

Microbial communities through the lens of high throughput sequencing, data integration and metabolic networks analysis

developing computational approaches to better understand microbial assemblages

Haris Zafeiropoulos

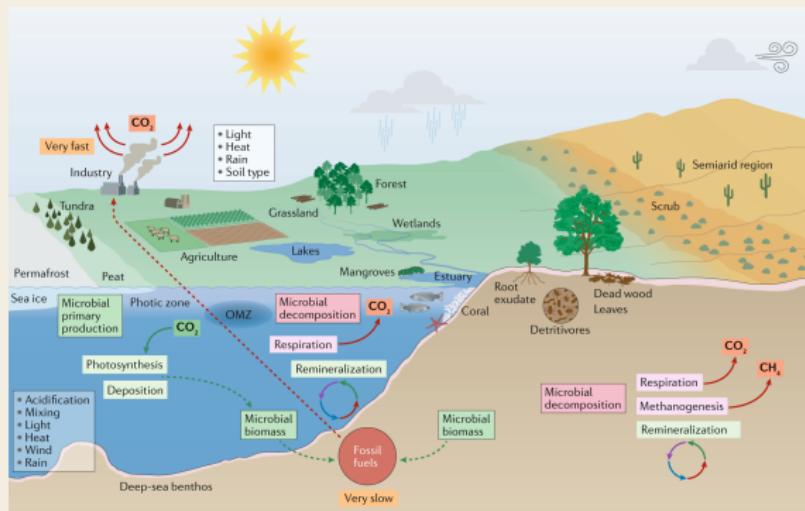
PhD candidate



- 1. Chapter 1:** Introduction
- 2. Chapter 2:** Software development to establish quality HTS-oriented bioinformatics methods for microbial diversity assessment
 - 2.1** PEMA: a flexible Pipeline for Environmental DNA Metabarcoding Analysis of the 16S/18S ribosomal RNA, ITS & COI marker genes
 - 2.2** The Dark mAtteR iNvestigator (DARN) tool: getting to know the known unknowns in COI amplicon data
- 3. Chapter 3:** PREGO: a literature- and data-mining resource to associate microorganisms, biological processes, and environment types
- 4. Chapter 4:** A New MCMC Algorithm for Sampling the Flux Space of Metabolic Networks
- 5. Chapter 5:** Deciphering the functional potential of a hypersaline marsh microbial mat community
- 6. Chapter 6:** Os and 1s in marine molecular research: a regional HPC perspective
- 7. Chapter 7:** Conclusions

Microbial ecology & biogeochemical cycles

a corner-stone for life on earth



- composition
- functions
- interactions

→ power biogeochemical cycling

Figure from: Nature Reviews Microbiology 17.9 (2019): 569-586.

Main questions regarding a microbial community for a deeper understanding of such assemblages

Community
structure
who

Functional
potential
what

Microbial
interactions
why / how

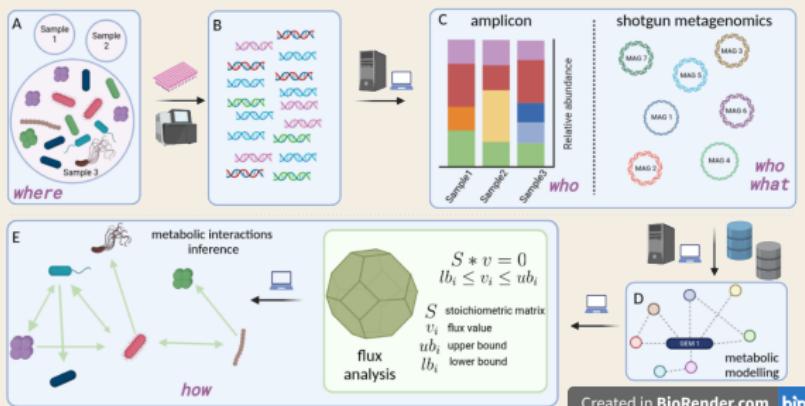
everyone is everywhere

zero-sum game

the entangled bank

Reverse ecology

transforming ecology into a high-throughput field



community ecology studies with no *a priori* assumptions about the organisms under consideration by exploiting advances in systems biology and genomic metabolic modeling

High Throughput technologies

a new era bringing its own challenges

- biology-oriented issues
- technology-oriented issues
- computing requirements
- multiple channels of information



Aims and objectives

- to enhance the analysis of microbiome data by building algorithms and software that address limitations and on-going computational challenges
- to exploit state-of-the-art methods to identify taxa and functions that play a key part in microbial community assemblages in hypersaline sediments

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eDNA metabarcoding for biodiversity assessment

Marker genes

1. **16S rRNA**: Bacteria, Archaea
 2. **12S rRNA**: Vertebrates
 3. **18S rRNA**: Small eukaryotes, Metazoa
 4. **ITS**: Fungi
 5. **COI**: Eukaryotes
 6. ***rbcl***: Plants
 7. ***dsrb***: Bacteria, Archaea
 8. ...

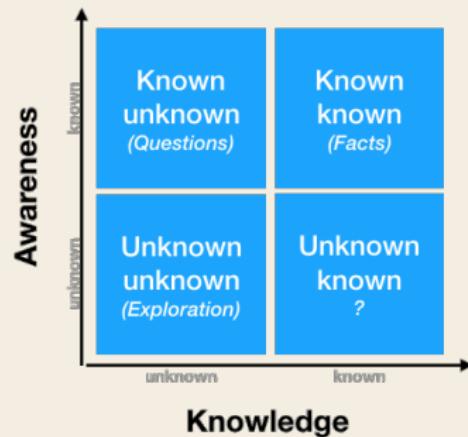
Methodology



- Sampling
 - Extraction
 - Bioinformatics
 - Biodiversity analysis

Bioinformatics challenges

for the analysis and the interpretation of amplicon data



PEMA: a flexible Pipeline for Environmental DNA Metabarcoding Analysis of the 16S/18S ribosomal RNA, ITS & COI marker genes

Aim of the study and contribution

To build an open source pipeline that bundles state-of-the-art bioinformatics tools for all necessary steps of amplicon analysis and aims to address:

- one-stop-shop for several marker genes & approaches
- easy-to-set & easy-to-use
- scalable
- flexible

Methods / Implementation

PEMA coding insights

```
for(int i : range(1,  
    in := "in_$i.tx  
    sys date > $in  
  
    out := "out_$i.t  
    task( out <- in  
        sys echo Tas  
    }  
,
```

Big-
DataScript
programming
language



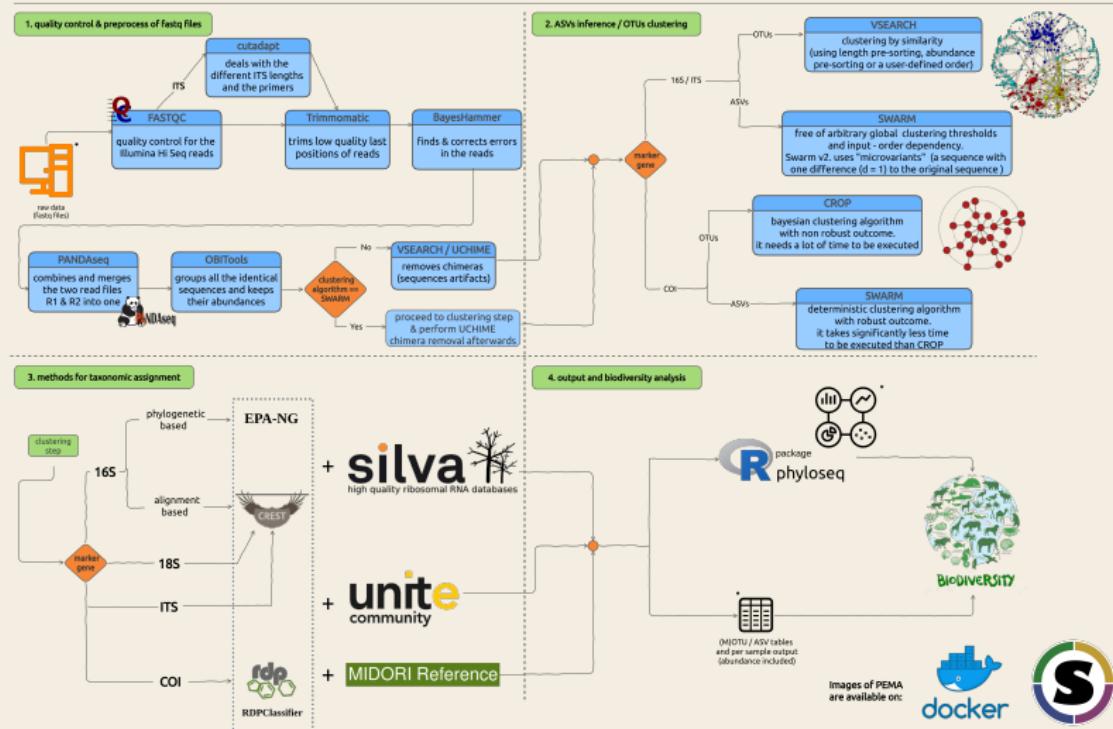
Containerization

High performance
computing

Results: PEMA features

an overview

PEMA in a nutshell



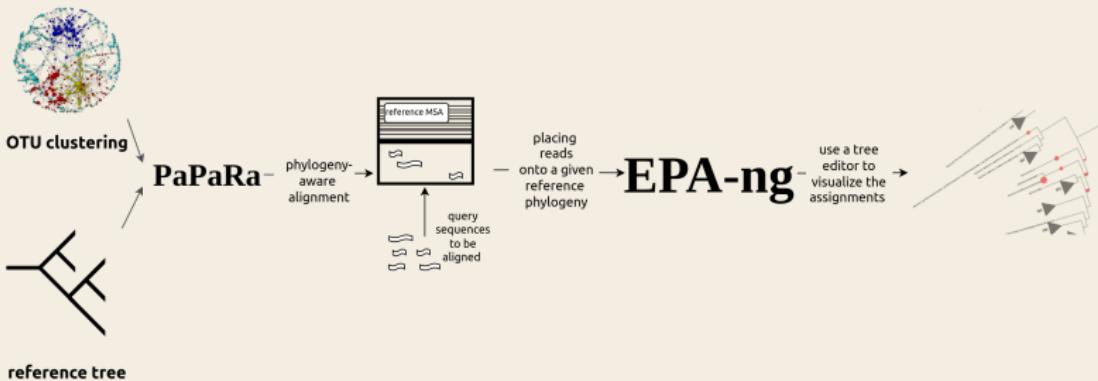
Results: PEMA features

phylogeny-based taxonomy assignment for the case of 16S rRNA gene

A. create reference tree



B. phylogeny-based taxonomy assignment



Results: tuning effects

in mock communities

Mock communities using the 16S rRNA gene and multiple parameter sets
 (identification at the genus level):

mock community of Gohl et al. (2016) KAPA protocol	Swarm (d = 1 strict = 0.8 no singletons)	Swarm (d = 1 strict = 0.8 with singletons)	Swarm (d = 3 strictness = 0.6 no singletons)	Swarm (d = 3 strictness = 0.8 with singletons)	Swarm (d = 10 strictness = 0.8 with singletons)	Swarm (d = 10 strictness = 0.8 no singletons)	Swarm (d = 25 strictness = 0.6)	Swarm (d = 25 strictntess = 0.8)	Swarm (d = 30 strictness = 0.6)	Swarm (d = 30 strictness = 0.8)
P	12	15	18	18	15	17	17	17	17	17
P	2	2	21	11	6	5	5	4	5	5
N	8	5	2	2	5	3	3	3	3	3
REC (TP / TP+FP)	0.86	0.88	0.46	0.62	0.71	0.77	0.77	0.81	0.77	0.77
EC (TP / TP+FN)	0.6	0.75	0.9	0.9	0.75	0.85	0.85	0.85	0.85	0.85
F1 (2 * (PREC * REC) / (PREC+REC))	0.71	0.81	0.61	0.73	0.73	0.81	0.81	0.83	0.81	0.81

mock community of Gohl et al. (2016) KAPA protocol	vsearch (id =0.95 strict = 0.8)	vsearch (id =0.97 strictness = 0.8)	vsearch (id =0.99 strictness = 0.8)
TP	11	12	12
FP	3	3	3
FN	9	8	8
PREC (TP / TP+FP)	0.79	0.80	0.80
REC (TP / TP+FN)	0.55	0.6	0.6
F1 (2 * (PREC * REC) / (PREC+REC))	0.65	0.69	0.69

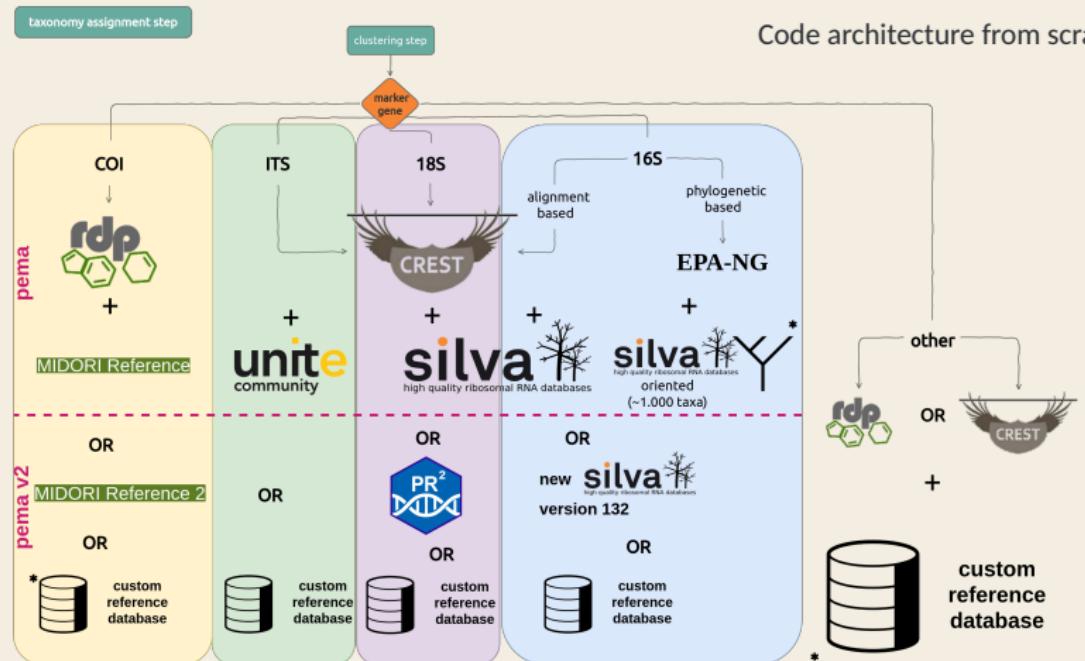
Results: Tuning effects

in real-world data

Using the *Bista et al.* dataset (COI) and multiple d values of the Swarm algorithm

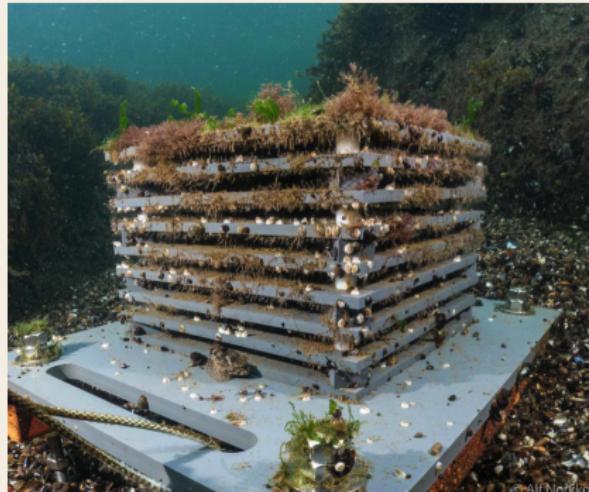
Parameter	$d = 1$	$d = 2$	$d = 3$	$d = 10$	$d = 13$
MOTUs after pre-process and clustering steps	83,791	59,833	33,227	7,384	4,829
MOTUs after chimera removal	80,347	57,863	32,539	7,339	4,796
Non-singleton MOTUs	6,381	4,947	2,658	1,914	1,634
Assigned species	62	83	86	86	84
Execution time (h)	2:01:35	2:09:49	1:51:44	2:17:26	2:31:15

PEMA v.2 addressing some of the challenges



Illustrations with an asterisk are from the Noun Project.

Latest PEMa version *addressing the challenges of the community*



ASSEMBLE 
ASSOCIATION OF EUROPEAN MARINE BIOLOGICAL LABORATORIES EXPANDED

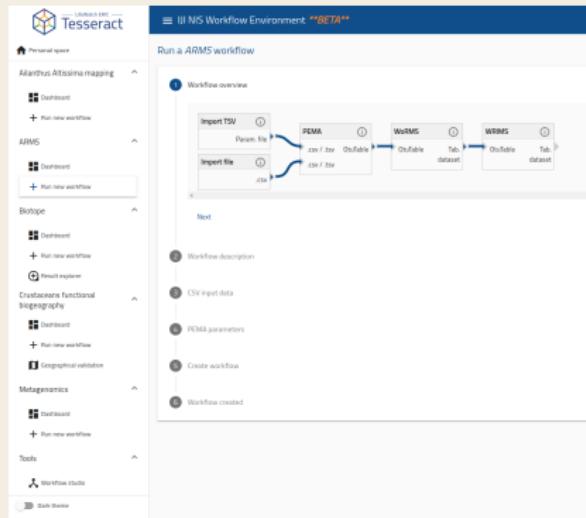
MBON
Marine Biodiversity
Observation Network

pema:v.2.1.4 includes:

1. analysis of 12S rRNA data now supported ([12S Vertebrate Classifier v2.0.0-ref database](#))
2. PR2 as an alternative reference database for the case of 18S rRNA
3. the ncbi-taxonomist tool was added to return the NCBI Taxonomy Id of the taxonomies found

Moving at the large scale

PEMA @ infrustuctures



1. Web - interface make analysis even easier
2. researchers without access to HPC/clouds etc are now able to run big scale analyses
3. Combine with other tools

Conclusions

on PEMA and eDNA metabarcoding

- PEMA is accurate, execution-friendly and fast pipeline
- tuning is essential in metabarcoding analyses
- sequencing a mock community along with your samples can be of great help in parameter tuning
- computing resources required range; infrastructures may benefit studies with great number of samples and CLI non-familiar users

The Dark mAtteR iNvestigator (DARN) tool: getting to know the known unknowns in COI amplicon data

Aim of the study and contribution

To build a framework for extracting non-target, potentially unassigned (or assigned with low confidence) sequences from COI environmental sequence samples, hereafter referred to as “dark matter” as per Bernard et al. (2018).

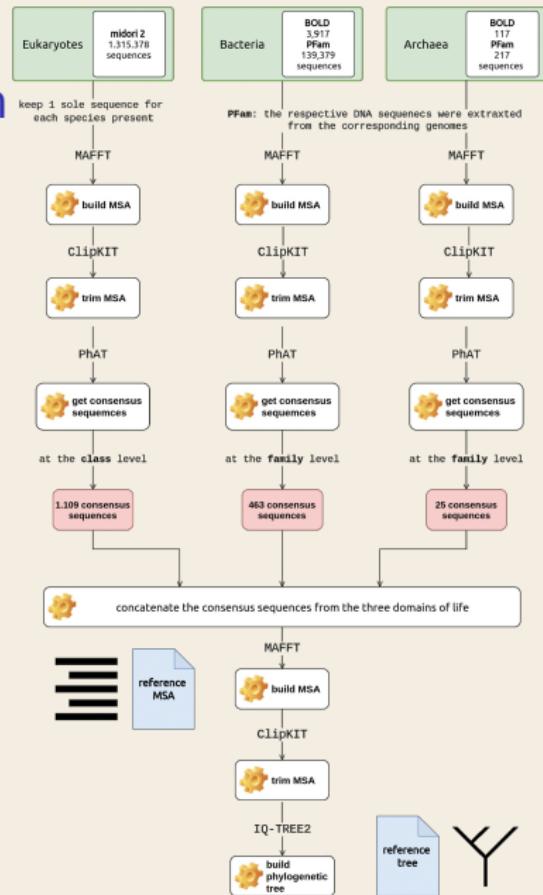
We argue that the vast majority of these sequences represent microbial taxa, such as bacteria and archaea

Methodology / Implementation

Dark mAtteR iNvesigator

In COI amplicon studies,
a great number of OTUs/ASVs retrieved
either have no hits or
their hit has a low confidence

DARN estimates to what extent
the OTUs/ASVs retrieved in an
environmental sample represent
target taxa or not



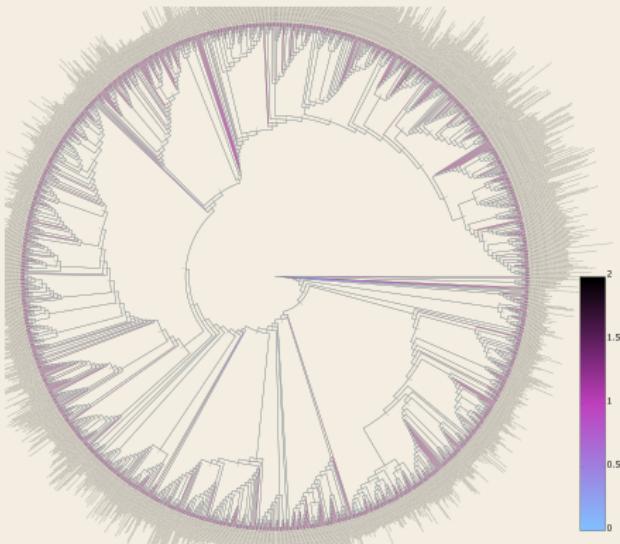
Methodology / Implementation

sequences retrieved

Resources	bacteria		archaea	
	# of sequences	# of strains	# of sequences	# of strains
BOLD	3,917	2,267	117	117
PFam-oriented	9,154	4,532	217	115
Total unique entries	11,421	6,798	334	201

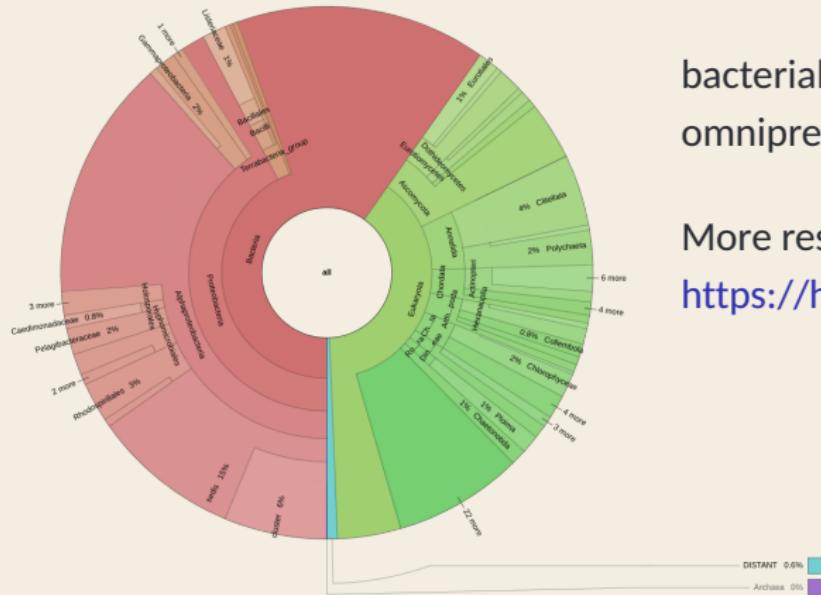
Methodology / Implementation

Phylogenetic tree of the COI consensus sequences retrieved



the consensus sequences have been placed in their corresponding taxonomic branches, proving the tree valid

Results: DARN using real-world data with multiple sample types, primers, PCR protocols and bioinformatics pipelines



bacterial sequences are omnipresent in COI amplicon data

More results at:

<https://hariszaf.github.io/darn/>

Conclusions

on DARN and COI amplicon studies

- bacteria, algae, fungi etc. was verified to be present in COI amplicon data
- bacteria make up a significant proportion of sequences generated in COI based eDNA metabarcoding datasets
- dark matter seems to be particularly common in eDNA as compared to bulk samples
- DARN supports quality control and further investigation of the unassigned OTUs/ASVs and allows researchers to better understand the known unknowns

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Chapter 3: PREGO: a literature- and data-mining resource to associate microorganisms, biological processes, and environment types

Aim of the study and contribution

To build a hypothesis generation resource based on associations between:

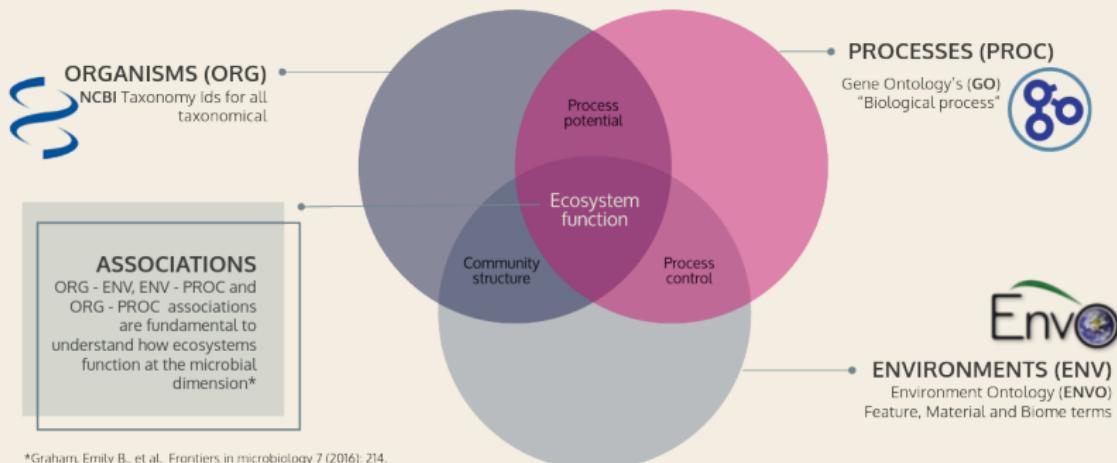
- *organisms* and the *environments* they inhabit
- *organisms* and the *biological processes* they are involved with
- *processes* and the *environments* where they occur

To this end, associations among such terms were exported from:

- the publicaly available literature
- genome and omics' studies, their results and their corresponding metadata

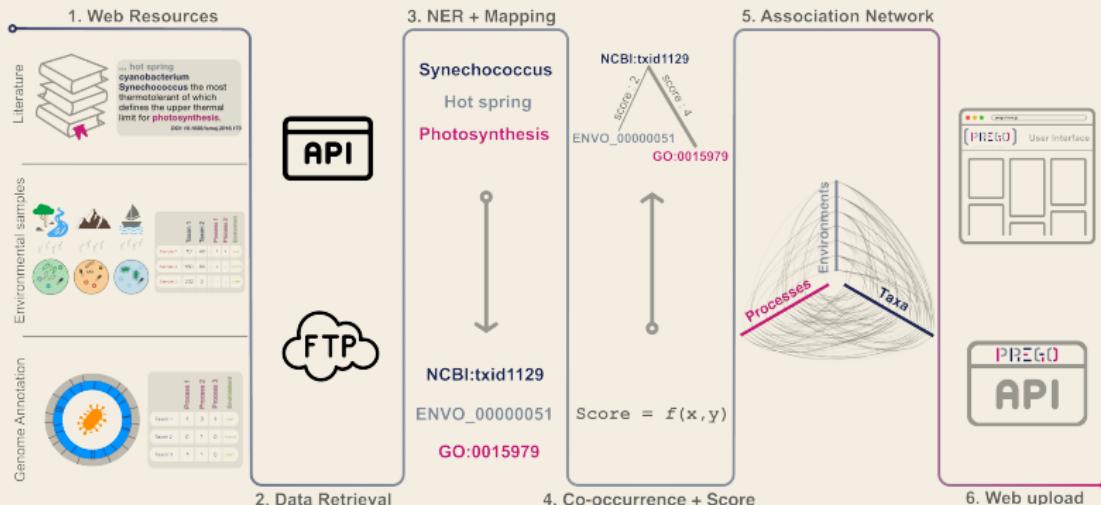
PREGO and its referring terms

the fundamental role of ontologies



Methods / Implementation

3 channels of information - 1 framework



Named Entity Recognition & the literature channel

exporting associations from publicly available publications

Identification of potentially important pathways missing from the model

EXTRACT	x
Protein	
Chemical compound	
Organism	
Environment	
Tissue	
Disease/phenotype	
Gene Ontology term	

From the metagenomic bins, we were able to identify two **metabolic processes** that were not previously included in the model. A number of MAGs (bin.59, bin.15, bin.73) clustered to the KEGG genomes of **freshwater sulfur**-oxidizing autotrophs capable of denitrification, *Sulfuritalea hydrogentivorans* [41], and *Sulfuricella denitrificans* [42]. These MAGs contained the diagnostic genes for **carbon fixation** (*rbcLS*), **sulfur** cycling (*dsrAB*), and denitrification (*nosZ*). One MAG (bin.59) also clustered with **iron** oxidizing autotroph *Sideroxydans lithotrophicus* **ES-1**. Bin.59 is the most relatively abundant bin from 17 to 21 m depth. Thus, if this MAG is associated with **iron** oxidation, it also contains **sulfur**-cycling genes that add to metabolic flexibility, which was previously observed [40]. The model did not include **sulfide oxidation** with **nitrate**, so it is unclear from the current model predictions where this process is expected to occur within the water column to compare to the MAG distributions.

Example text from Arora-Williams et al. Microbiome 6.1 (2018): 1-16.

The Environmental Samples and the Annotated Genomes and Isolates channels the role of metadata

Sample metadata [-]



Collection date:	11/1/11
Elevation:	200
Environment (biome):	soil
Environment (feature):	nosZ
Environment (material):	soil DNA
Environmental package:	MIGS/MIMS/MIMARKS.soil
Geographic location (depth):	15-20cm
Instrument model:	454 GS FLX Titanium
Investigation type:	metres-survey
NCBI sample classification:	410658
Project name:	EcoFINDERS

Project Information	
Cultured	No
Ecosystem	Environmental
Ecosystem Category	Aquatic
Ecosystem Subtype	Oceanic
Ecosystem Type	Marine

MG-RAST ID	name	biome	feature	material	sample	library	location	country	coordinates	download
mgm4702467.3	06032015b_S2_L001_R2_001	Large lake biome	lake	water	mgs485560	mg485562	Cincinnati	USA	39.11, -84.5	
mgm4702469.3	06052015a_S3_L001_R2_001	Large lake biome	lake	water	mgs485566	mg485568	Cincinnati	USA	39.11, -84.5	
mgm4702471.3	06032015a_S1_L001_R2_001	Large lake biome	lake	water	mgs485554	mg485556	Cincinnati	USA	39.11, -84.5	

MG-RAST
metagenomics analysis server

Co-mentioning and scoring scheme

which are the most worthy and relevant associations

- genome annotation oriented associations: fixed scores
- associations in the *Environmental Samples* channel are scored based on the number of samples they co-occur.
- similarly, in the *Literature* channel, based on the number of publications

		Y = y		
		Yes	No	Total
X = x	Yes	$c_{x,y}$	$c_{x,0}$	$c_{x..}$
	No	$c_{0,y}$	$c_{0,0}$	$c_{0..}$
	Total	$c_{.,y}$	$c_{.,0}$	$c_{..}$

Environmental samples score:

$$score_{x,y} = 2.0 * \sqrt{\frac{c_{x,y}}{c_{.,y}}} \quad (1)$$

Associations between entities of PREGO after metadata retrieval and co-occurrence analysis

Channel	Source	Environments		Taxonomy	Taxa		Taxa
		- Processes	- Functions		- Environments	- Processes	- Function
Literature	MEDLINE			Strains	69,968	590,630	384,079
	PubMed -	883,997	422,579	Species	778,877	3,501,635	1,961,920
	PMC OA			Total	1,669,608	7,969,310	4,613,827
	MG-RAST amplicon			Strains	13,645		
				Species	39,007	-	-
				Total	53,439		
Environmental samples	MG-RAST metagenome			Strains	262,106		8,626,328
			620,846	Species	103,913	-	10,715,548
				Total	372,301		19,950,096
	MGnify amplicon			Strains	18	-	
				Species	30,122	351	-
				Total	111,976	2,097	
Annotated Genomes and Isolates	JGI IMG isolates			Strains	8,229		3,461,693
				Species	42,141	-	13,216,559
				Total	50,888		16,821,850
	STRUO			Strains			1,803
				Species	-	-	4,070,195
				Total			4,079,312
	BioProject			Strains	3,263	7,473	
				Species	4,187	4,294	
				Total	7,641	12,169	
	All			Strains	357,229	598,103	12,473,903
		883,997	1,043,425	Species	998,247	3,506,280	29,964,222
				Total	2,265,853	7,983,576	45,465,085

A real case hypothesis generation scenario

Posidonia and its microbiome



What about *Posidonia* ?

Literature suggests that

Planctomycetes

and especially *Blastopirellula*

and *Rhodopirellula*

are commonly

found in its microbiome.

Why so ?

Let us have a look [here!](#)

Conclusions

on PREGO and its associations

- Literature
- similar number of molecular functions in all cases indicates the robustness of the main metabolic processes required for life
- , the number of environmental types that have been associated with members of each phylum varies, as a phylum may be universally present, while others could be strongly niche-specific

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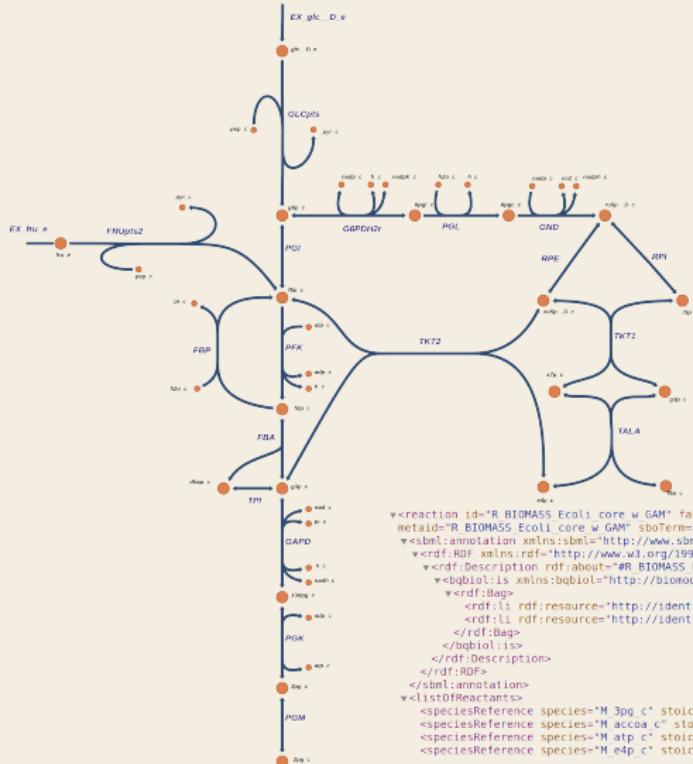
Chapter 4: A New MCMC Algorithm for Sampling the Flux Space of Metabolic Networks

Aim of the study and contribution

Flux sampling is a computationally intensive task, especially as the dimension of the polytopes derived from the metabolic model under study increases.

Microbial genome-scale models correspond to relatively low dimensional polytopes, but that is not the case for models integrating multiple GEMs. Further, more often than not, metabolic models of a host and a microbe is. Therefore, to allow flux sampling at high dimensional polytopes we introduce a Multi-phase Monte Carlo Sampling (MMCS) algorithm.

Metabolic modelling and the biomass function



Metabolic models allow us to move from a metabolic map to mathematical structures the study of which may provide fundamental biological insight

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Chapter 5: Deciphering the functional potential of a hypersaline marsh microbial mat community

Aim of the study and contribution

To exploit state-of-the-art methods to identify taxa and functions that play a key part in microbial community assemblages in hypersaline sediments

Both amplicon and metagenome analysis was conducted to investigate the composition and the functional potential of microbial communities from the Tristomo marsh (Karpathos island, Greece)

Tristomo swamp in Karpathos

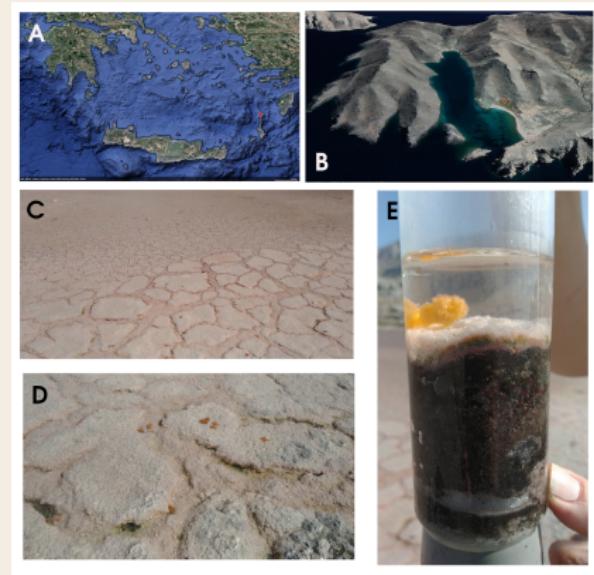
a seasonal brackish water marsh formed at the edge of a small plain

Type of samples:

- from clearly observed mats, top - bottom layers
- from not clar, no slicing
- aggregate samples

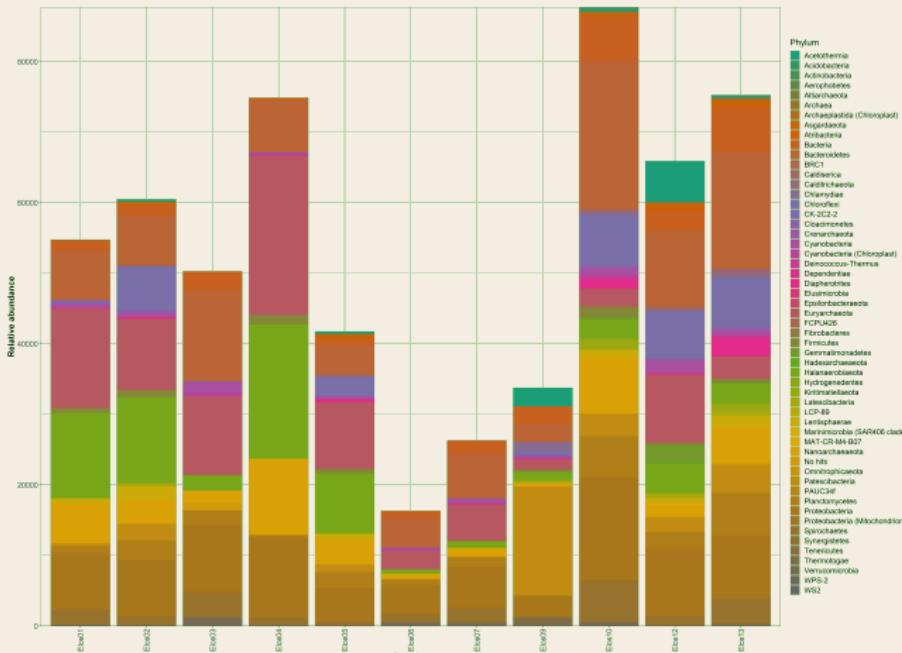
Sampling time points:

- July 2018
- November 2019



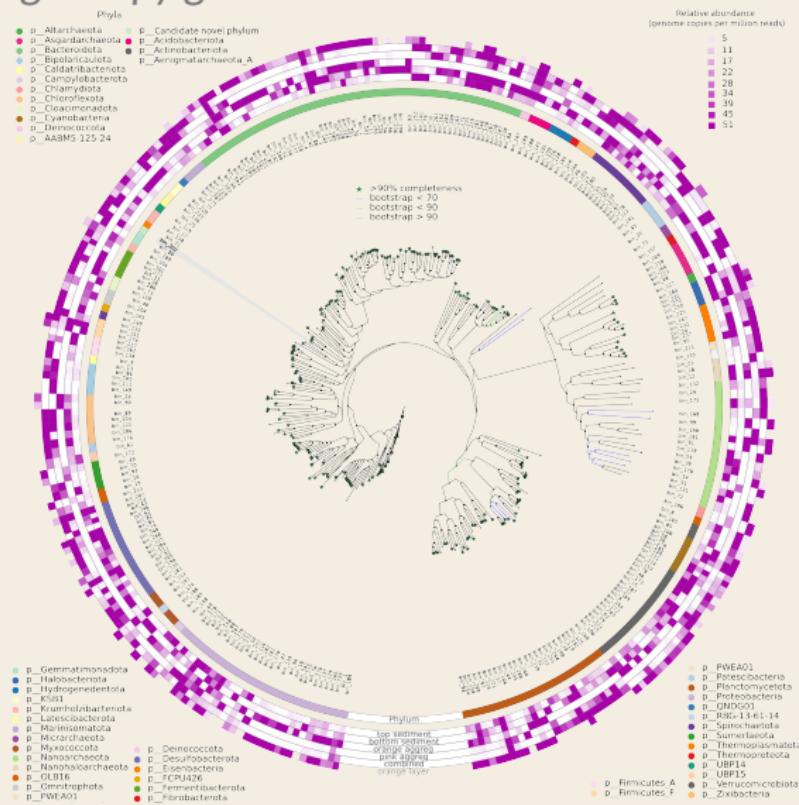
Abundances of the main microbial taxa, at the phylum level

based on 16S rRNA amplicon data

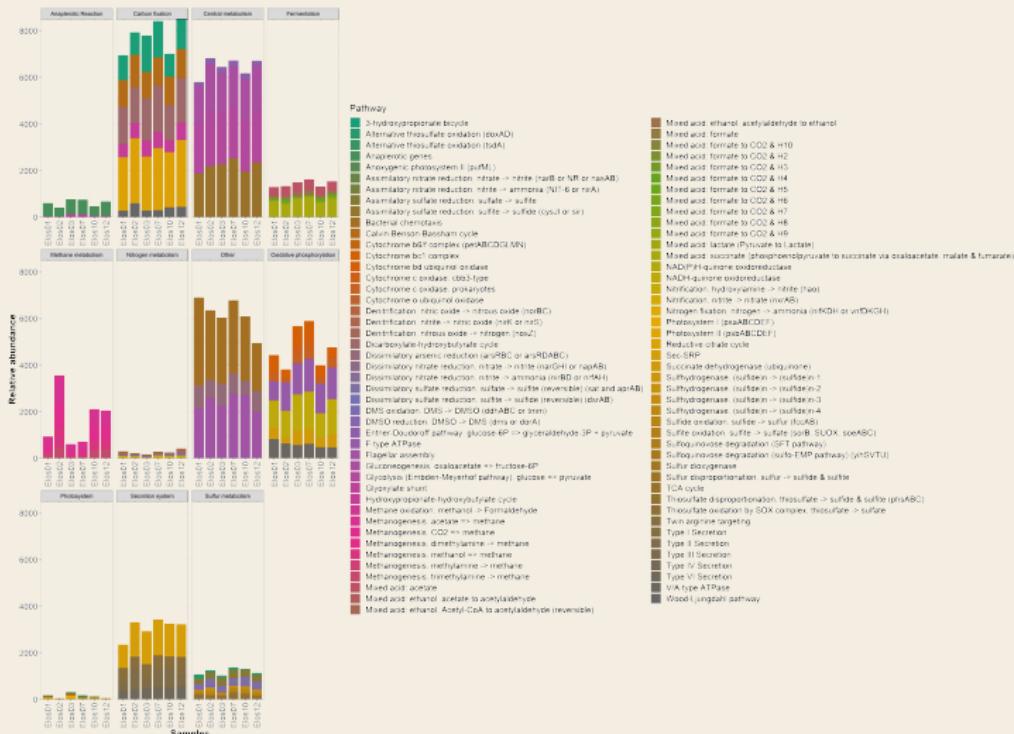


MAGs phylogeny

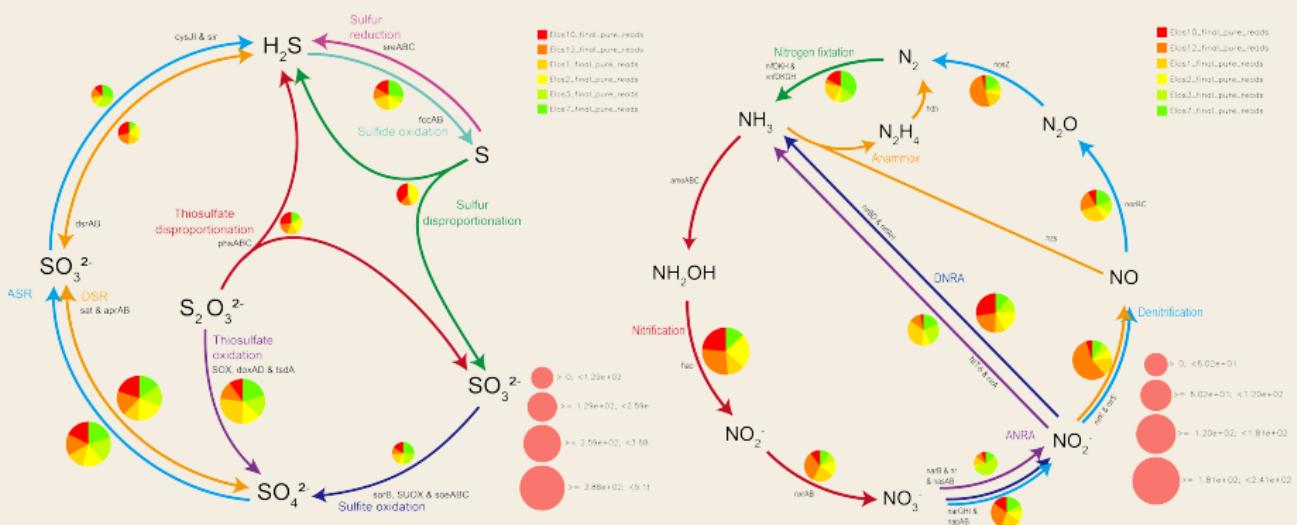
based on 25 single-copy genes



Metabolic pathways per biogeochemical cycle and their relative abundance at each sample



The S and the N cycle using KEGG annotation terms



Conclusions

on the

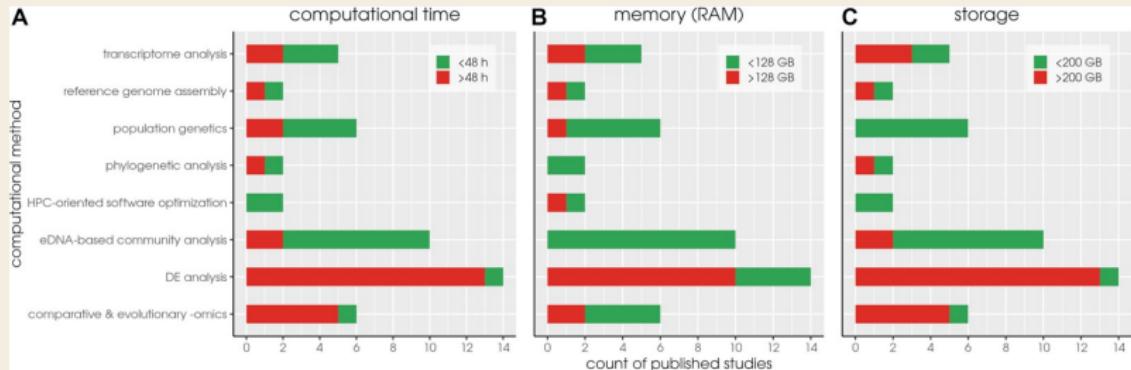
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Chapter 6: Os and 1s in marine molecular research: a regional HPC perspective

Aim of the study and contribution

To present insights from a thorough analysis of the research supported by the IMBBC HPC facility and some of its latest usage statistics in terms of resource requirements, computational methods, and data types as well as how the latter contributed in shaping the facility along its lifespan

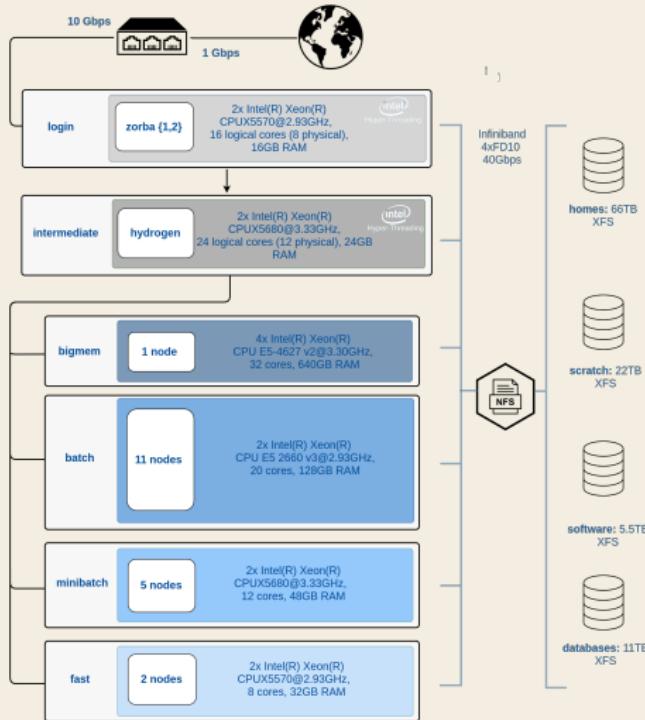
Computational requirements for trivial bioinformatic tasks



Red bars denote published research with high resource requirements
of the various computational methods employed at the IMBBC HPC facility

Zorbas: the HPC facility of IMBBC

a Tier 2 (regional) HPC facility



Block diagram of the
Zorba architecture

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Chapter 7: Conclusions

- Bioinformatics approaches enhance microbial diversity assessment based on HTS data
- Containerization technologies and e-infrastructures provide the means for computational capacity and reproducibility
- High quality metadata enable efficient exploitation of sequencing data in a meta-analysis level

Chapter 7: Conclusions

and future perspectives

- Markov Chain Monte Carlo approaches enable flux sampling in high-dimensional polytopes
- Hypersaline mats host a great range of novel taxa & their functioning might be subject to anaplerotic reactions

Perspective for future work

"a combination of quantitative high - throughput experiments and predictive metabolic models can elucidate the genotype - phenotype map of microbial metabolic strategies" providing great insight on the evolvability of metabolic decisions and on how such decisions affect microbial coexistence in the communities.

Wrap-up

software tools



a pipeline for eDNA metabarcoding analysis

github.com/hariszaf/pema



github.com/hariszaf/darn



github.com/lab42open-team/ github.com/GeomScale/dingo
the prego* repositories



Publications

- [1] **Zafeiropoulos, H.**, Paragkamian, S., Ninidakis, S., Pavlopoulos, G.A., Jensen, L.J. & Pafilis, E. (2022). PREGO: a literature- and data-mining resource to associate microorganisms, biological processes, and environment types. *Microorganisms* 10(2), 293.
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- [3] Chalkis, A., Fisikopoulos, V., Tsigaridas, E. & **Zafeiropoulos, H.** (2021). Geometric algorithms for sampling the flux space of metabolic networks, *37th International Symposium on Computational Geometry (SoCG 2021)*.
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- [8] Garza, D.R., Gonze, D., **Zafeiropoulos, H.**, Liu, B. & Faust, K., (2022) Metabolic models of human gut microbiota: advances and challenges (*under review at Cell systems*)
- [9] Paragkamian, S., Sarafidou, G., ..., **Zafeiropoulos, H.**, Arvanitidis, C., Pafilis, E. & Gerovasileiou, V. Automating the curation process of historical literature on marine biodiversity using text mining: the DECO workflow (*accepted in Frontiers in Marine Science*)

Acknowledgments

funding & grants



Acknowledgments

people and more

My promtors:

Dr. Pafilis E.

Prof. Nikolaou Chr.

Prof. Ladoukakis

the rest of my 7-member committee:

Prof. Dina Lika

Prof. Panagiotis Sarris

Prof. Jens Carlsson

Prof. Karoline Faust

Special thanks to:

PhD Paragkamian S.

Dr. Gargan L.

Dr. Hintikka S.

Mr. Ninidakis St.

Mr. Potirakis Ant.

Dr. Chalkis A.

Dr. Fisikopoulos V.

Prof. Tsigaridas E.

My mojo:

Dr. Pavlouri C.

My corner:

Would not be here
if it was not with you.



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