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FIRST CHARACTERIZATION OF THE MICROBIAL COMMUNITIES OF THE BLUE HOLE OF FAANU MUDUGAU, MALDIVES

PRIMA CARATTERIZZAZIONE DELLE COMUNITÀ MICROBICHE DEL BLUE HOLE DI FAANU MUDUGAU, MALDIVE

Abstract - *Blue holes, vertical water-filled openings in carbonate rock, exhibit complex ecologies and water chemistry. To date, the Maldivian Faanu Mudugau Blue Hole is the only known blue hole in the Indian Ocean. The compositions and functions of the microbial communities inhabiting this blue hole are still poorly studied. We analyzed the prokaryotic communities from the water column and from a microbial filamentous mat. Several molecular approaches and metabolic analyses were carried out to characterize the microbial communities in this extreme environment. Four zones with different depth-related microbial communities were identified. A strong metabolic activity was observed along the entire water column. The oxic-anoxic interface exhibited the highest microbial diversity and metabolic activity. In total, 48 metagenome-assembled genomes were recovered from the filamentous mat, predominantly composed of Chloroflexota, Proteobacteria, Desulfobacterota, and Planctomycetota phyla. However, none of these MAGs have been classified to species level, suggesting the discovery of new uncharacterized species.*

Key-words: *Environmental gradients, marine cave, metagenomic assembled genomes, microbial ecology, Maldives*

Introduction - Blue holes are subsurface voids that develop several meters underwater in carbonate banks and islands (Mylroie *et al.*, 1995). Blue holes may contain marine, fresh, or mixed-chemistry waters, influenced by tides and/or bottom water sources. Due to the limited water exchange within these systems, chemical and physical gradients are generated. Some blue holes are characterized by extreme conditions, such as anoxia and high concentrations of hydrogen sulphide (Canganella *et al.*, 2004, 2007; He *et al.*, 2020), thus being inhabited by peculiar microorganisms (Canganella and Wiegel, 2011). To date, the Faanu Mudugau Blue Hole in the Maldives (3°55.507'N, 72°56.559'E) is the only one known in the Indian Ocean. It opens on a lagoon floor at 30 m depth, with an entrance of 70 m diameter, and descends to 85 m depth (Fig. 1a). Along its vertical to overhanging walls, in some lateral recesses or ledges at 50 m depth, there are speleothems (*i.e.*, stalactites and stalagmites), as proof of the karstic origin of the cave (Colantoni *et al.*, 2003).

In the Faanu Mudugau Blue Hole, the physico-chemical composition of the water exhibits significant variation along the depth gradient. A transition zone (TZ) occurs at about 50 m depth, where temperature (above: 29.4±0.01°C, TZ: 27.4±1.21°C, below: 24.98±0.53°C), salinity (above: 34.37±0.02, TZ:34.74±0.25, below: 35.18±0.08) and pH (above: 8.27±0.01, TZ: 7.81±0.26, below: 7.42±0.05) change. Below 50 m depth, oxygen concentration collapses (above: 6.18±0.03 mg/L, TZ: 3.62±2.30 mg/L, below: 0.07±0.00 mg/L), while hydrogen sulphide substantially increase (above: 0.40±0.01

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mg/L, TZ: 0.42±0.04 mg/L, below: 0.55±0.02 mg/L) (Colantoni *et al*., 2003; Cutroneo *et al*., 2023).

In this paper we provide a first characterization of the microbial communities of the Faanu Mudugau Blue Hole along the water column and across the oxic-anoxic interface that separates a shallow layer of normal marine water from a deep sulphide-rich layer.

Materials and methods – All samples were collected by scuba divers using sterile Falcon tubes or plastic bottles. The samples were then treated with RNA later, stored at room temperature, and analyzed upon arrival in the lab. The microbial component of the blue hole was collected by sampling water every 10 m from the surface down to 80 m depth and by scraping microbial mats from the rocky walls at 50 m and 70 m depth. Water samples were inoculated into the AN MicroPlate and GEN III MicroPlate of the BIOLOG system to analyse the metabolic response of both anaerobic and aerobic microbial communities (respectively) along the water column. According to the biochemical properties of carbon sources, the substrates in both microplates were assigned to five categories: i) amino acids; ii) carbohydrates; iii) carboxylic acids, esters, and fatty acids; iv) hexose acids; and v) polyols. Unique assays were present in both microplates, such as the antibiotic resistance in GEN III MicroPlate and the use of nucleotides as a carbon source in AN MicroPlate. Water samples were then treated appropriately for investigation with Scanning Electron Microscope (SEM) and Denaturing Gradient Gel Electrophoresis (DGGE). Metabolic diversity was measured using the Shannon Index (H', with natural logarithm). The data matrix was submitted to Correspondence Analysis (CA) to explore the microbial phenotypic fingerprints along the depth gradient. The microbial biodiversity was investigated in water samples by DGGE, whereas the molecular microbial community structure was based on 16S rDNA gene sequences of excised bands from DGGE. In addition, 16S rDNA PCR amplicon library was generated from genomic DNA extracted from bacterial mat samples. The library obtained was sequenced using an Ion Torrent (PGM) Platform (Thermo Fisher Scientific, MA). Raw reads were then imported into the "Quantitative Insights into Microbial Ecology (QIIME 2)" software (v. 2020-11) for dereplication and clustering into operational taxonomic units (OTUs). OTUs were picked with De novo approach with a default identity of 99%. Taxonomy assignment of representative sequences was done against the Silva database release 138 trained with amplification primers to target the V4 region of the 16S rRNA gene. Meanwhile, the metagenomic shotgun sequences obtained from the filamentous microbial mats were treated with Metawrap tool. Metagenome-assembled genomes (MAGs) were taxonomically classified using the Genome Taxonomy Database (GTDB) (Chaumeil *et al*., 2020).

Results - A strong microbial metabolic response is observed along the entire water column of the Faanu Mudugau Blue Hole. The phenotypic fingerprint obtained by GEN III Microplates indicate that the utilization of all five types of carbon sources by aerobic microbes exhibits an increasing trend in the oxic-layer, reaching the maximum values at 50 m depth. Conversely, the results from AN MicroPlate analyses indicate that the utilization of all carbon sources by anaerobic bacteria is virtually absent in the first 40 meters, reaches the maximum value at 50 m depth, and then decreases in the anoxic-layer. This implies that the aerobic metabolism occurs exclusively from the surface down to 50 m depth, while anaerobic metabolism starts at 50 m depth and remains widely expressed down to the bottom of the cavity. Both metabolisms exhibit their peaks at a depth of 50 meters (Fig. 1b), suggesting that the oxic-anoxic interface represents a highly metabolically active zone. Overall, anaerobic metabolic activity along the whole

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water column is higher than the aerobic one (0.09 OD vs 0.06 OD, respectively). The same pattern is exhibited by the metabolic diversity, which is higher in the anoxic layer and peaks at 50 m depth ($H'_{50m}=4.6$).
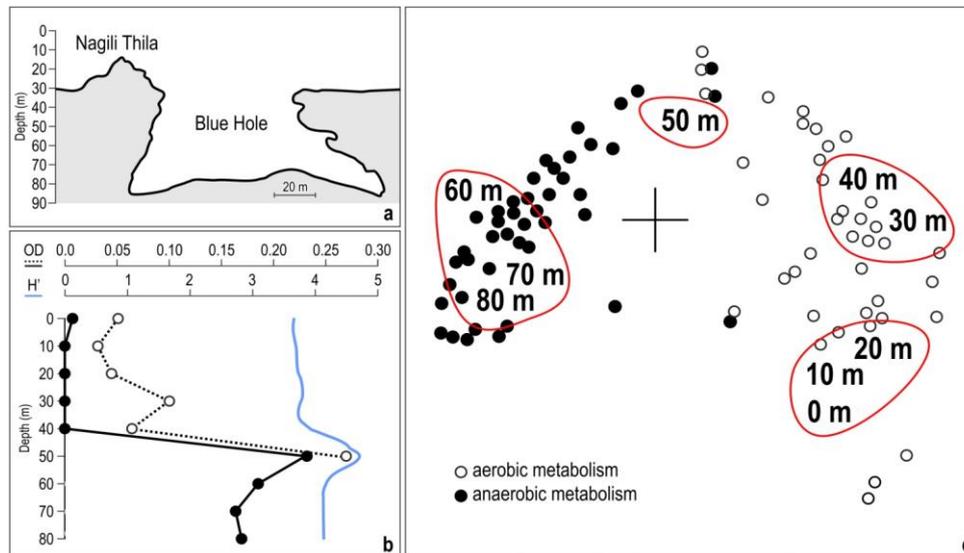

Fig. 1 – **a**) The Blue Hole of Faanu Mudugau (section); **b**) Depth distribution of both aerobic (white dots) and anaerobic (black dots) microbial metabolic response (OD: optical density) and Shannon diversity (H') (blue line); **c**) Multivariate plot from Correspondence Analysis of phenotypic fingerprints, with the four depth-related microbial communities.
a) *Il Blue Hole di Faanu Mudugau (sezione);* **b**) *Distribuzione della profondità, delle risposte metaboliche microbiche, sia aerobiche (punti bianchi) che anaerobiche (punti neri), espressa in densità ottica (OD) e diversità di Shannon (H') (linea blu);* **c**) *Proiezione sul piano individuato dai primi due assi dell'Analisi delle Corrispondenze sulle impronte fenotipiche, con evidenziate le quattro comunità microbiche legate alla profondità.*

Only the first axis of the CA is significant ($p \leq 0.05$, Lebart's test): all the points of the Faanu Mudugau Blue Hole phenotypic fingerprints are ordered horizontally according to the depth gradient (Fig. 1c). Four depth-related zones with different communities can be recognized. Between 0 and 20 m depth, outside the blue hole, there is a complex microbial community characterized mostly by Gamma-Proteobacteria (such as *Thioalkalivibrio* and *Thioploca*) and some Delta-Proteobacteria. Between 30 and 40 m depth, in the oxic-layer of the blue hole, the microbial community is mostly characterized by Alpha-Proteobacteria (*Rhodovibrio*-related and methanotrophs). In the oxic-anoxic interface at 50 m, a peculiar community of both aerobic and anaerobic (obligate or facultative) species is identified: in the water, the most abundant bacteria are Delta-Proteobacteria and sulphate-reducing bacteria (such as Desulfobacteriaceae, *Desulfonanticus* and *Synteophus*), followed by Bacteroidetes; on the walls, the community is composed by Proteobacteria (especially Alpha- and Gamma-Proteobacteria: 45%), Desulfobacterota (7%) and Bacteroidota (6%). In the anoxic water layer between 60 and 80 m depth, Alpha-Proteobacteria, Sphingomonadales and Cyanobacteria are dominant (Canganella et al., 2007).
A total of 48 MAGs were obtained from the filamentous mat (Fig. 2), with 7 of them belonging to Archaea, each representing a distinct phylum. In contrast, the bacterial MAGs were predominantly composed of Chloroflexota, Proteobacteria, Desulfobacterota, and Planctomycetota. Notably, none of these bacterial groups have been categorized at the species level, indicating the potential identification of previously

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unrecognized species. However, the estimation of the abundances revealed that the most abundant taxa belonged to the class of the *Dehalococcoidia,* which accounted for about 26%.
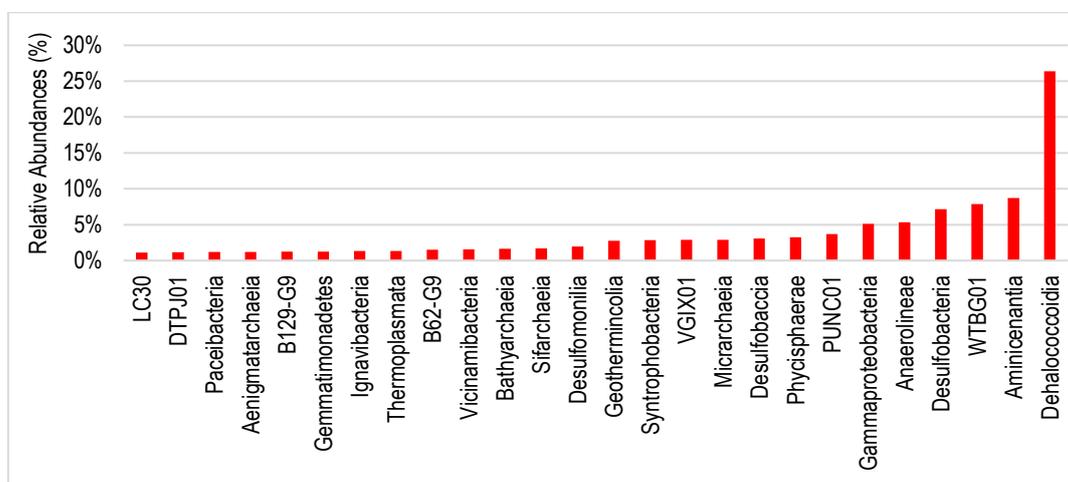

Fig. 2 –   Relative abundances of MAGs in the filamentous microbial mat metagenome.
Abbondanze relative dei MAG nel metagenoma del feltro microbico filamentoso.

Conclusions - The oxic-anoxic interface of the Faanu Mudugau Blue Hole represents a unique environment where a peculiar microbial community thrives, generating high metabolic activity and diversity. This result is consistent with the observations of He et al. (2020) which characterized the microbial communities along the water column of the Sansha Blue Hole (South China Sea), stating that the oxygen gradient defined ecologically specialized microbial communities especially at the oxic-anoxic interface.
Blue holes can be seen as natural laboratories that allow for the ecological study of extreme environments. Further investigations on the Faanu Mudugau Blue Hole are needed to better understand the structure and functioning of this peculiar ecosystem.

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