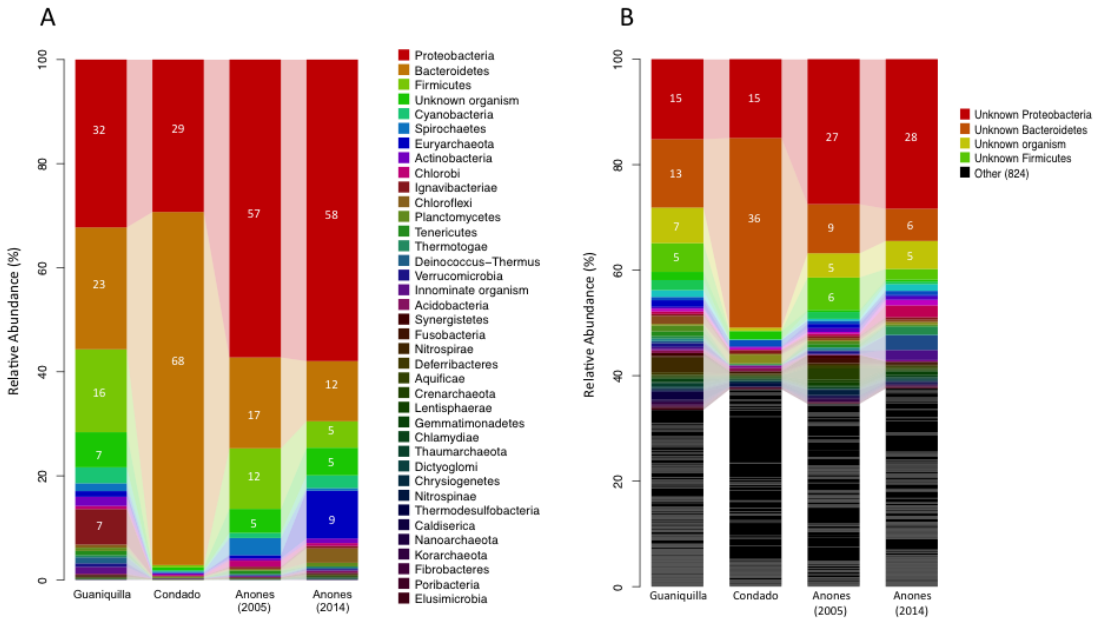
852

853 **Figure 1. Community diversity of the three lagoons sampled**. (A) Nonpareil 3.0
854 curves of the Guaniquilla, Condado, Anones-2005 and Anones-2014 lagoon
855 metagenomes, showing the estimated average coverage of the corresponding
856 microbial communities (dots) and the amount of sequencing required to achieve 95%
857 or 99% coverage (dashed lines on the top). Note that curves to the right require more
858 sequencing effort in order to reach high coverage, therefore, the corresponding
859 communities are more diverse. (B) Rarefaction curves of Guaniquilla, Condado,
860 Anones-2005 and Anones-2014 lagoon metagenomes based on 16S rRNA gene
861 fragments recovered. The graph shows the number of unique OTUs per number of
862 sequences analyzed.

863

864

865



866

867 **Figure 2**. **Taxonomic classification of the abundant organisms present in the**
868 **metagenomes of this study**. (A) Phylum-level and (B) genus-level taxonomic
869 classification of the lagoon microbial communities. The graphs are based on MyTaxa
870 analysis of assembled contigs. Numbers indicate relative abundance of each phylum,
871 based on the total reads of the metagenomic dataset (minimum abundance shown is
872 5%).

873

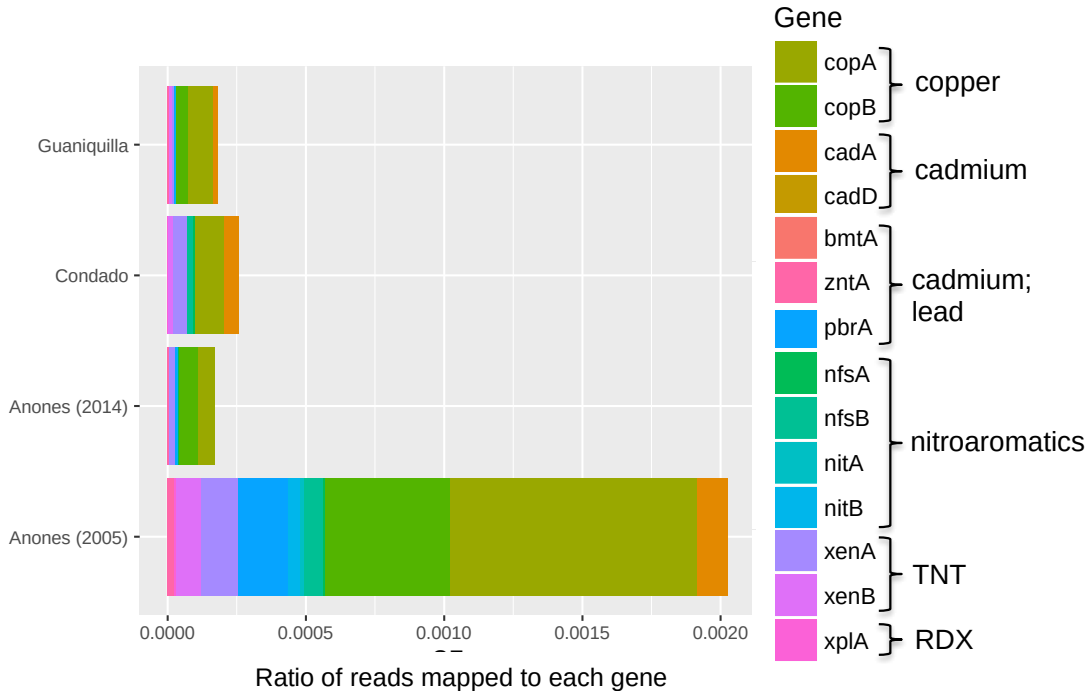


Figure 3. Relative abundance of biodegradation genes involved in explosive and heavy metal (Pb, Cd and Cu) biotransformations in the metagenomes of this study. Relative abundance was estimated as the fraction of metagenomic reads assigned to each specific gene, divided by the number of total reads in each metagenome.

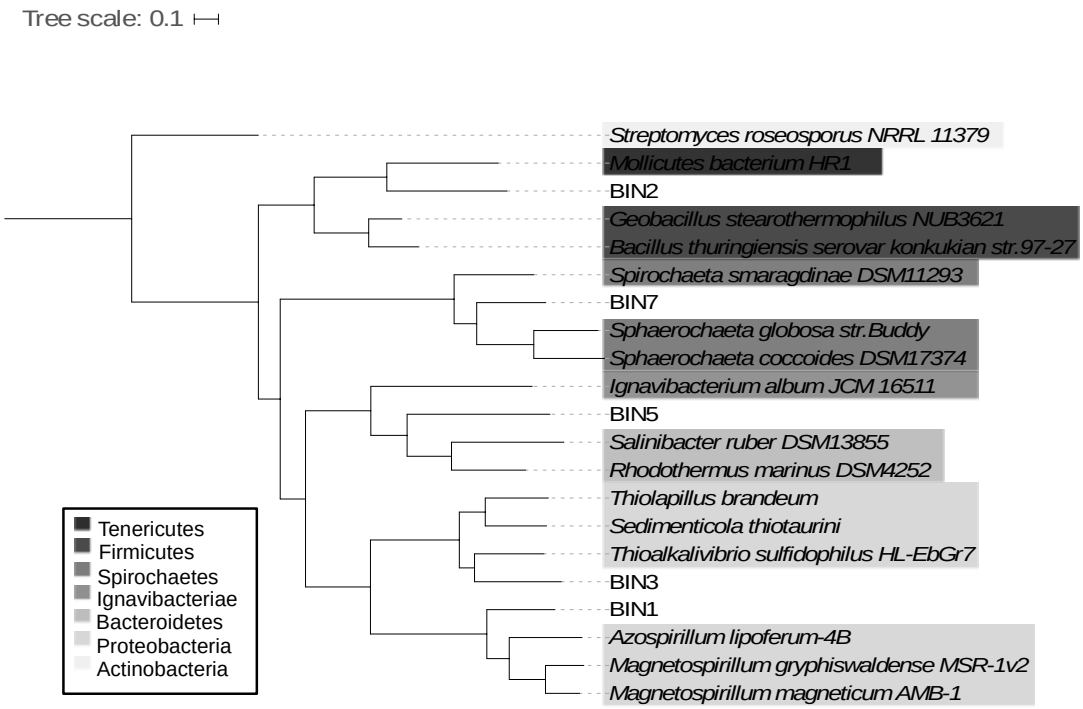


Figure 4. **Phylogenetic relationships among recovered genomes (MAGs) from the metagenomes of this study and selected reference genomes**. The phylogeny is based on a maximum likelihood analysis of the concatenated alignment of 57 single copy genes using RAxML and color-coded by phylum.

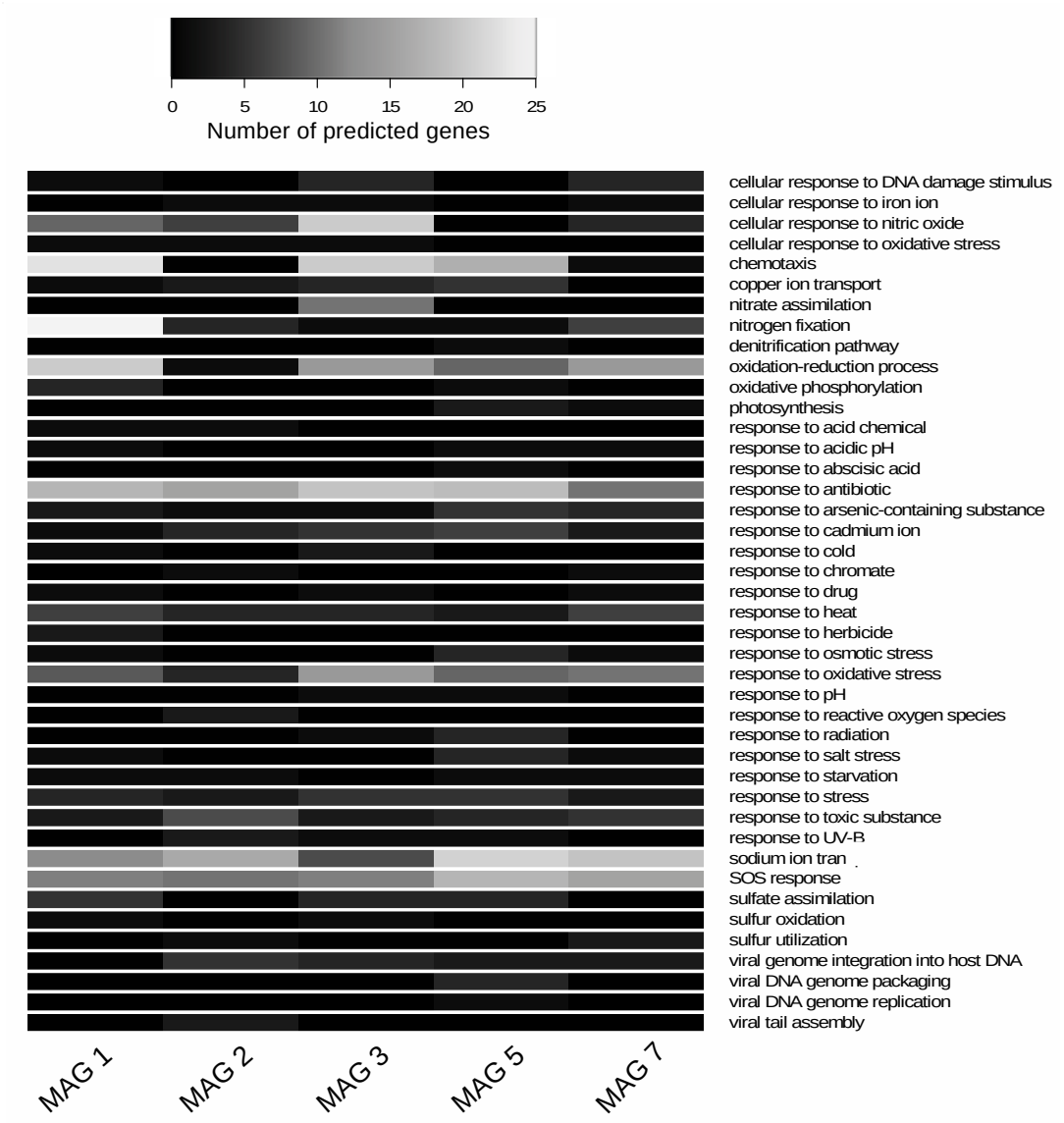


Figure 5. Functional gene annotation of MAGs based on the SwissProt database. Heatmap shows the counts (i.e., number of genes found; see scale bar on top) of gene functional categories (rows) in each metagenome of this study (columns). For each functional category shown, all genes assigned to this function in SwissProt were used to estimate the counts.

Table 1. Physical and chemical properties of the four sediment samples from each lagoon.

Lagoon (sample)	Date	pH	Temperature (°C)	Salinity (%)	Pb (µg/g)	Cd (µg/g)	Cu (µg/g)	RDX (µmoles/kg)	2,6-DNT (µmoles/kg)
Anones	2005	7.75	ND[a]	3.8	15.788	0.748	21.320	7.164	10.516
Anones	2014	7.10	35.7	7.1	17.664	0.585	63.382	< 0.2µM	< 0.1µM
Condado	2013	7.54	ND[a]	0	34.898	0.258	31.218	< 0.2µM	< 0.1µM
Guaniquilla	2013	7.12	ND[a]	7.0	9.321	0.263	17.714	< 0.2µM	< 0.1µM

[a] Not determined.

Table 2. Genomes recovered from Anones-2005 Lagoon by binning with MaxBin and statistics.

MAG	Average Coverage	Genome Size (MB)	Completeness (%)	Contamination[a] (%)	Estimated Genome Size[b] (MB)	Estimated Relative Abundance[c] (%)	AAI[d] (%)	Anones-2014	Condado	Guaniquilla
MAG 1	37.6	3.1	100.0	1.9	3.1	12	*Magnetospirillum magneticum* AMB 1 (50.21%)	No matches	No matches	No matches
MAG 2	20.6	2.3	89.7	3.1	2.6	4.8	*Mollicutes* bacterium HR1 (45.82%)	No matches	No matches	Related population
MAG 3	20.1	3.2	99.1	0.9	3.3	6.5	*Thioalkalivibrio sulfidiphilus* HL EbGr7 (50.2%)	No matches	Related population	No matches
MAG 5	9.7	3.9	86.9	4.0	4.5	3.9	*Rhodothermus marinus* DSM 4252 (42.73%)	No matches	No matches	No matches
MAG 7	6.2	2.7	83.2	9.0	3.2	1.7	*Spirochaeta smaragdinae* DSM 11293 (50.01%)	No matches	No matches	Related population

[a] Posible quimeras.
[b] Represents genome size divided by completeness.
[c] Represents genome size times the coverage times read length divided by total of reads of metagenome.
[d] Closest relative based on Average Amino Acid Identity.

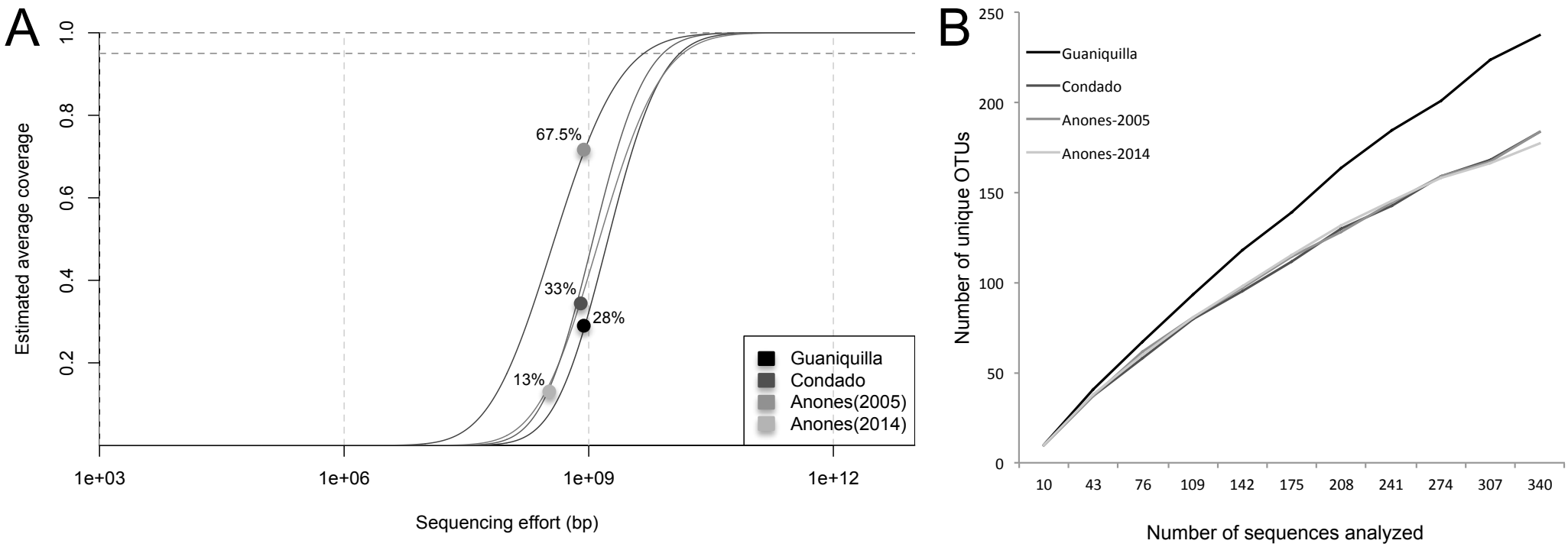


Figure 1. Community diversity of the three lagoons sampled. (A) Nonpareil 3.0 curves of the Guaniquilla, Condado, Anones-2005 and Anones-2014 lagoon metagenomes, showing the estimated average coverage of the corresponding microbial communities (dots) and the amount of sequencing required to achieve 95% or 99% coverage (dashed lines on the top). Note that curves to the right require more sequencing effort in order to reach high coverage, therefore, the corresponding communities are more diverse. (B) Rarefaction curves of Guaniquilla, Condado, Anones-2005 and Anones-2014 lagoon metagenomes based on 16S rRNA gene fragments recovered. The graph shows the number of unique OTUs per number of sequences analyzed.

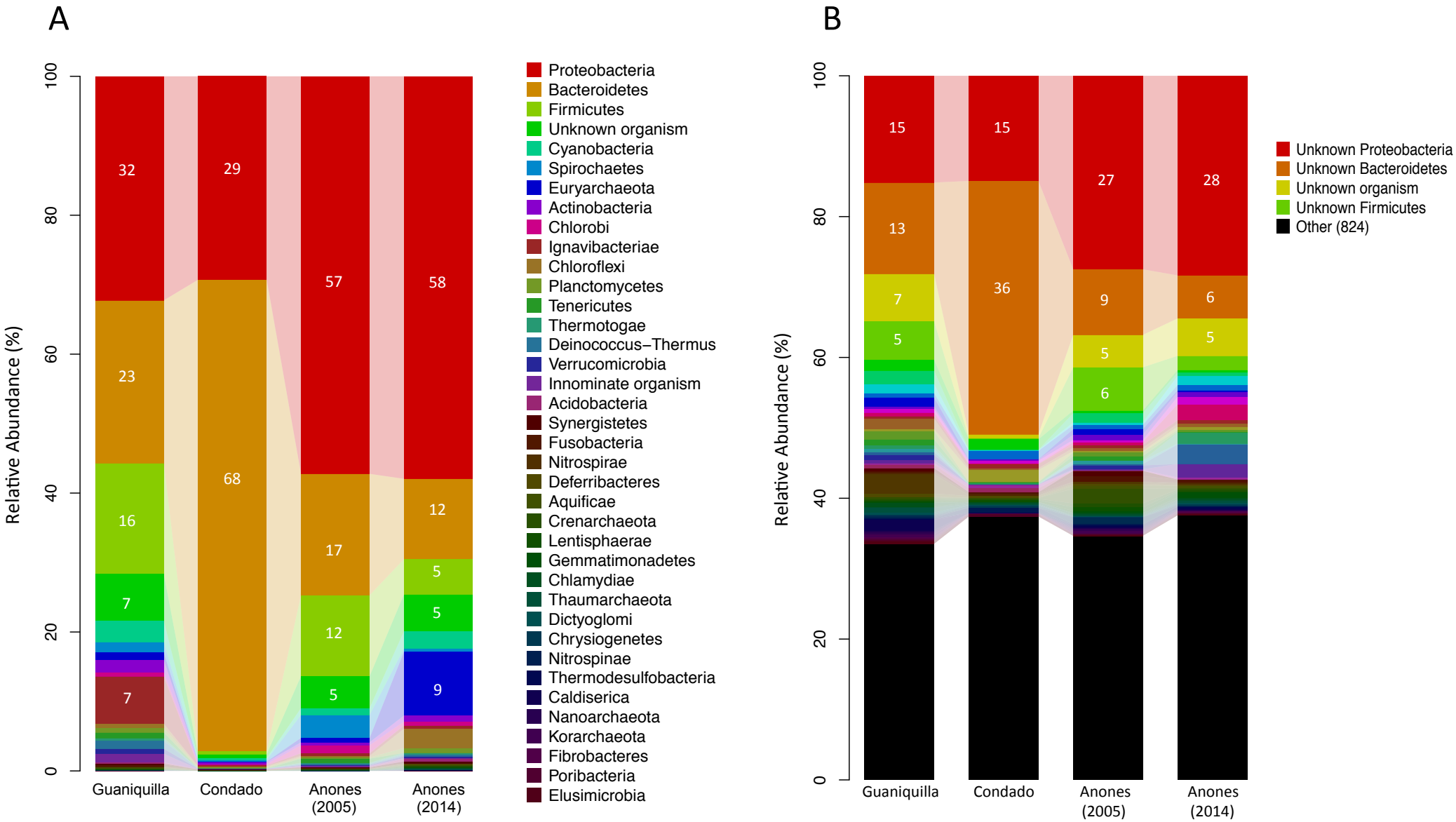


Figure 2. **Taxonomic classification of the abundant organisms present in the metagenomes of this study**. (A) Phylum-level and (B) genus-level taxonomic classification of the lagoon microbial communities. The graphs are based on MyTaxa analysis of assembled contigs. Numbers indicate relative abundance of each phylum, based on the total reads of the metagenomic dataset (minimum abundance shown is 5%).

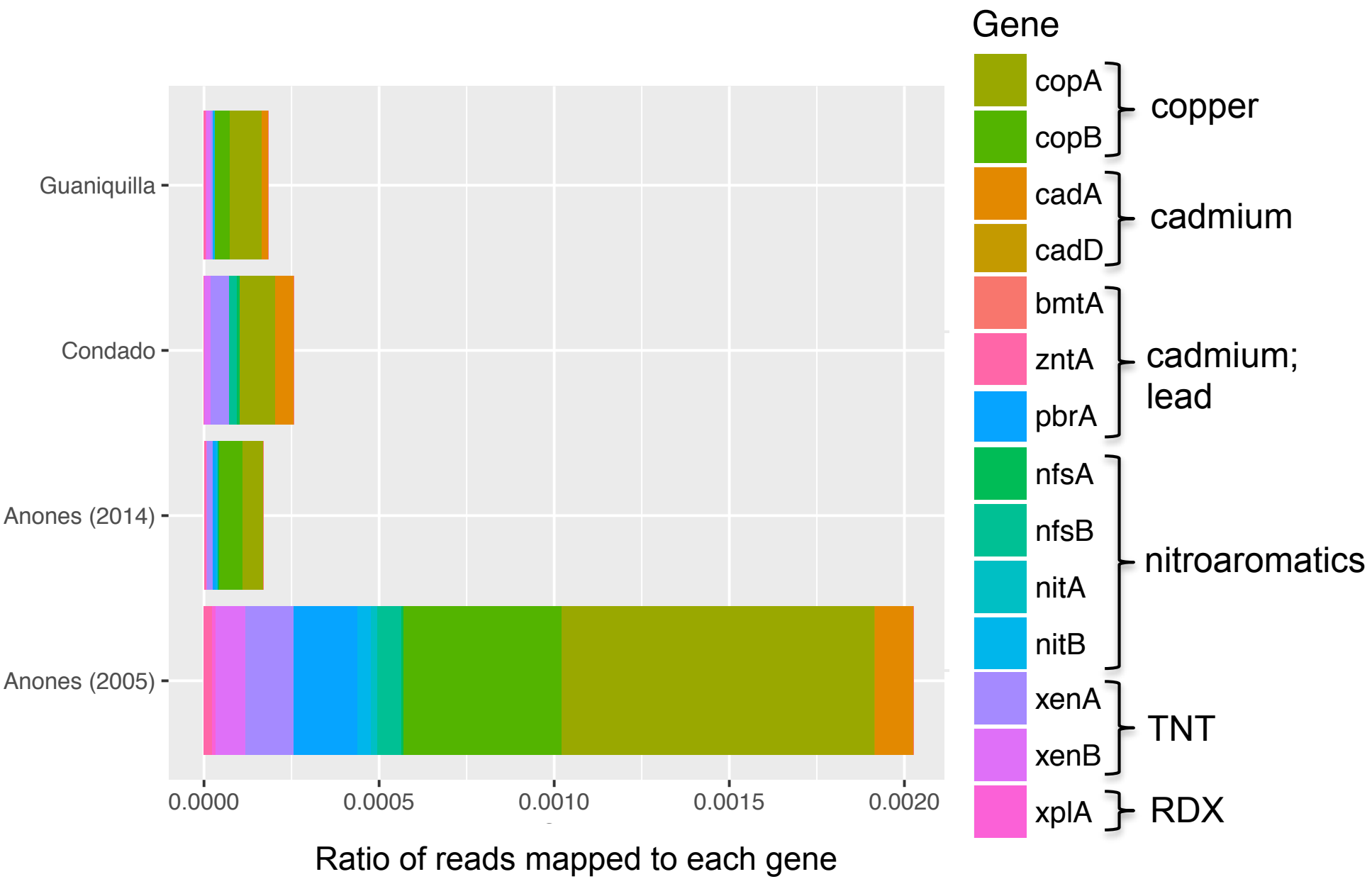


Figure 3. Relative abundance of biodegradation genes involved in explosive and heavy metal (Pb, Cd and Cu) biotransformations in the metagenomes of this study. Relative abundance was estimated as the fraction of metagenomic reads assigned to each specific gene, divided by the number of total reads in each metagenome.

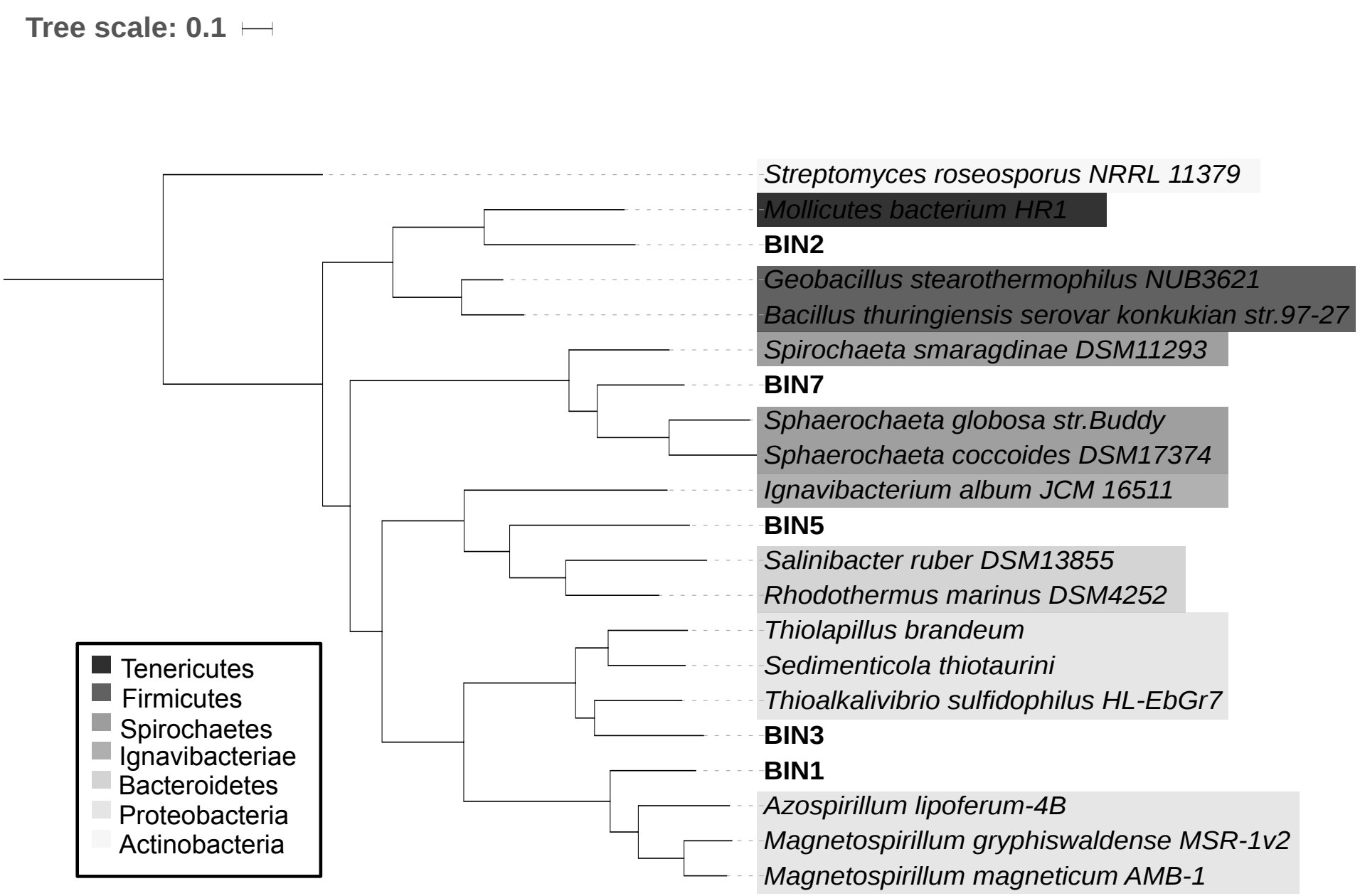


Figure 4. Phylogenetic relationships among recovered genomes (MAGs) from the metagenomes of this study and selected reference genomes. The phylogeny is based on a maximum likelihood analysis of the concatenated alignment of 57 single copy genes using RAxML and color-coded by phylum.

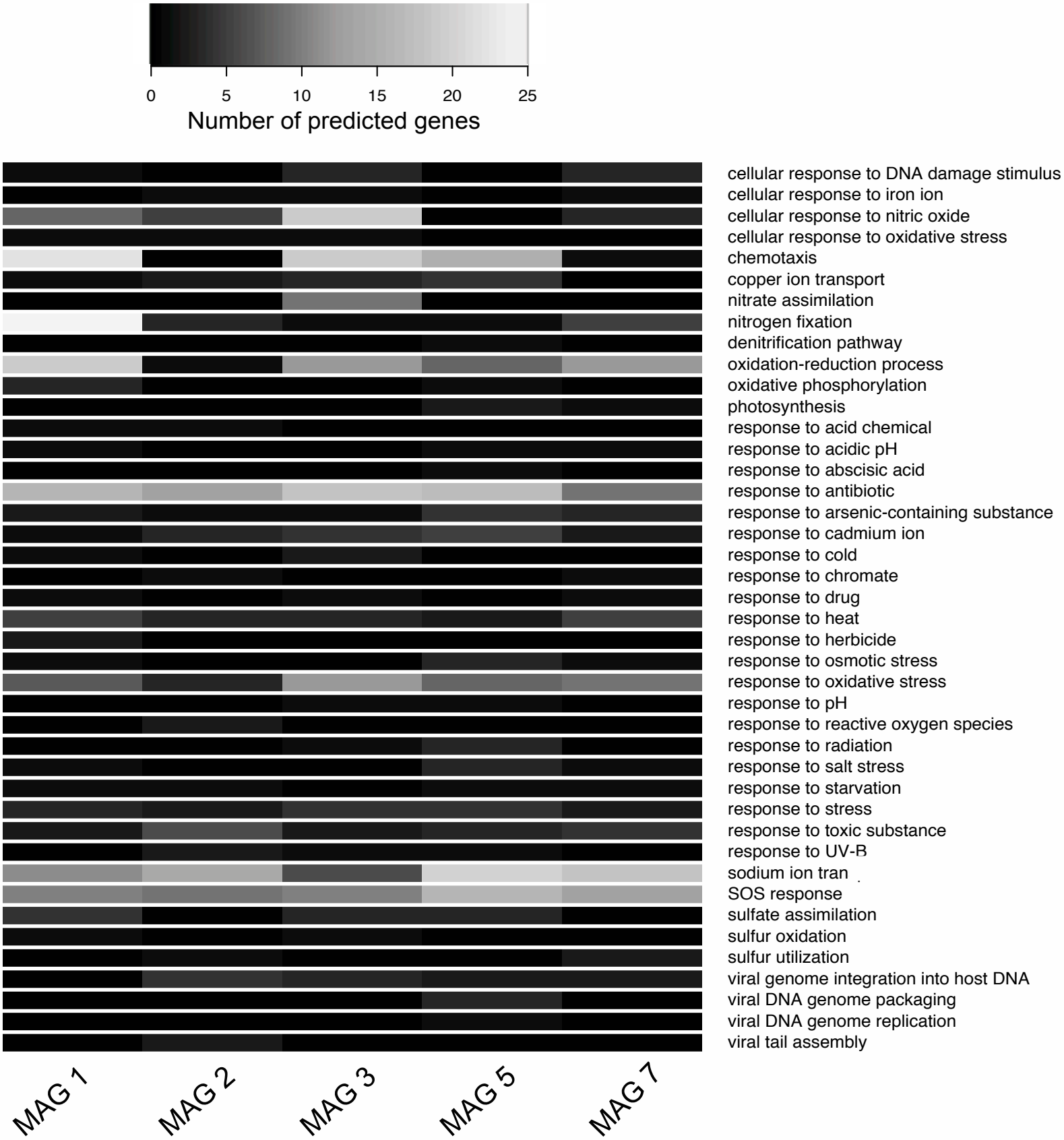


Figure 5. Functional gene annotation of MAGs based on the SwissProt database. Heatmap shows the counts (i.e., number of genes found; see scale bar on top) of gene functional categories (rows) in each metagenome of this study (columns). For each functional category shown, all genes assigned to this function in SwissProt were used to estimate the counts.