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

developing computational approaches to better understand microbial assemblages

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Microbial ecology & biogeochemical cycles

a corner-stone for life on earth

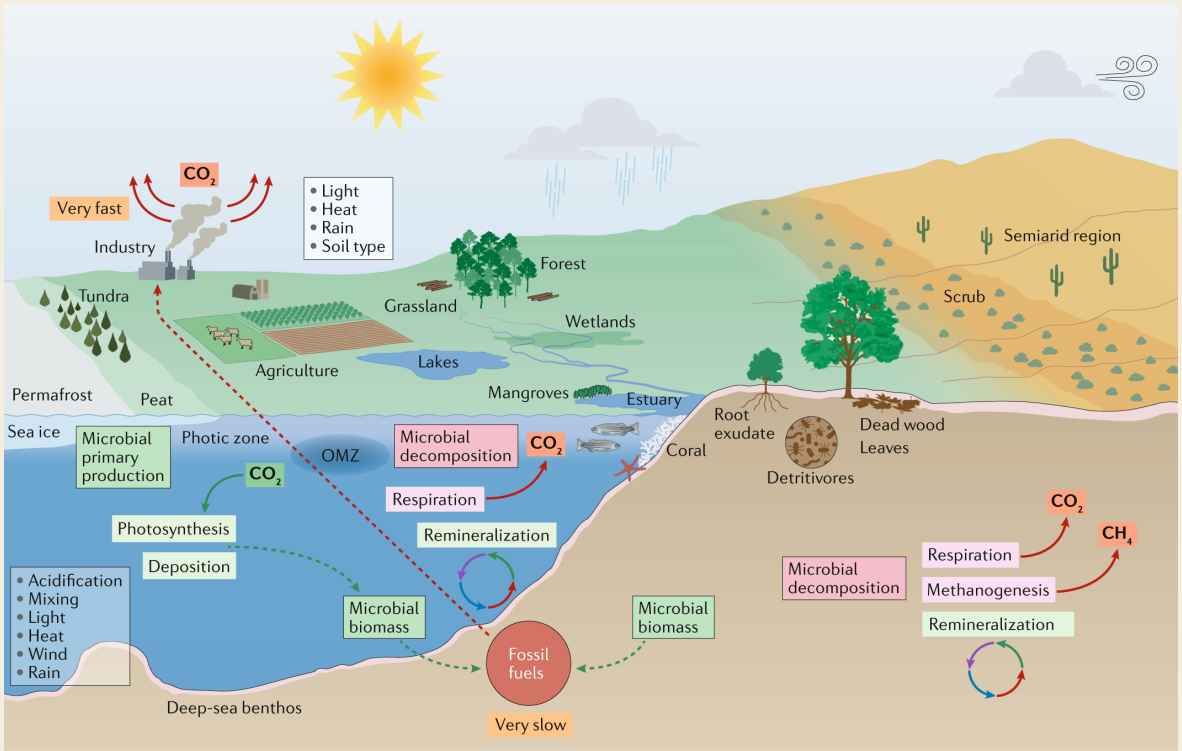


Figure from: Nature Reviews Microbiology 17.9 (2019): 569-586.

- composition
- functions
- interactions

→ power biogeo-chemical cycling

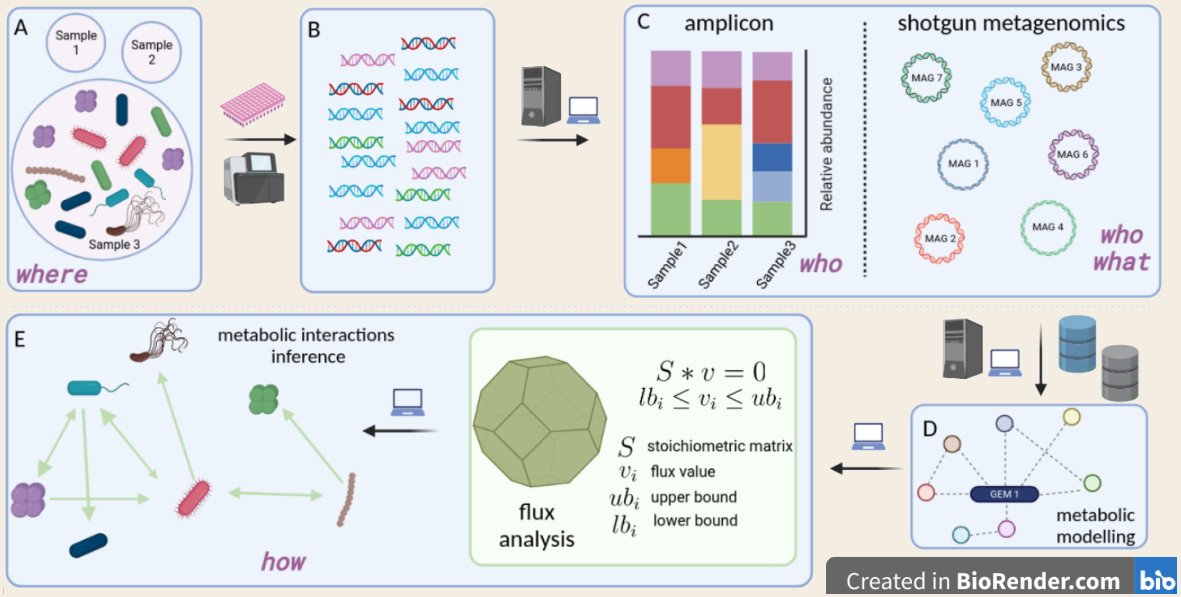
Main questions regarding a microbial community

for a deeper understanding of such assemblages

Community structure ***who***	Functional potential ***what***	Microbial interactions ***why / how***
eveyrone is everywhere	*zero-sum game*	*the entagnled 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
- techonology-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

eDNA metabarcoding

for biodiversity assessment

Marker genes

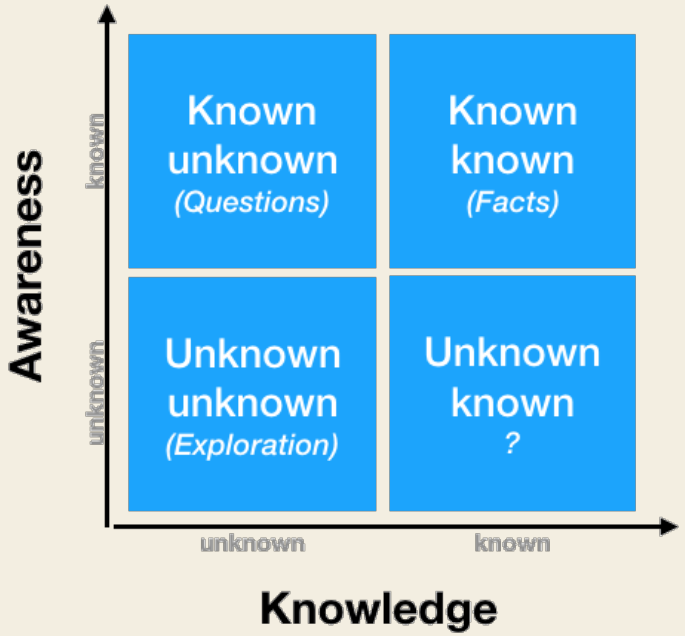
1. **16S rRNA:** Bacteria, Archaea
2. **12S rRNA:** Vertebrates
3. **18S rRNA:** Small eukaryotes, Metazoa
4. **ITS:** Fungi
5. **COI:** Eukaryotes
6. ***rbcl*:** Plants
7. ***dsrb*:** Bacteria, Archaea
8. ...

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
    }
}
```

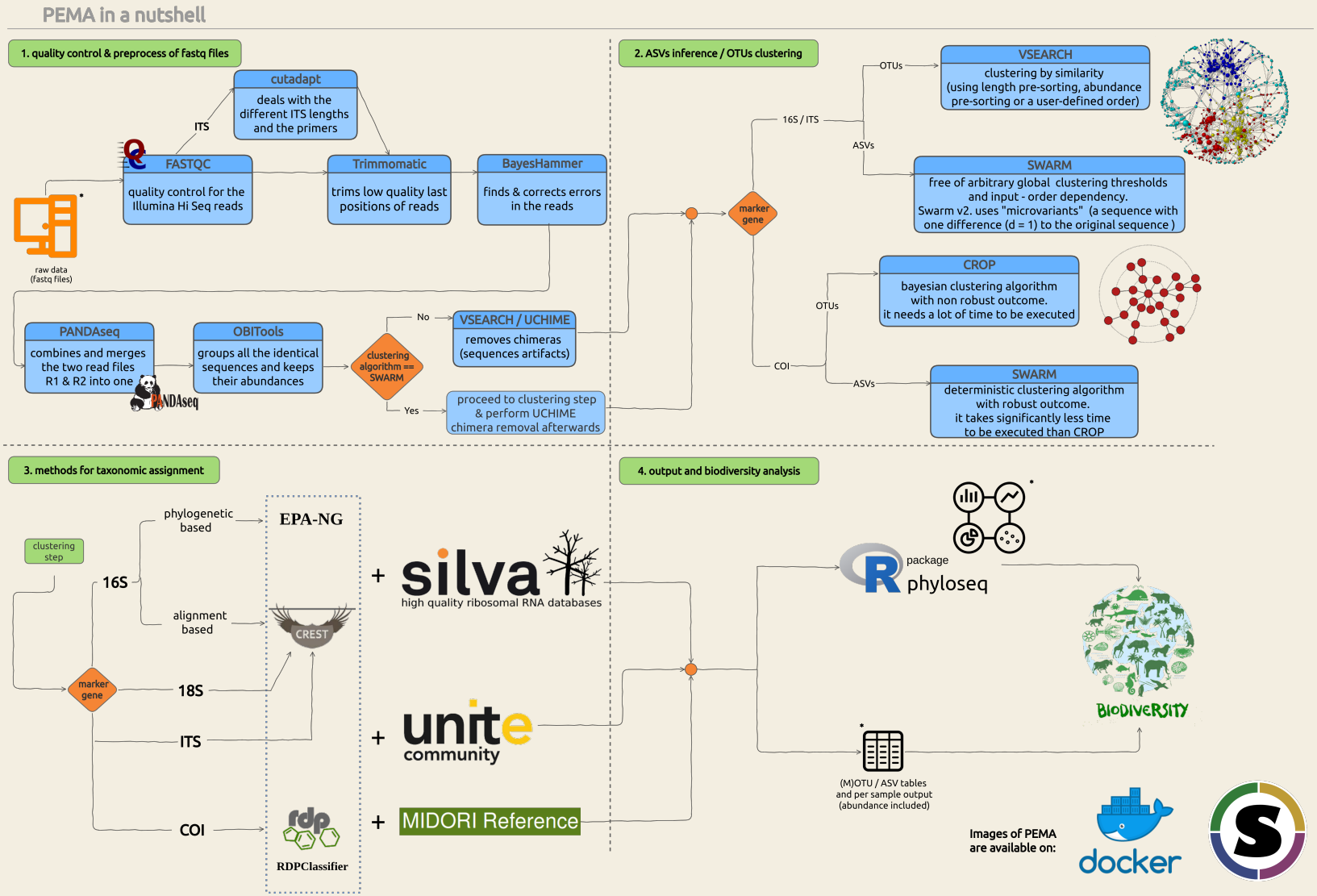
Big-DataScript programming language

Containerization

High performance computing

Results: PEMA features

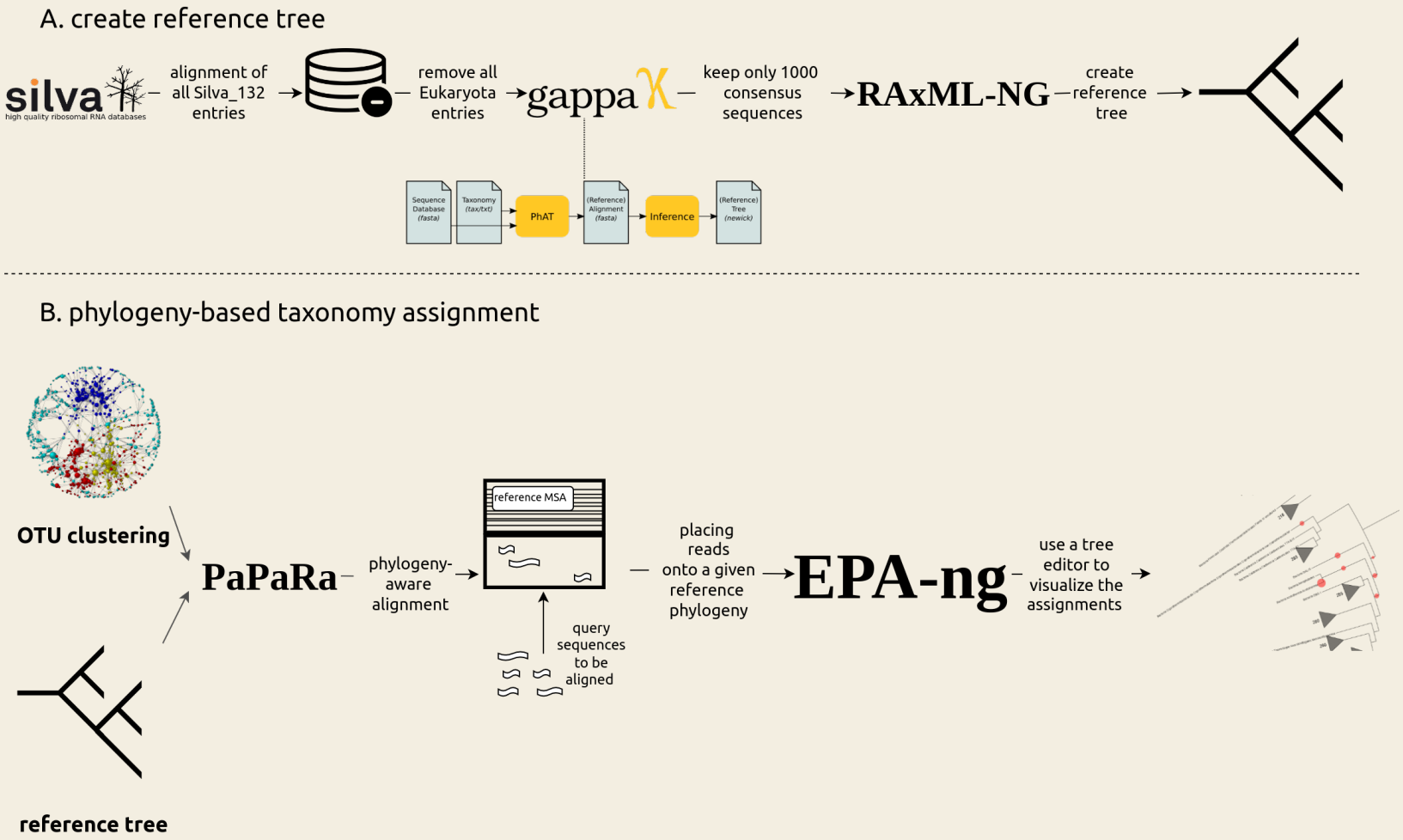
an overview



* Illustrations with asterisk are from the Noun Project

Results: PEMA features

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



Results: tuning effetcts

in mock communities

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

ock community of Gohl et al. (2016) APA 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
P	2		2	21	11	6	5	5	4	5
N	8		5	2	2	5	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
EC (TP / TP+FN)	0.6		0.75	0.9	0.9	0.75	0.85	0.85	0.85	0.85
1 (2 * (PREC * REC) / (PREC+REC)	0.71		**0.81**	0.61	0.73	0.73	0.81	0.81	0.83	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

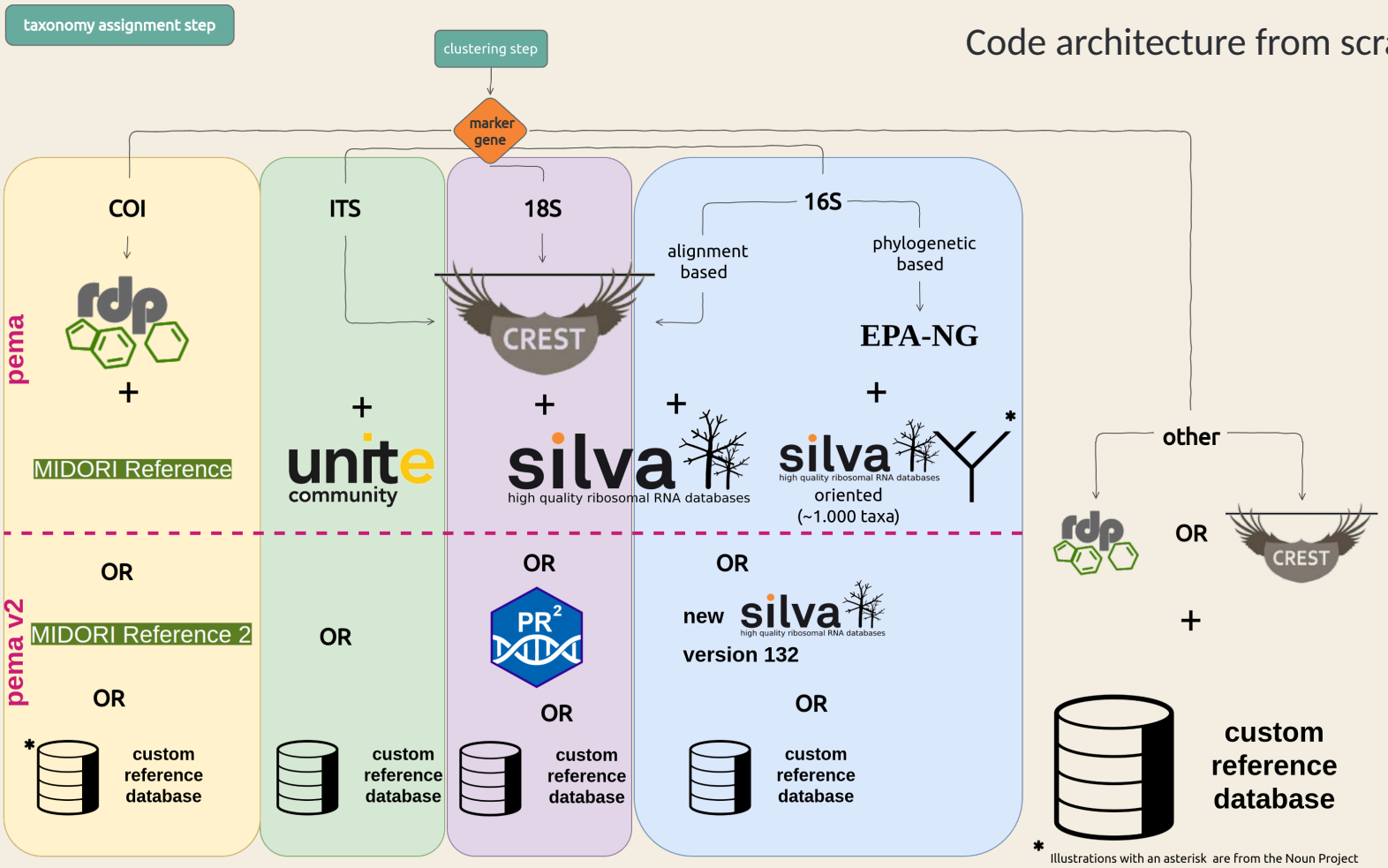
in real-world data

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



Code architecture from scratch!

Latest PEMA version

addressing the challenges of the community

ASSEMBLE
ASSOCIATION OF EUROPEAN MARINE BIOLOGICAL LABORATORIES EXPANDED

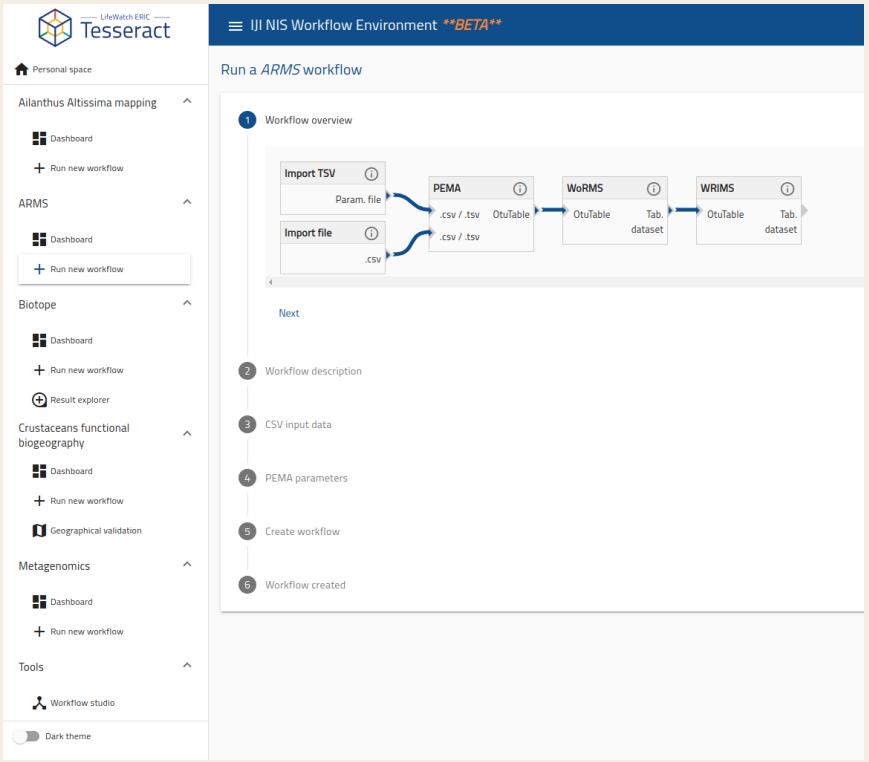
MBON
Marine Biodiversity Observation Network

`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; infrustuctures 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

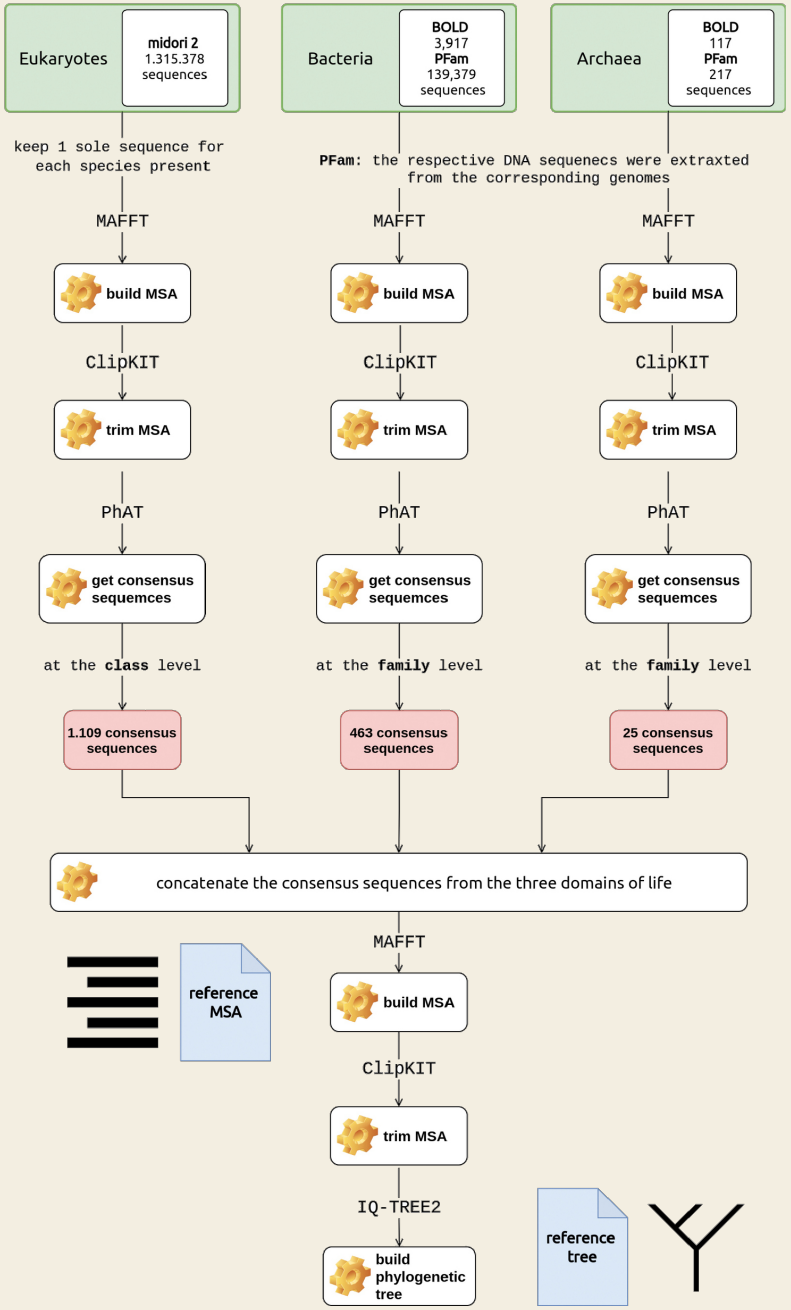


Figure from: 10.3897/mbmg.5.69657

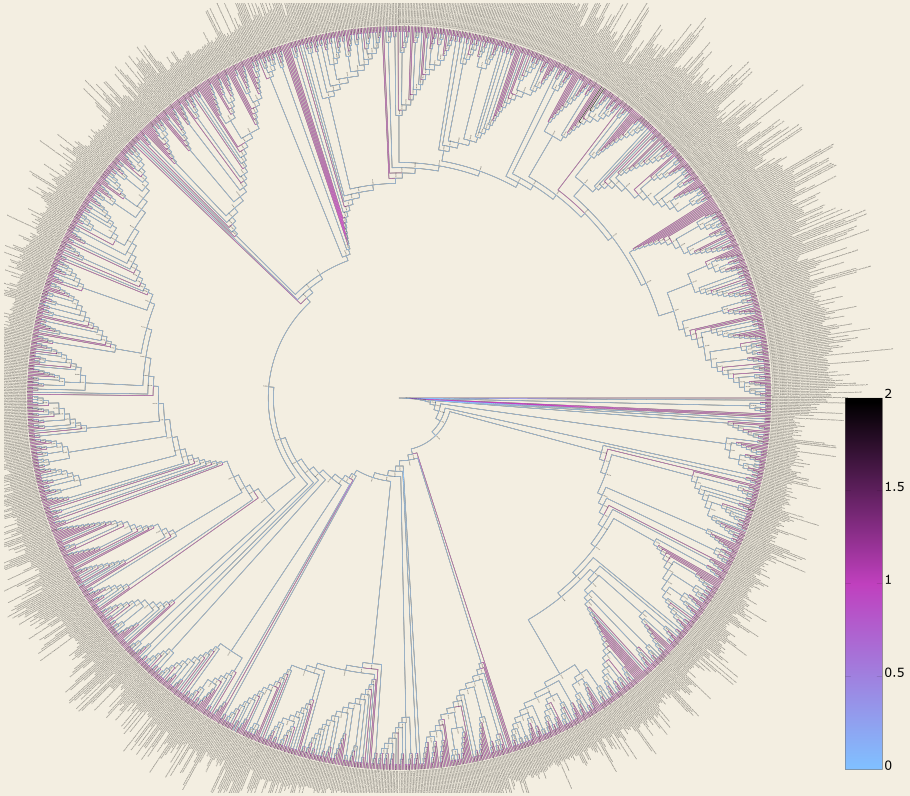
Methodology / Implementation

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

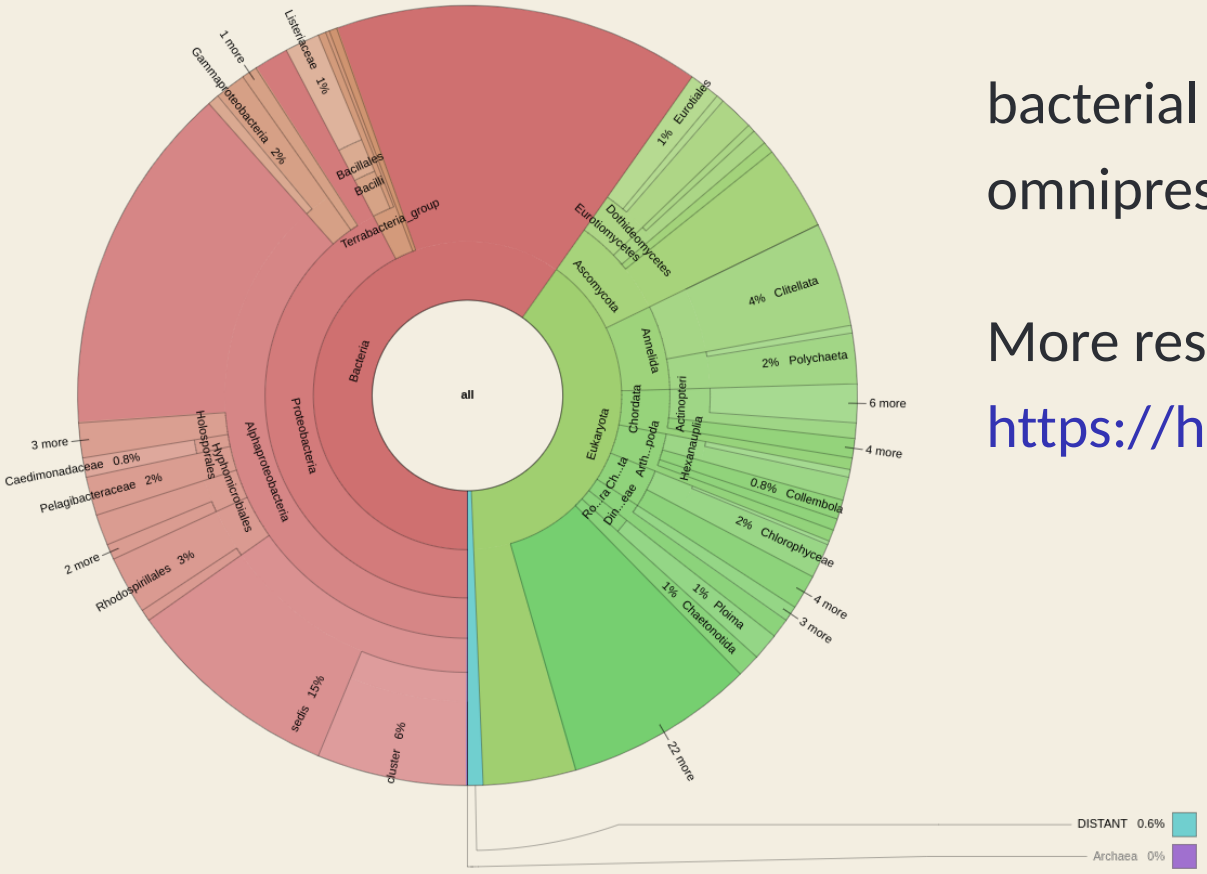
Phylogenetic tree of the COI consensus sequences retrieved



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 bioinormatics 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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    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
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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:

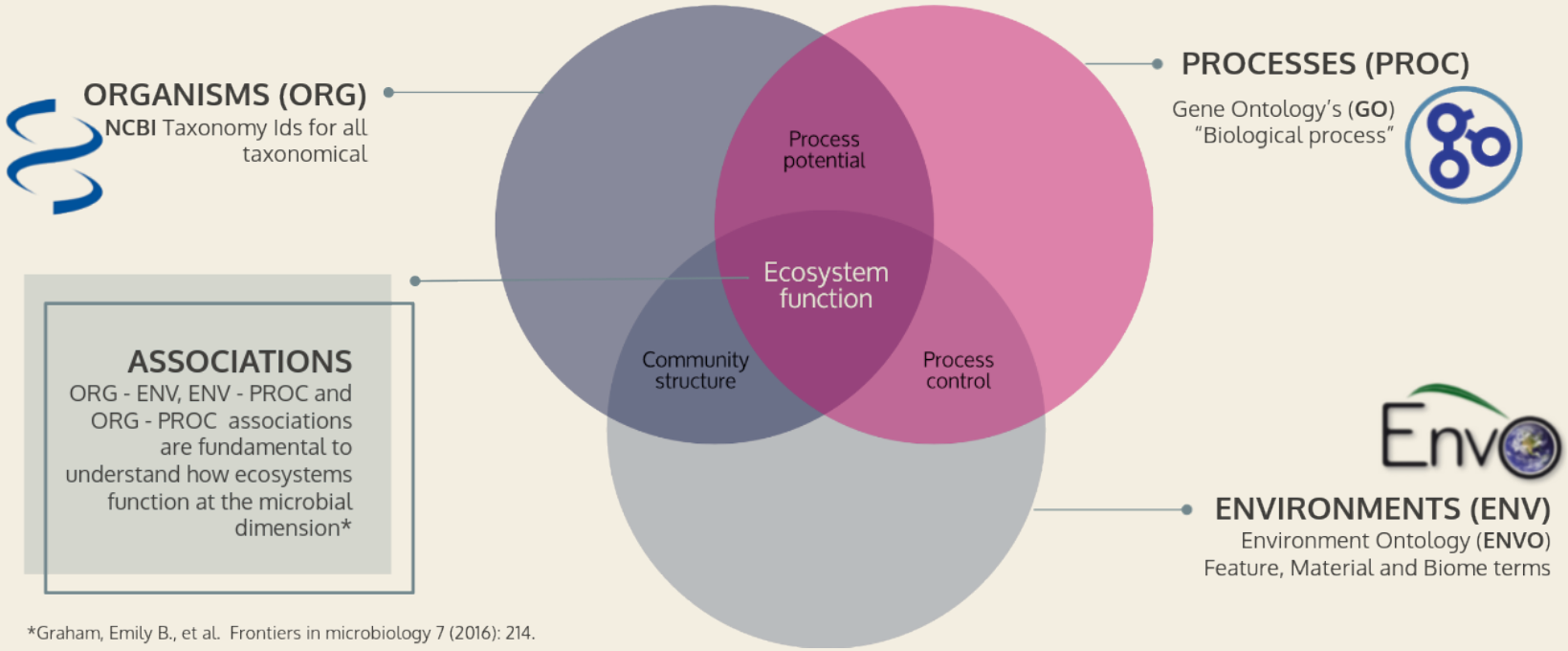
- *organisms* and the *environments* they inhabit
- *organisms* and the biological *processes* they are involved with
- *processes* and the *environments* where they occur

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

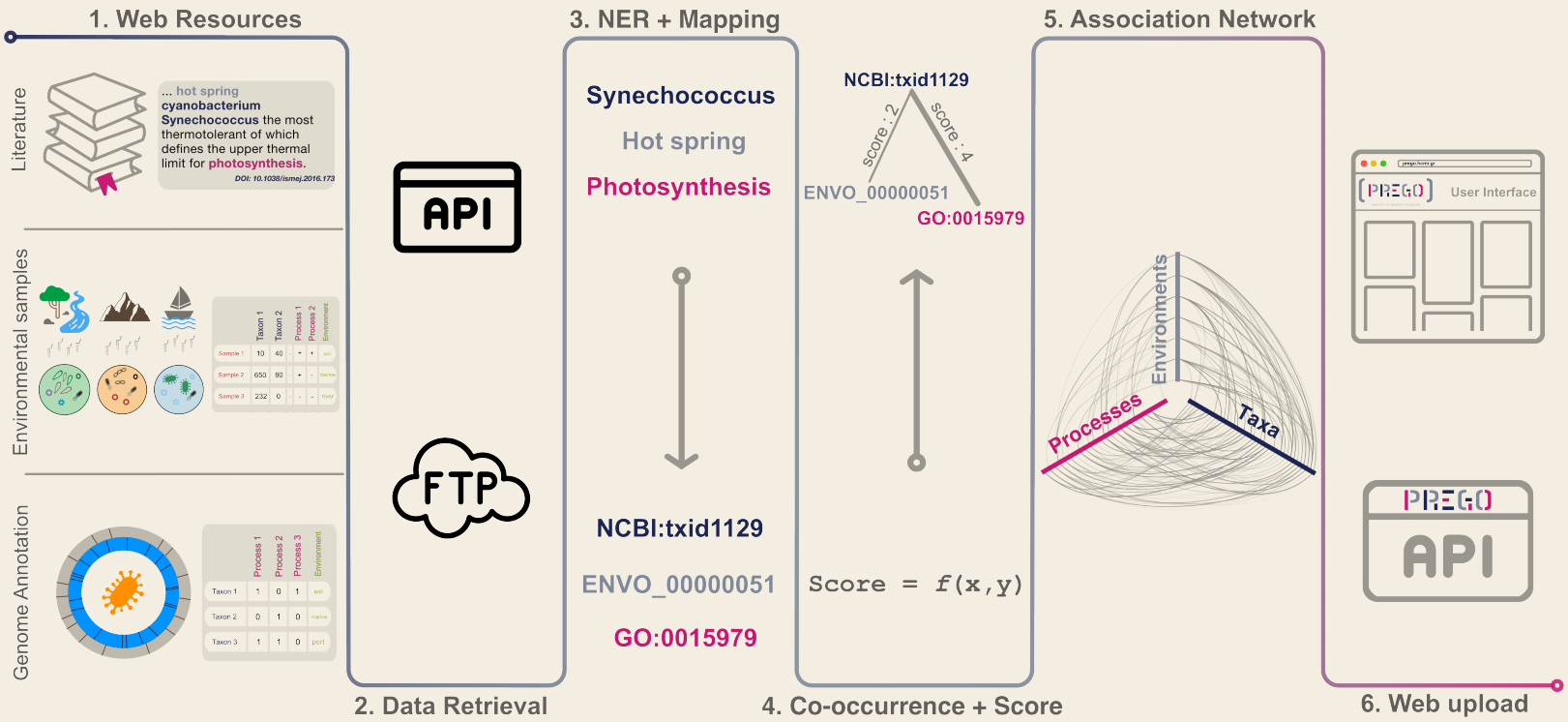
the fundamental role of ontologies



*Graham, Emily B., et al. Frontiers in microbiology 7 (2016): 214.

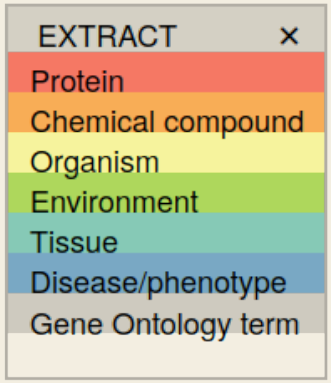
Methods / Implementation

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

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 hydrogenivorans* [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:	miens-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	mgl485562	Cincinnati	USA	39.11, -84.5	metadata submitted
mgm4702469.3	06052015a_S3_L001_R2_001	Large lake biome	lake	water	mgs485566	mgl485568	Cincinnati	USA	39.11, -84.5	metadata submitted
mgm4702471.3	06032015a_S1_L001_R2_001	Large lake biome	lake	water	mgs485554	mgl485556	Cincinnati	USA	39.11, -84.5	metadata submitted

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:

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

Associations between entities of PREGO

after metadata retrieveal and co-occurrence analysis

Channel	Source	Environments - Processes	Environments - Functions	Taxonomy	Taxa - Environments	Taxa - Processes	Taxa - Function
Literature	MEDLINE PubMed - PMC OA	883,997	422,579	Strains	69,968	590,630	384,079
				Species	778,877	3,501,635	1,961,920
				Total	1,669,608	7,969,310	4,613,827
Environmental samples	MG-RAST amplicon	-	-	Strains	13,645		
				Species	39,007	-	-
				Total	53,439		
	MG-RAST metagenome	-	620,846	Strains	262,106		8,626,328
				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	
	JGI IMG isolates	-	-	Strains	8,229		3,461,693
				Species	42,141	-	13,216,559
				Total	50,888		16,821,850
Annotated Genomes and Isolates	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	
Total	All	883,997	1,043,425	Strains	357,229	598,103	12,473,903
				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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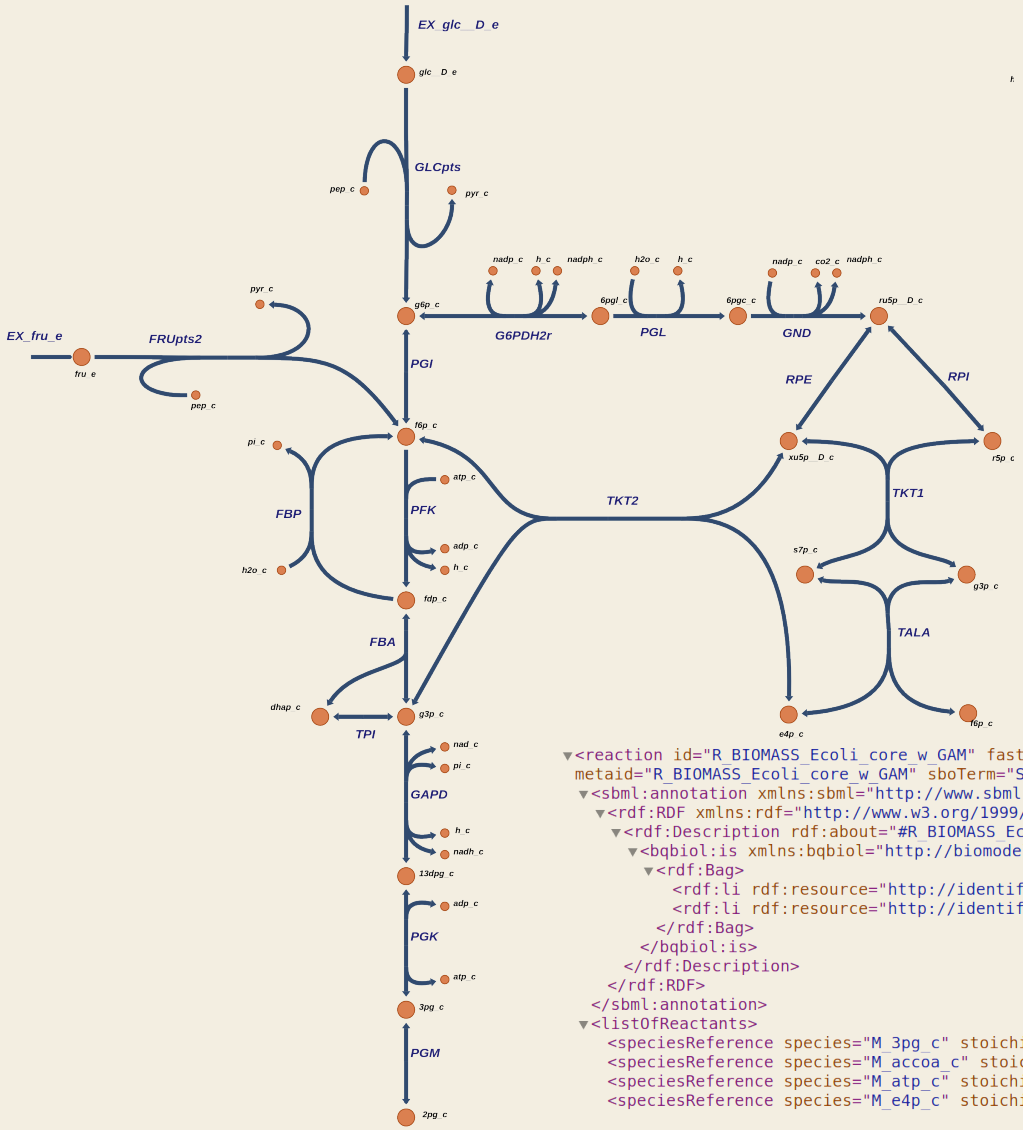
Chapter 4: A New MCMC Algorithm for Sampling the Flux Space of Metabolic Networks

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.
To allow flux sampling at high dimensional polytopes, such as those of multispecies communities or host-microbe cases, we introduce a Multi-phase Monte Carlo Sampling (MMCS) algorithm.

Metabolic modelling

and the biomass function




```
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```

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

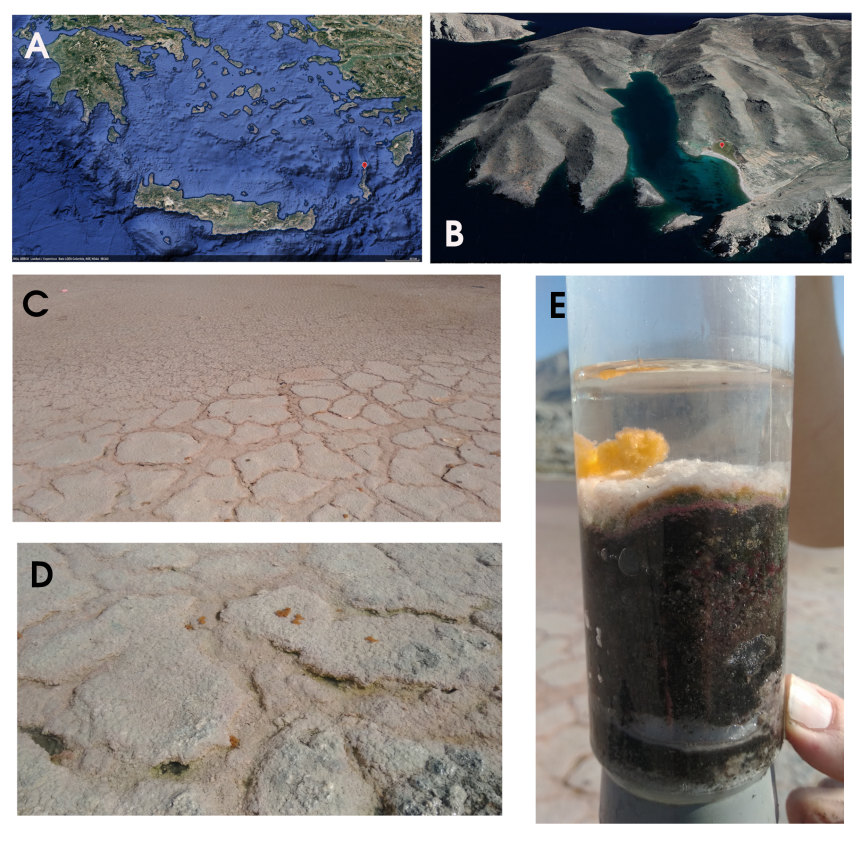
a seasonal brackish water marsh formed at the edge of a small plain

Type of samples:

- from clearly observed mats, top - bottom layers
- if no clearly observed mats samples with no slicing
- aggregate samples

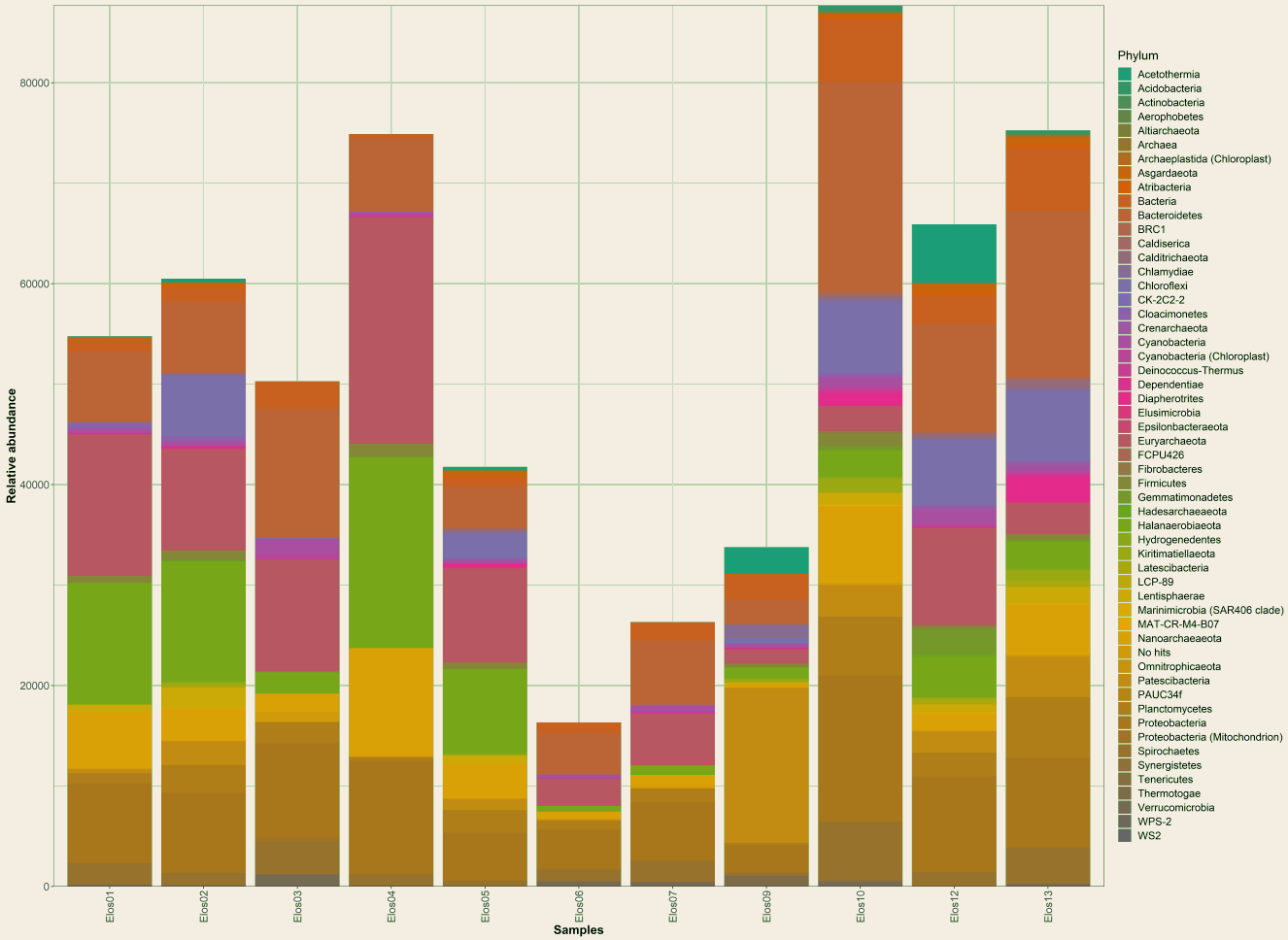
Sampling time points:

- July 2018
- November 2019



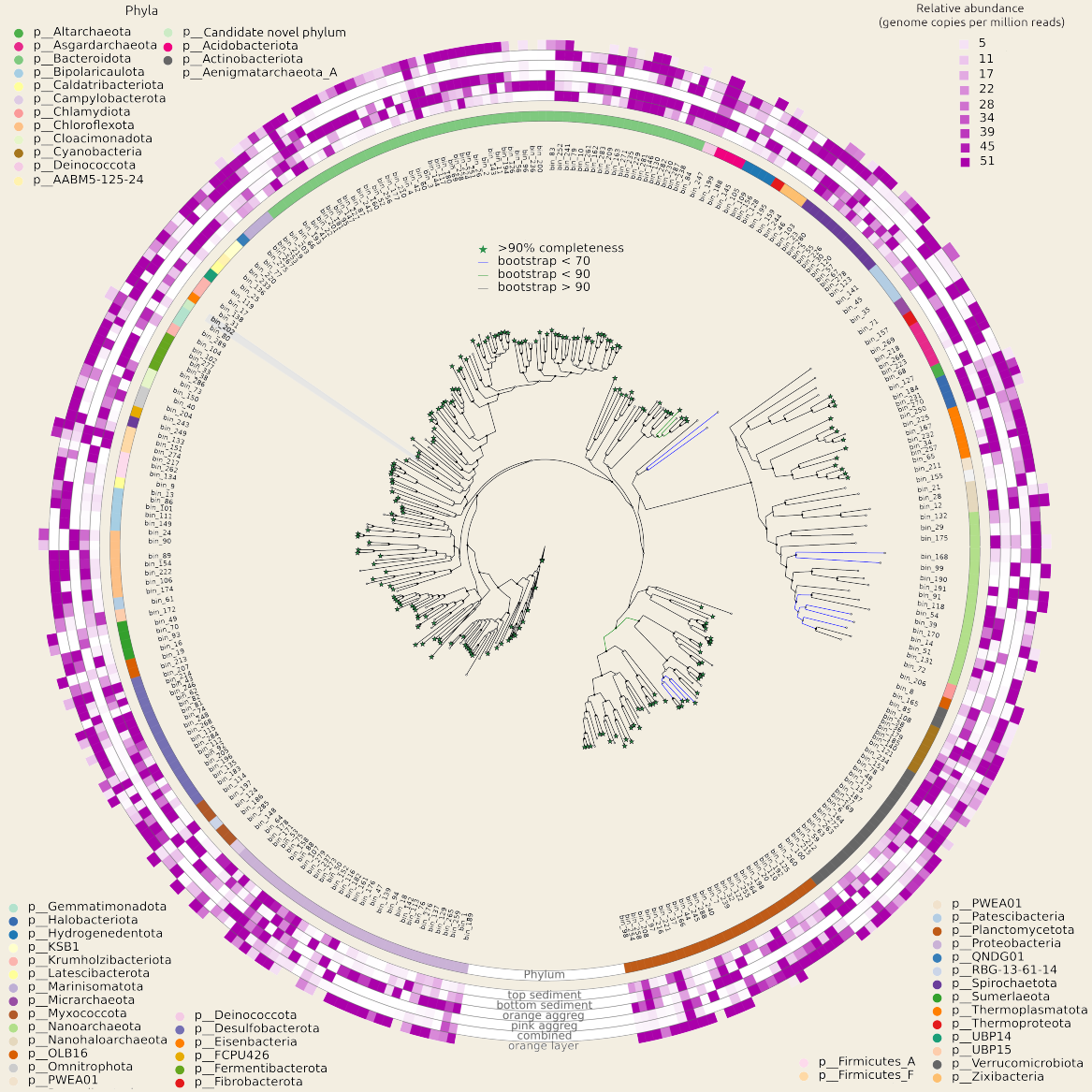
Abundances of the main microbial taxa, at the phylum level

based on 16S rRNA amplicon data



MAGs phylogeny

based on 25 single-copy genes

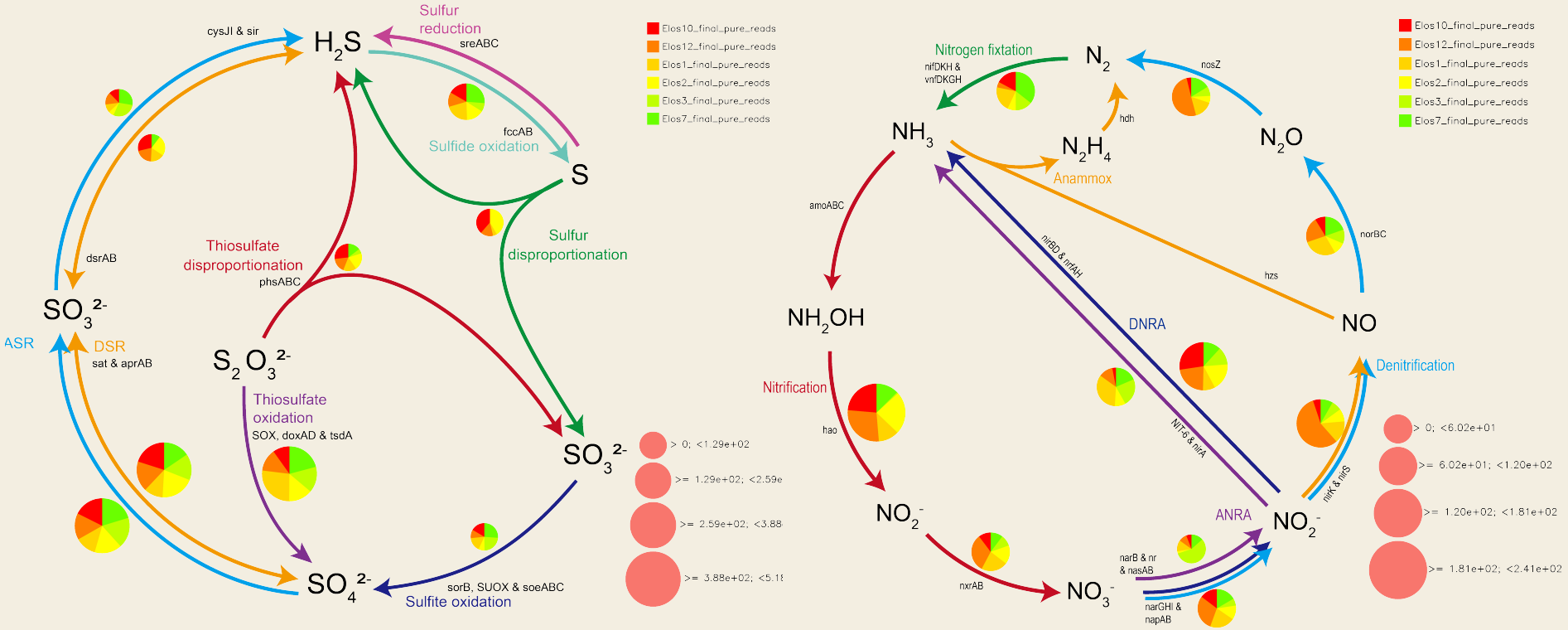


Metabolic pathways per biogeochemical cycle

and their relative abundance at each sample

The S and the N cycle

using KEGG annotation terms



Conclusions

on the

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:** 0s and 1s in marine molecular research: a regional HPC perspective
7. **Chapter 7: Conclusions**

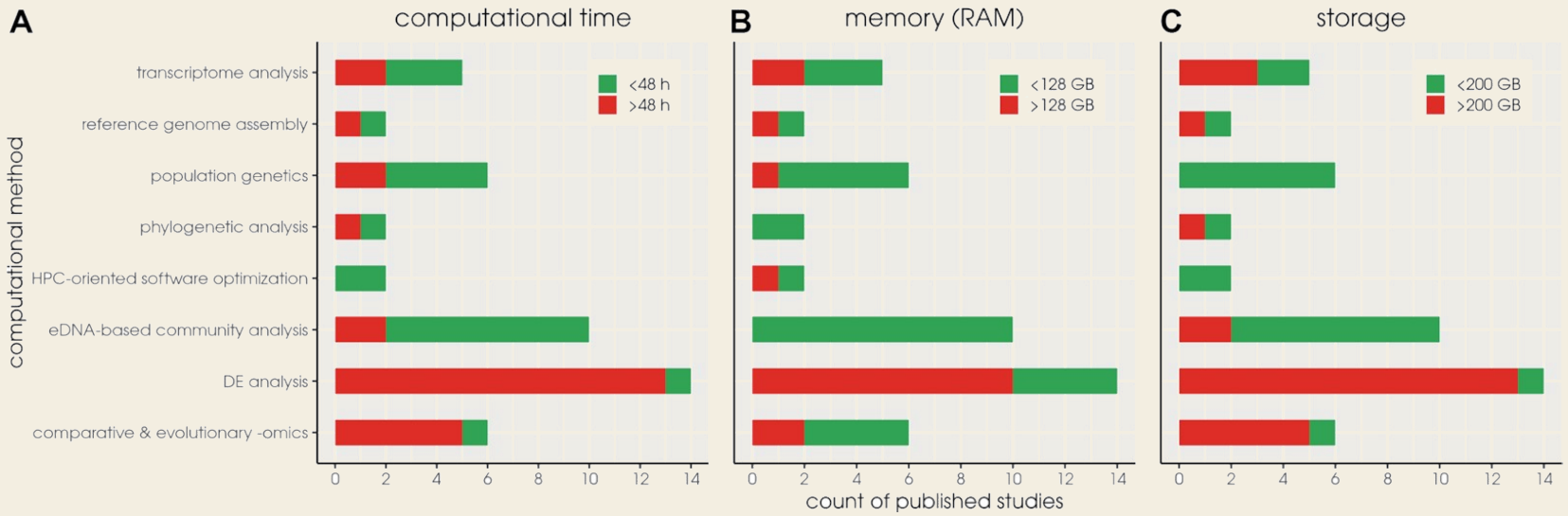
Chapter 6: 0s and 1s in marine molecular research: a regional HPC perspective

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

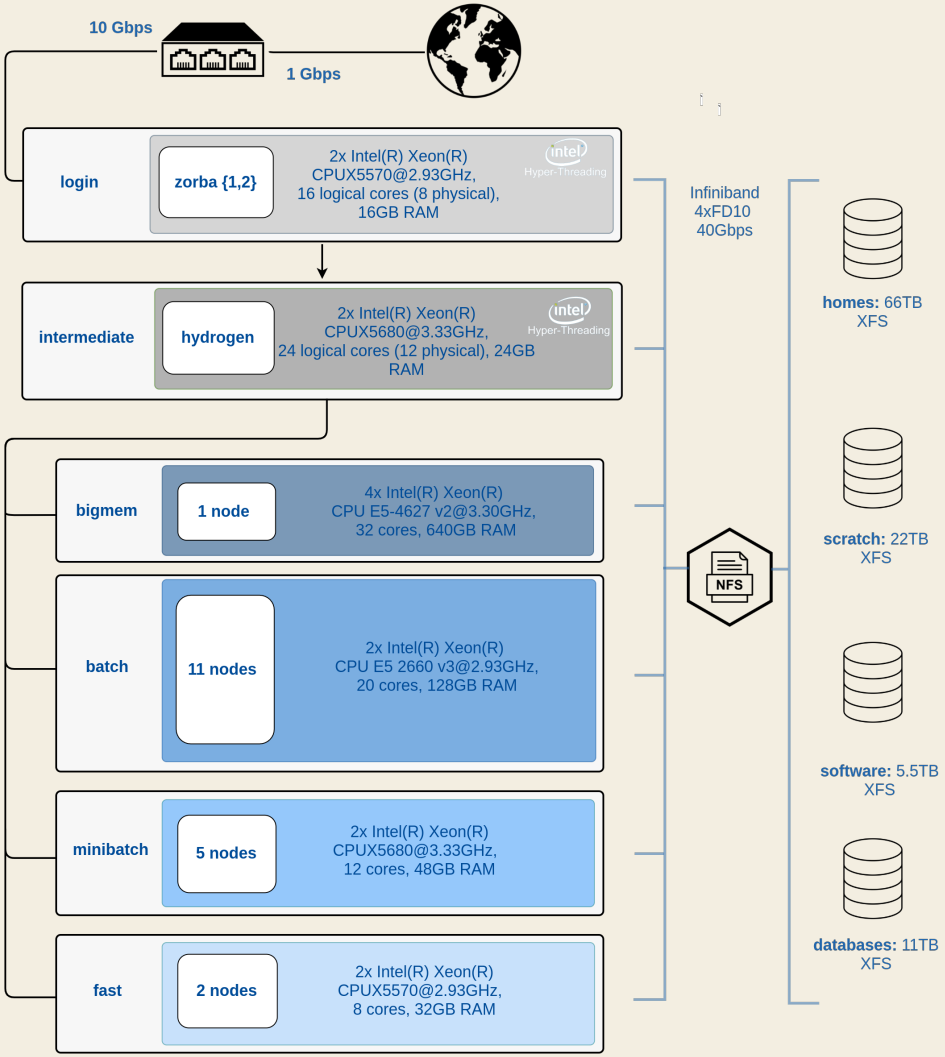
for trivial bioinformatic tasks



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 assesment 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

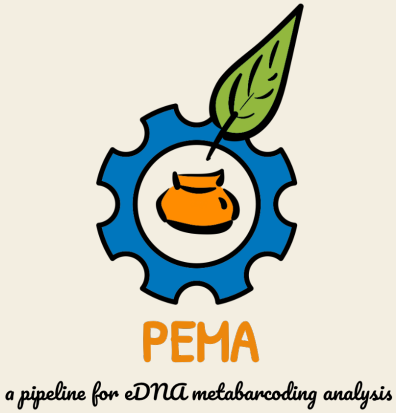
- Hypersaline mats host a great range of novel taxa & their functioning might be subject to anaplerotic reactions

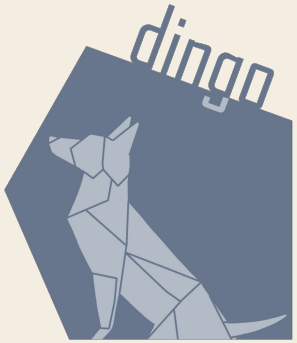
Perspective for future work

"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





github.com/hariszaf/pema

github.com/hariszaf/darn

github.com/lab42open-team/ the `prego*` repositories

github.com/GeomScale/dingo

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.

[2] **Zafeiropoulos, H.**, Gargan, L., Hintikka, S., Pavloudi, C. & Carlsson, J. (2021). The Dark mAtteR iNvestigator (DARN) tool: getting to know the known unknowns in COI amplicon data. Metabarcoding and Metagenomics, 5, e69657.

[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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[7] Pavloudi, C. & **Zafeiropoulos, H.** (2022) Deciphering the community structure and the functional potential of a hypersaline marsh microbial mat community ***(under review at FEMS Microbiology Ecology)***

[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

Google
Summer of Code

Acknowledgments

people and more

My promotors:

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. Pavloudi C.

My corner:

Would not be here

if it was not with you.

GeomScale