1st Conference on Green Chemistry and Sustainable Coatings

Pisa, Italy, 17th -18th June 2022

Study of the microbial communities associated with cultures of *Acartia tonsa* (Copepoda, Calanoida) and involved in the degradation of poly(butylene succinate-co-butylene adipate) (PBSA)

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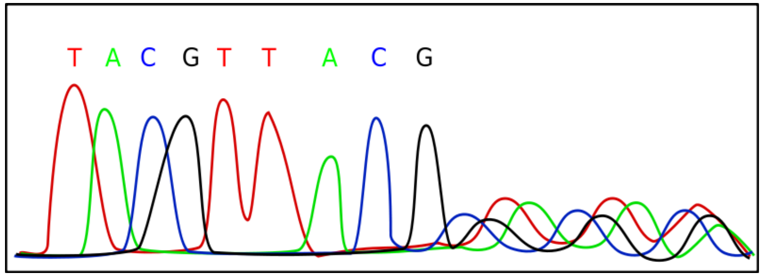
Horizon 2020
European Union Funding
for Research & Innovation

BBI JU

Bio-based Industries
Consortium

Aim of this work

Taxonomic and functional characterization of microbial communities associated with cultures of the marine calanoid copepod *Acartia tonsa:*



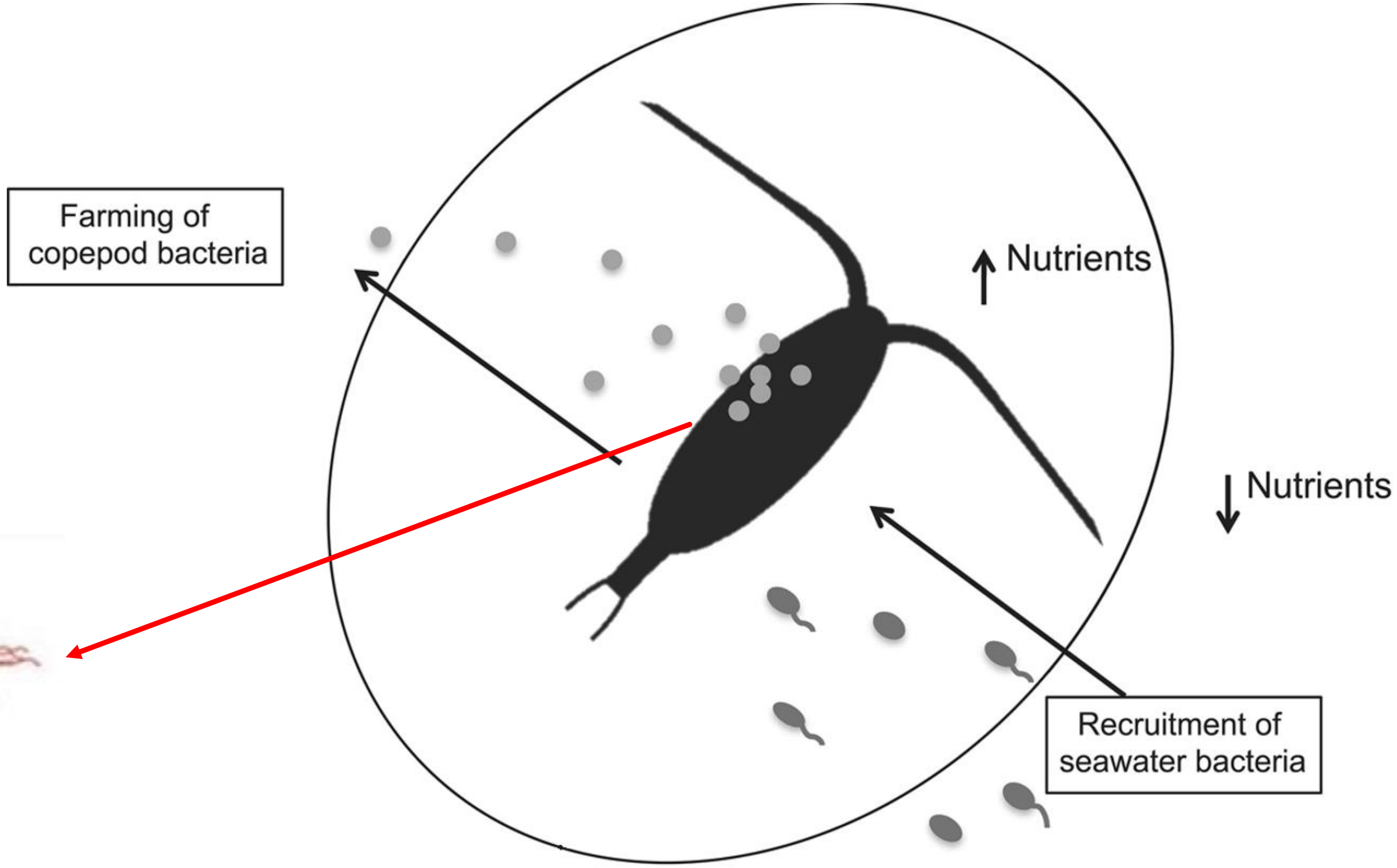
- *Culture-independent* approach

- *Culture-dependent* approach
- *Culture-independent* and *culture-dependent* approaches

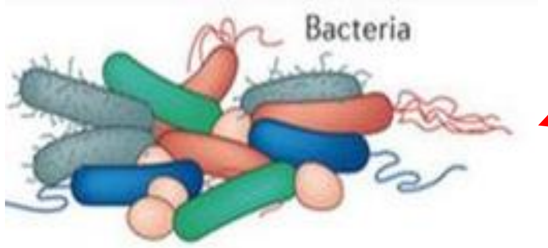
PBSA

Acartia tonsa: microbial hotspot

Zoosphere



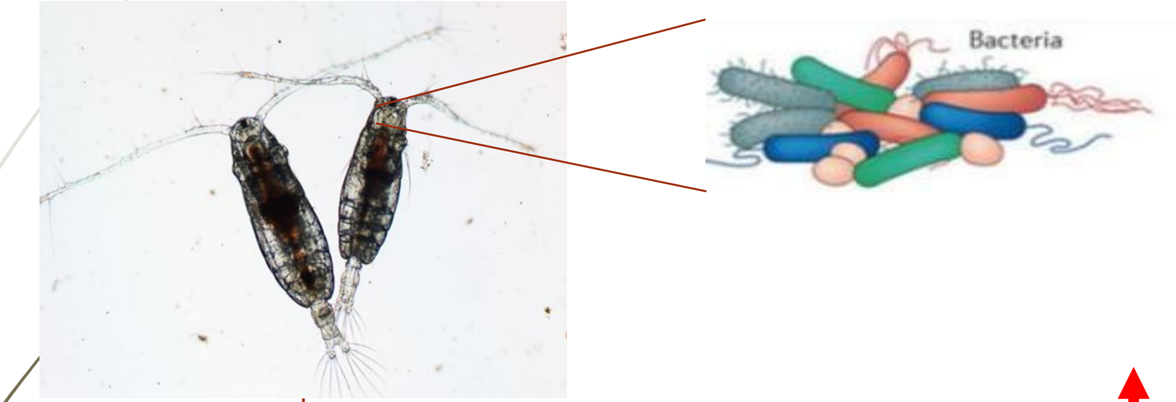
Core microbiome



Shoemaker M. K. *et al*., 2019

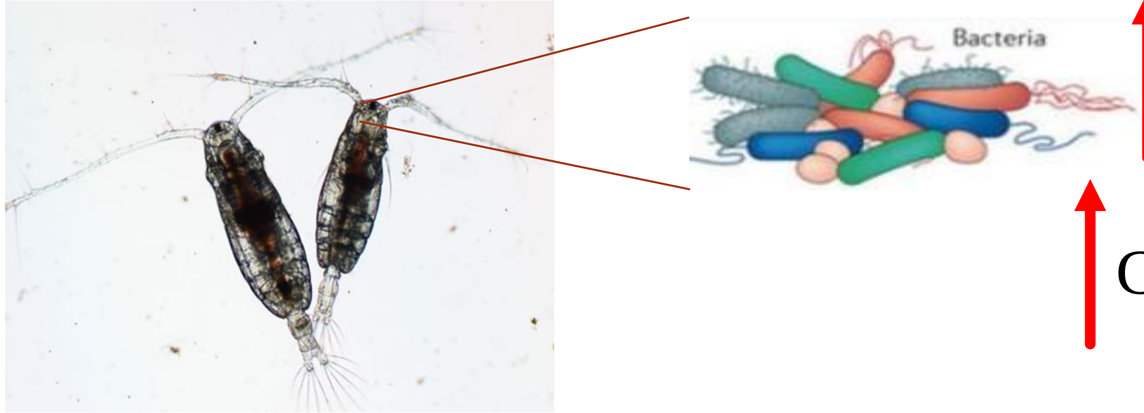
Functionality of microbial communities associated to *A.tonsa* carcasses

Newly formed carcasses



Degradation

Remains of carcasses

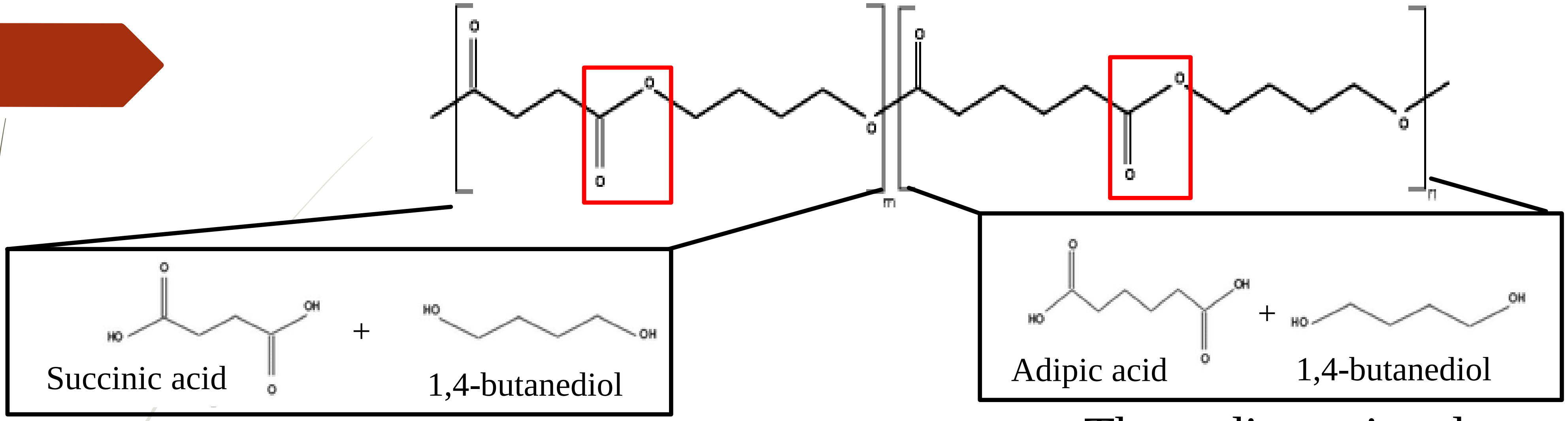


↑ Protease activities

↑ Lipase activities

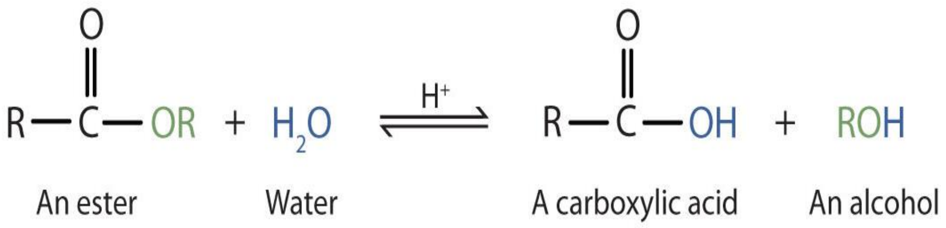
↑ Chitinase activities

poly(butylene succinate-co-butylene adipate)(PBSA)



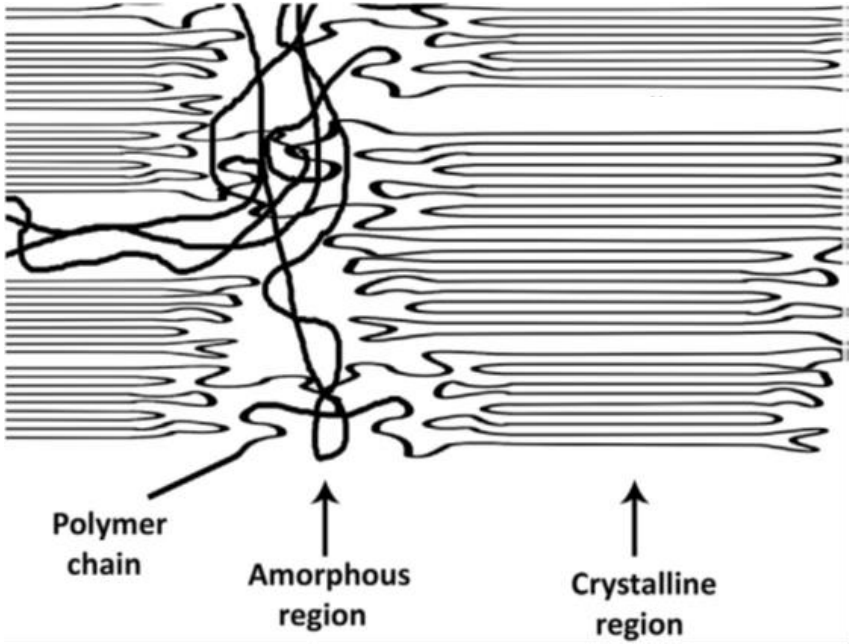
Shah A.A. *et al.*, 2014

Carboxyl-ester hydrolase



$$R-\overset{\overset{O}{\|}}{C}-OR + H_2O \overset{H^+}{\rightleftharpoons} R-\overset{\overset{O}{\|}}{C}-OH + ROH$$

Three-dimensional organization



Crawford C. B. *et al.*, 2017

Methods → *Acartia tonsa* colture

Rearing conditions:

- ✓ Filtered seawater → 0,22 µm
- ✓ Salinity → 30 psu
- ✓ Photoperiod → 14:10 h light: dark
- ✓ Temperature → 20±1 °C

ISPRA Laboratory colture

R. reticulata monoalgal diet

culture independent approach

metabarcoding rDNA16S and *predictive functional profiling*

"alive" Adults

New formed adults (VG0R)

Seven-day adults (VG7R)

Adults, artificially induced death (carcasses)

New formed carcasses (CG0R)

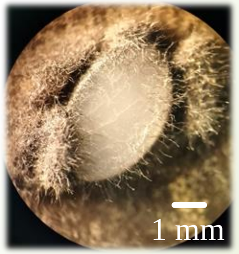
Four-day carcasses (CG4R)

Thirty-three-day carcasses (CG33R)

culture dependent approach

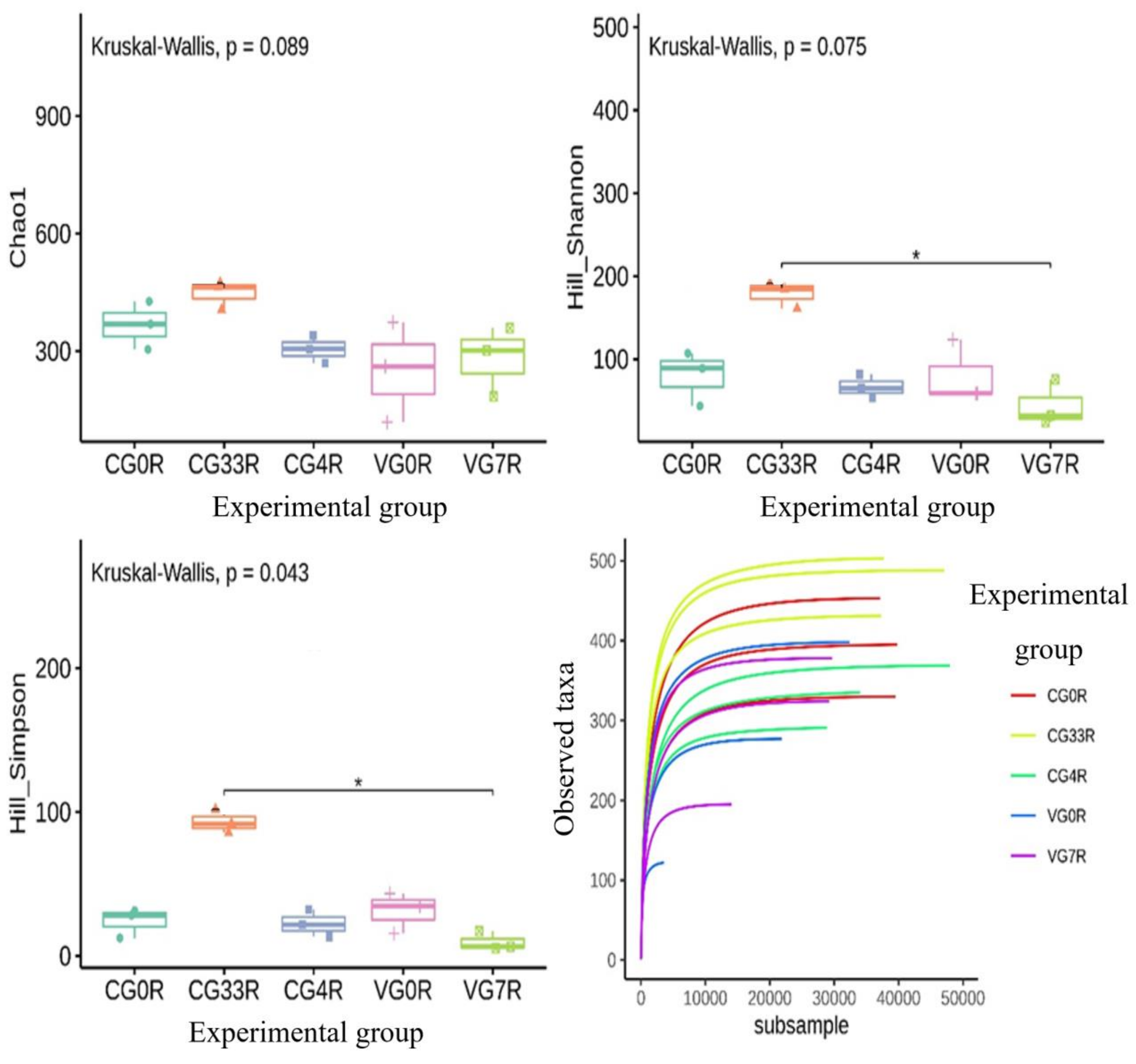
debris of *A.tonsa* carcasses

Bacterial and fungal morphotype isolation from PBSA

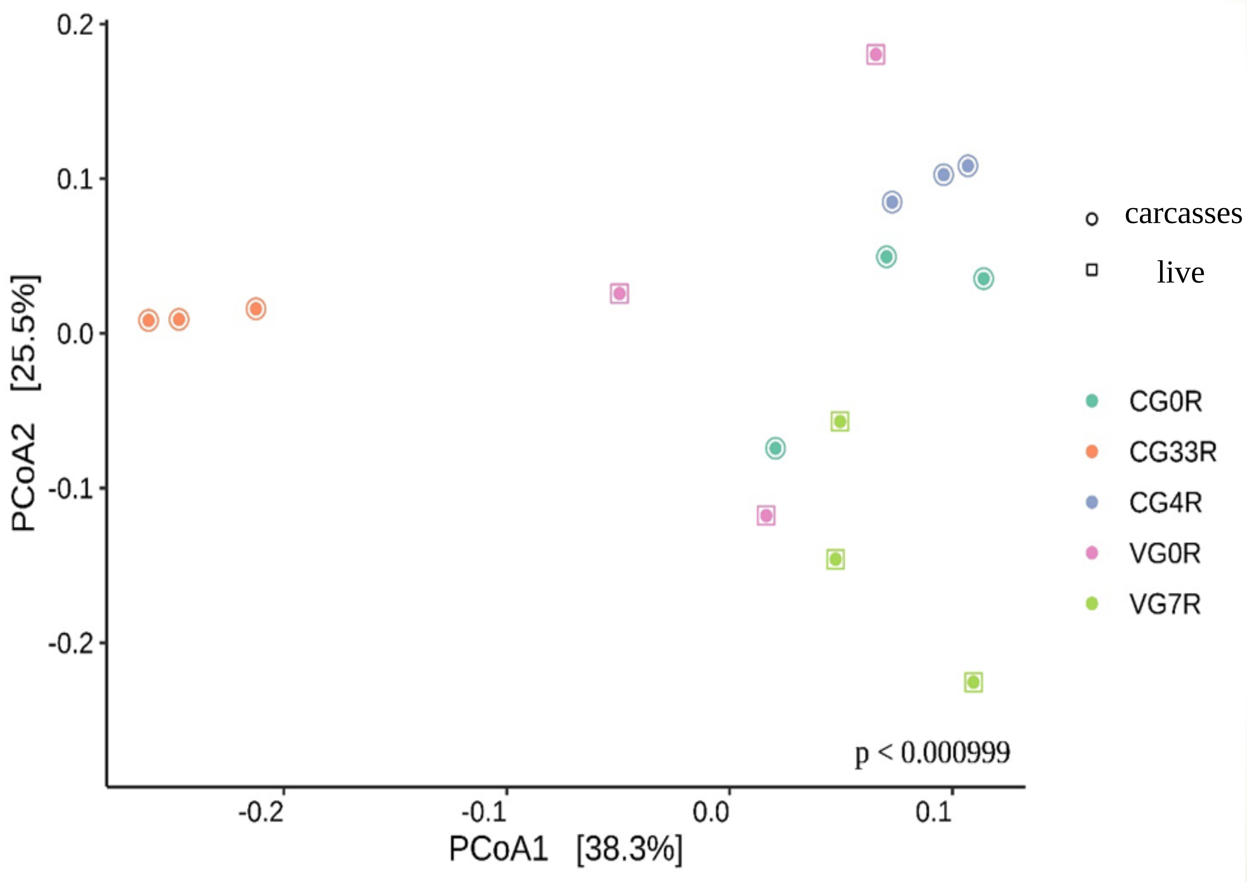


Results of *Culture-independent* approach

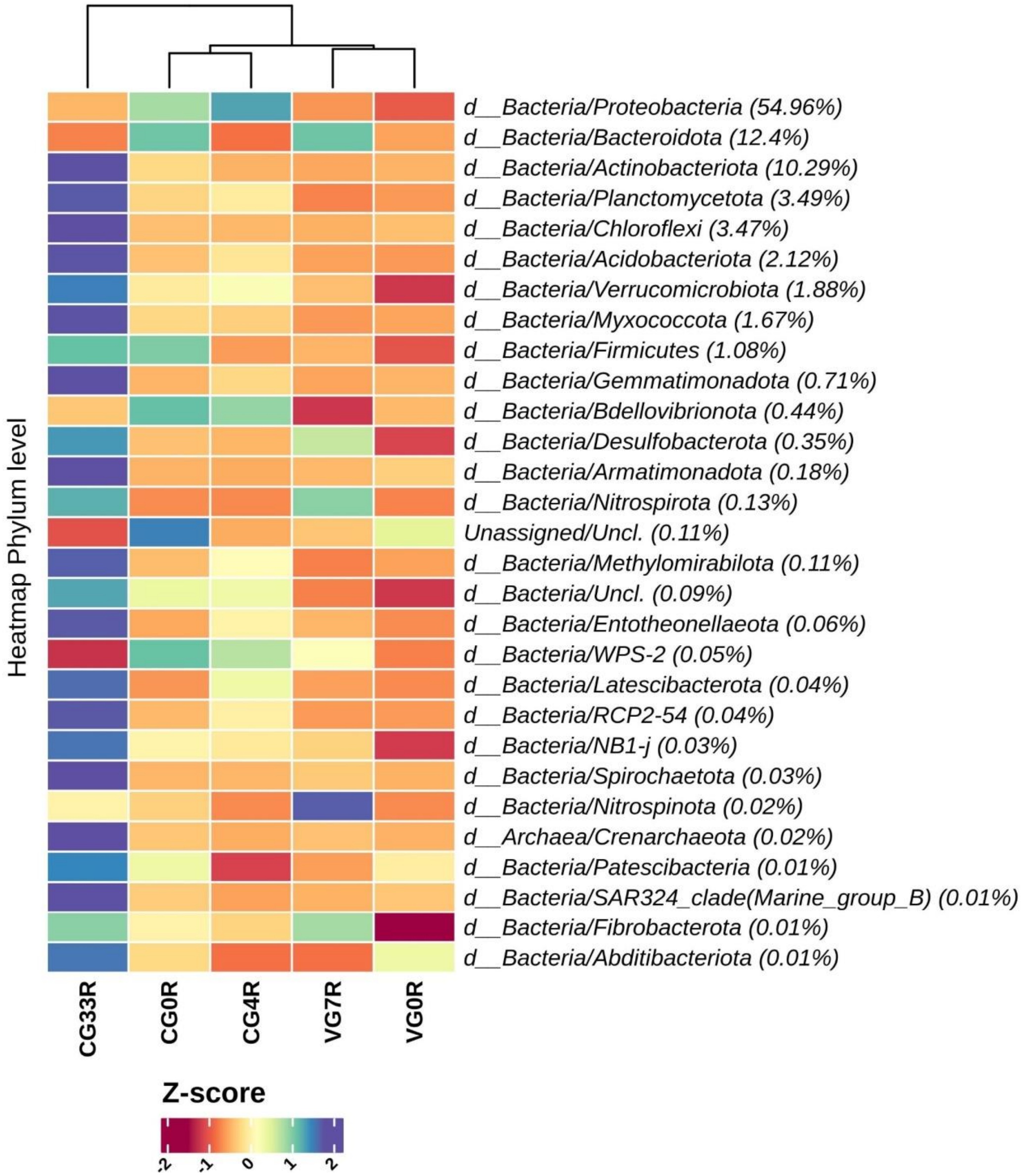
α-diversity

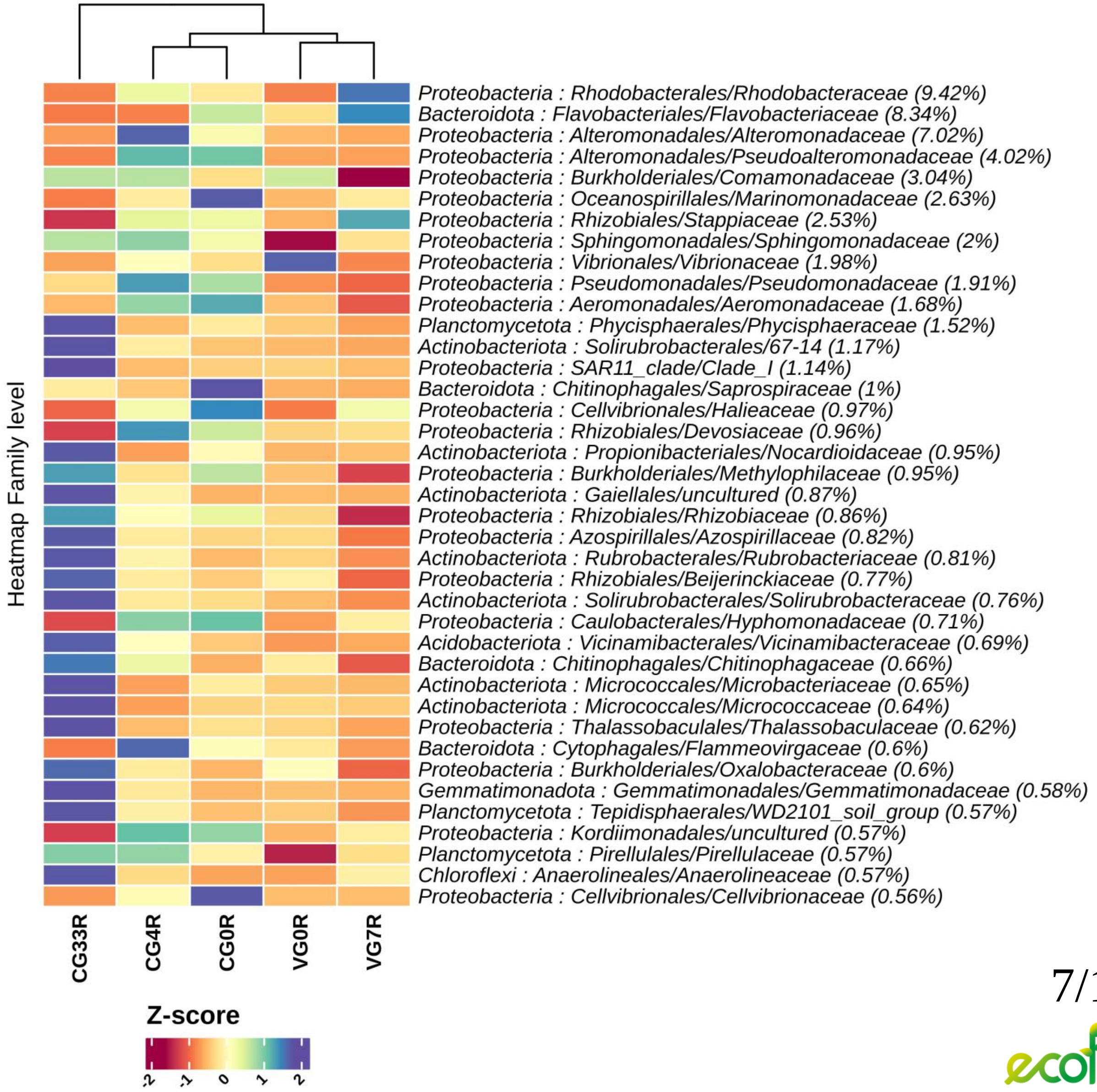


β-diversity



Results of *culture-independent* approach: Taxonomic profiling of bacterial community



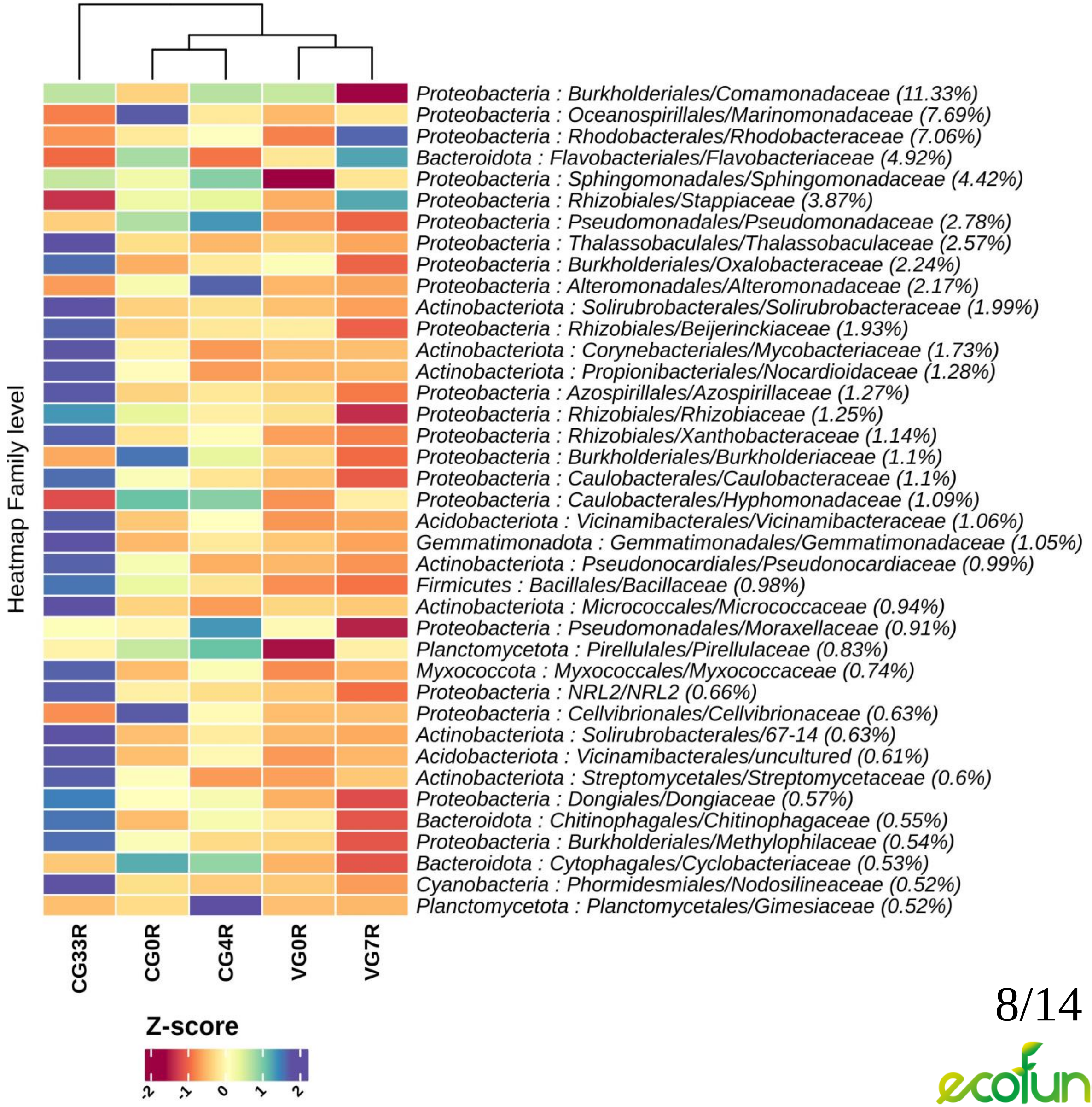


Results of *culture-independent* approach: *predictive functional profiling* of bacterial communities

Carboxylic-ester hydrolase activities

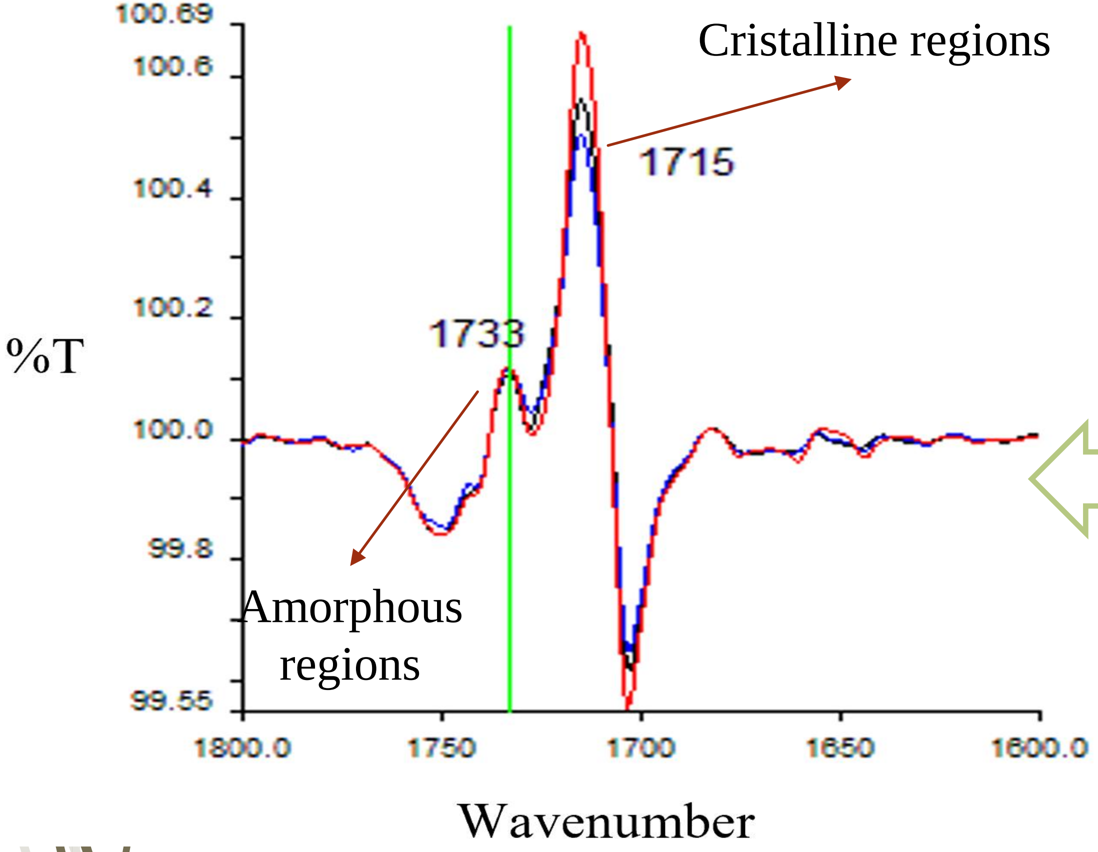
→ Carboxylesterase
→ Triacylglicerol lipase
→ Cutinase

PBSA degradation



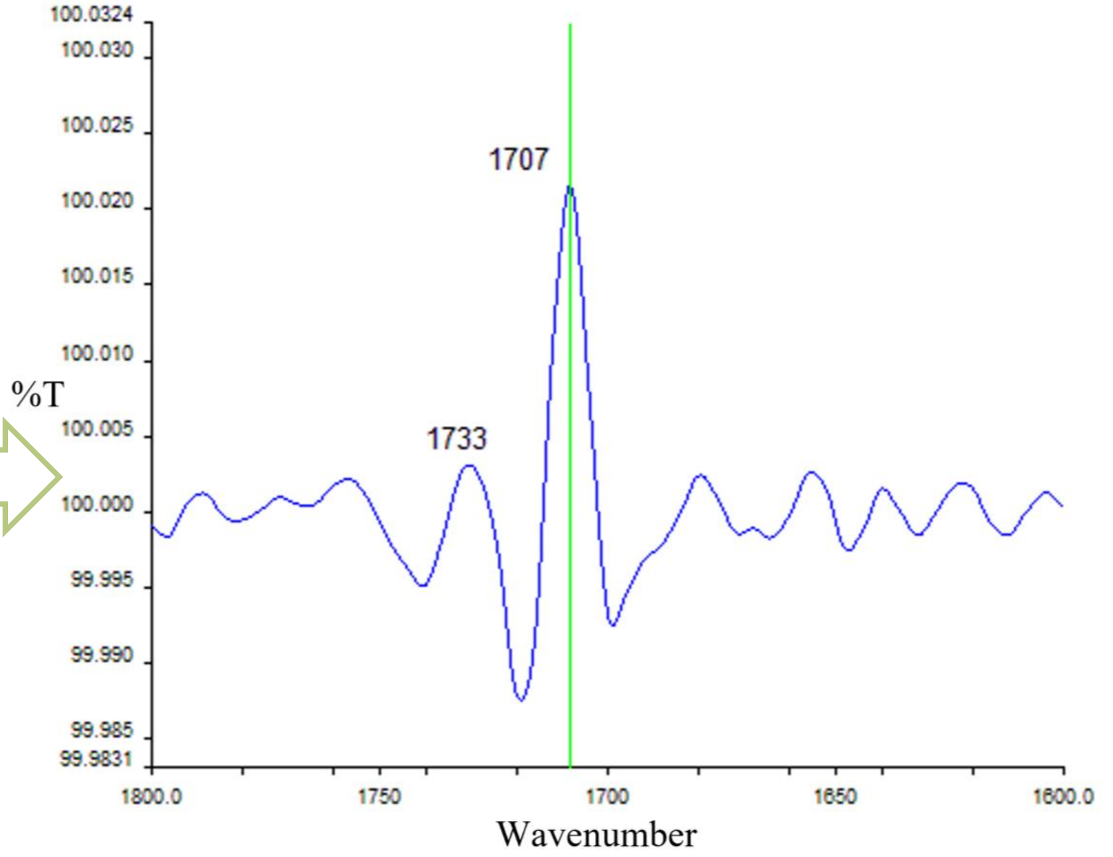
Results of *culture-dependent* approach: *Vibrio* sp.01 promotes the degradation of PBSA

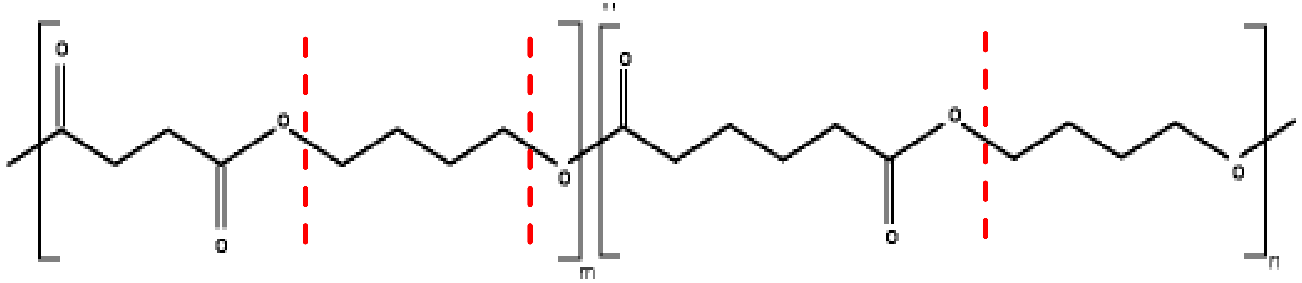
IR spectrum from non-inoculated granule



FTIR-ATR

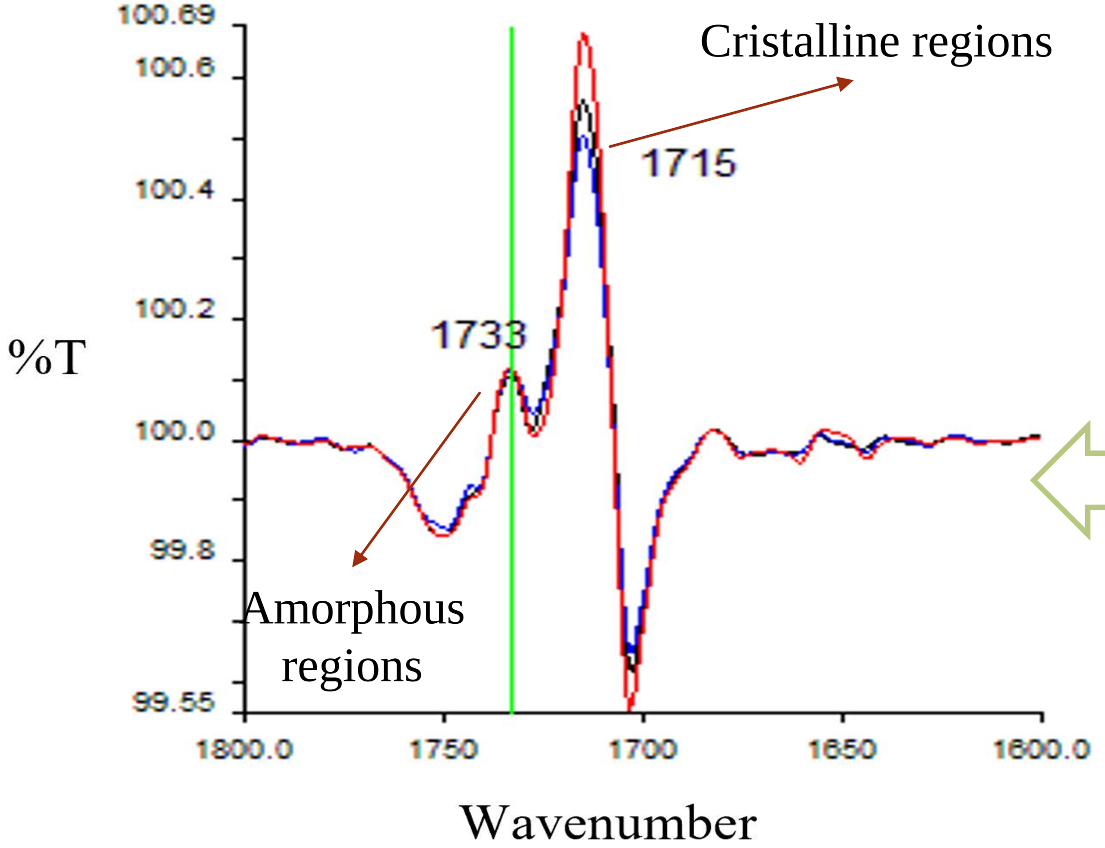
IR spectrum from 82 days inoculated granule





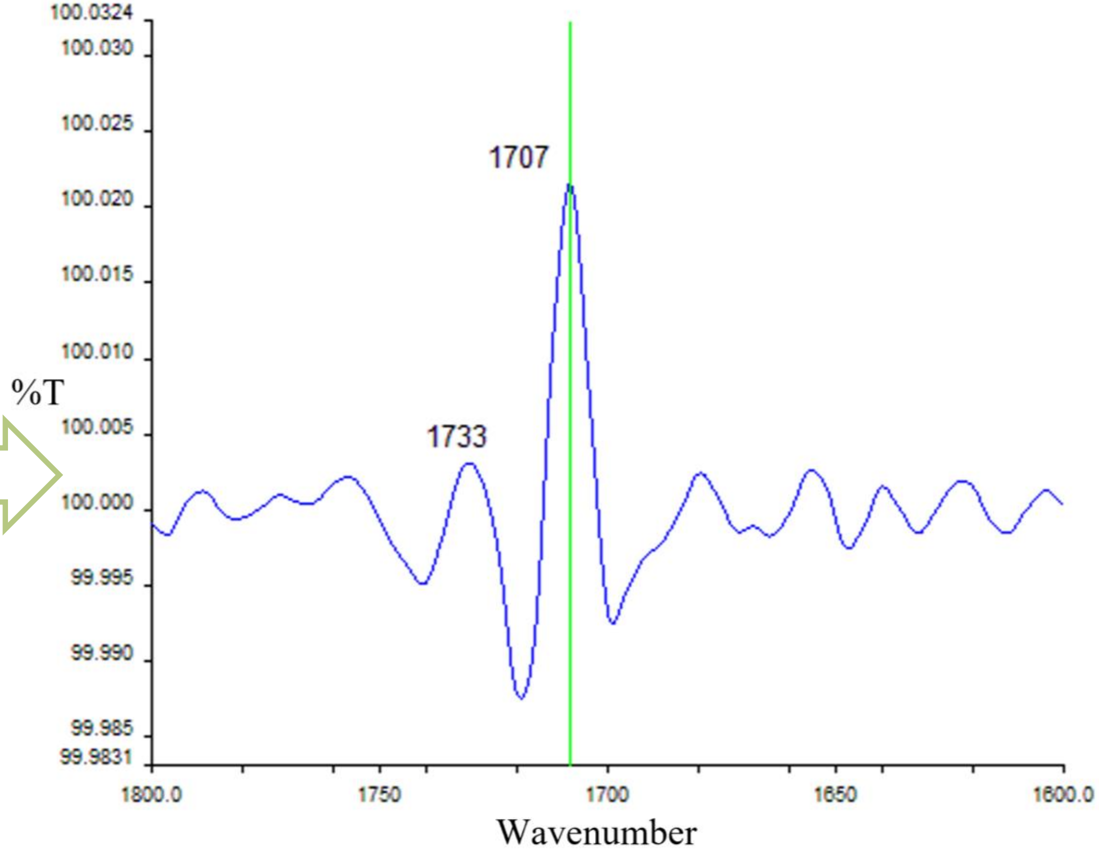
Results of *culture-dependent* approach: *Vibrio* sp.01 promotes the degradation of PBSA

IR spectrum from non-inoculated granule



FTIR-ATR

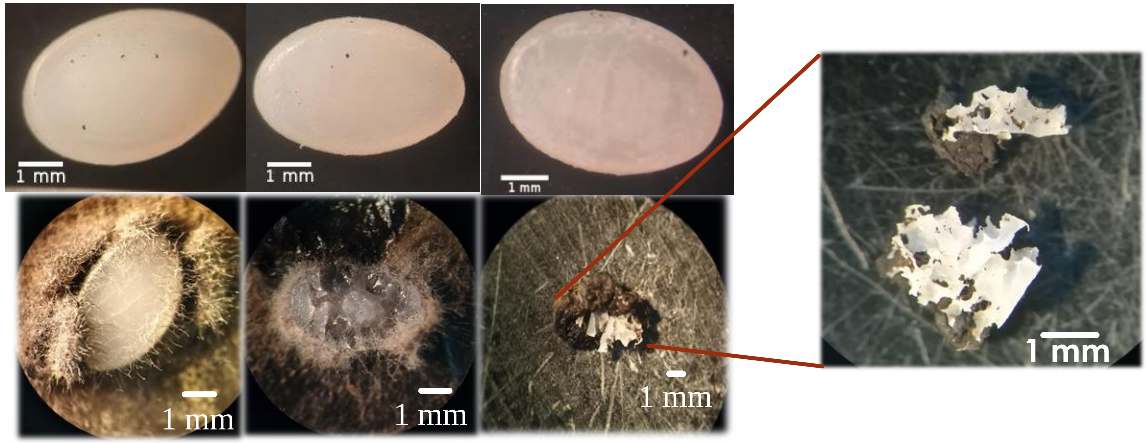
IR spectrum from 82 days inoculated granule

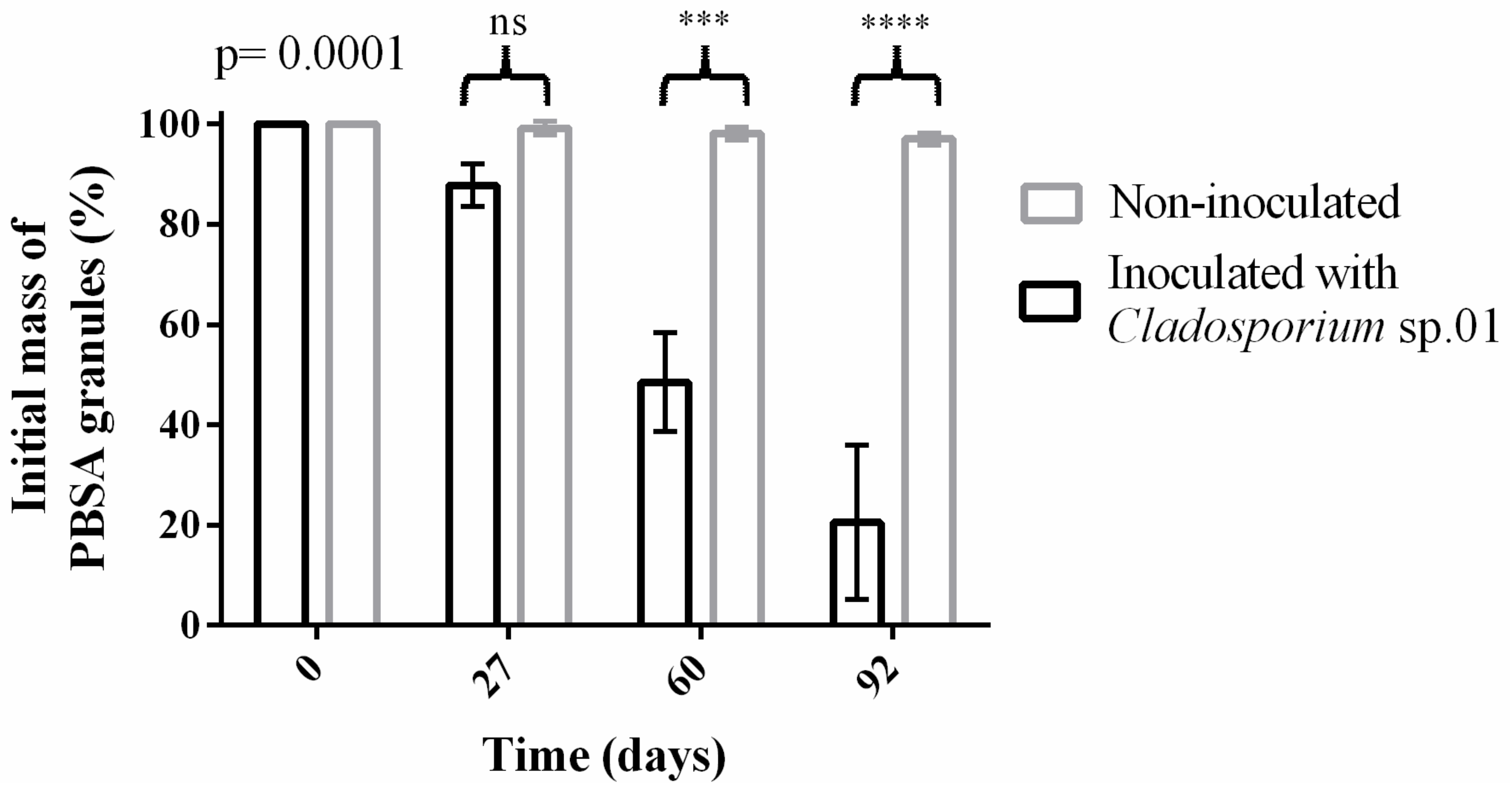


Carbonyl index

Uninoculated granule (82 days) (mean ± sd)	Inoculated granule with *Vibrio* sp. 01 (82 days) (mean ± sd)	t-test (independent samples)
15±0,3	**13,4±0,5**	**p=0,0085**

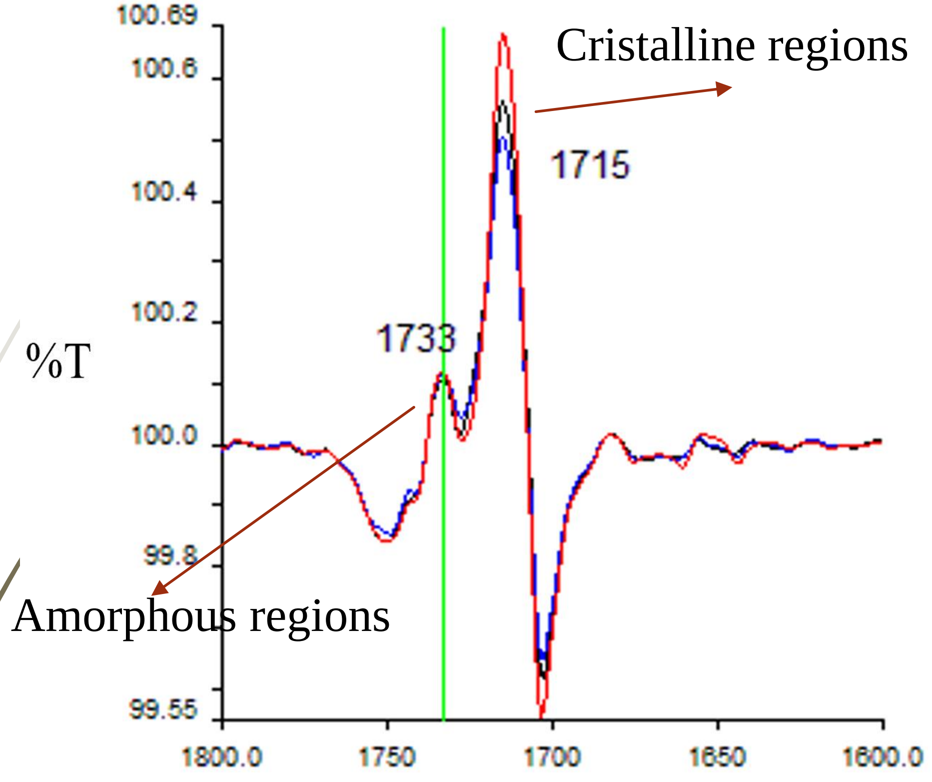
Results of *culture-dependent* approach: *Cladosporium* sp. 01 promotes the degradation of PBSA





Results of *culture-dependent* approach: IR spectrum of PBSA granule inoculated with *Cladosporium* sp.01

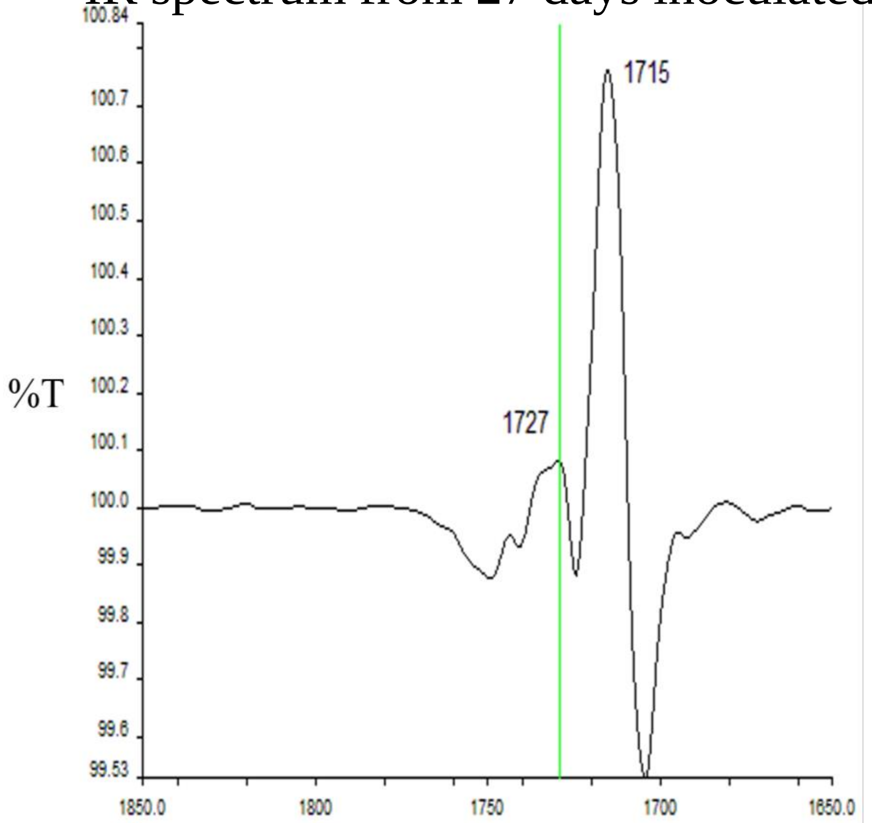
IR spectrum from non-inoculated granule



Cristalline regions

Amorphous regions

IR spectrum from 27 days inoculated granule



Carbonyl index

Uninoculated granule (60 days) (mean ± sd)	Inoculated granule with *Cladosporium* sp. 01 (60 days) (mean ± sd)	t-test (independent samples)
14,3±0,1	**11,6±0,8**	**p=0,0052**

Results overview

Culture-independent approach →

- ✓ «alive» adults→ transient associations
- ✓ Carcasses → stable associations and equidistribution of contribution to the PBSA degradation

Culture-dependent approach → PBSA as the only carbon source

I. *Vibrio* sp.01→ hydrolysis of ester bonds in the surface regions of PBSA

II. *Cladosporium* sp.01→ hydrolysis of ester bonds throughout PBSA and reduction of PBSA granule mass.

Conclusions and perspectives

Copepod

nursery of microorganisms that show enzymatic activities of interest

degradation of biopolymers with potential applications in the marine environment

- Genome sequencing of bacterial and fungal isolates.
- Set-up of petroleum-derived plastics experiments
- Refining *culture-dependent* approaches
- Extend a *culture-independent* approaches to fungi

DICI
DIPARTIMENTO DI INGEGNERIA
CIVILE E INDUSTRIALE

ISPRA
Istituto Superiore per la Protezione
e la Ricerca Ambientale

Sistema Nazionale
per la Protezione
dell'Ambiente

DIPARTIMENTO DI BIOLOGIA
UNIVERSITÀ DI PISA

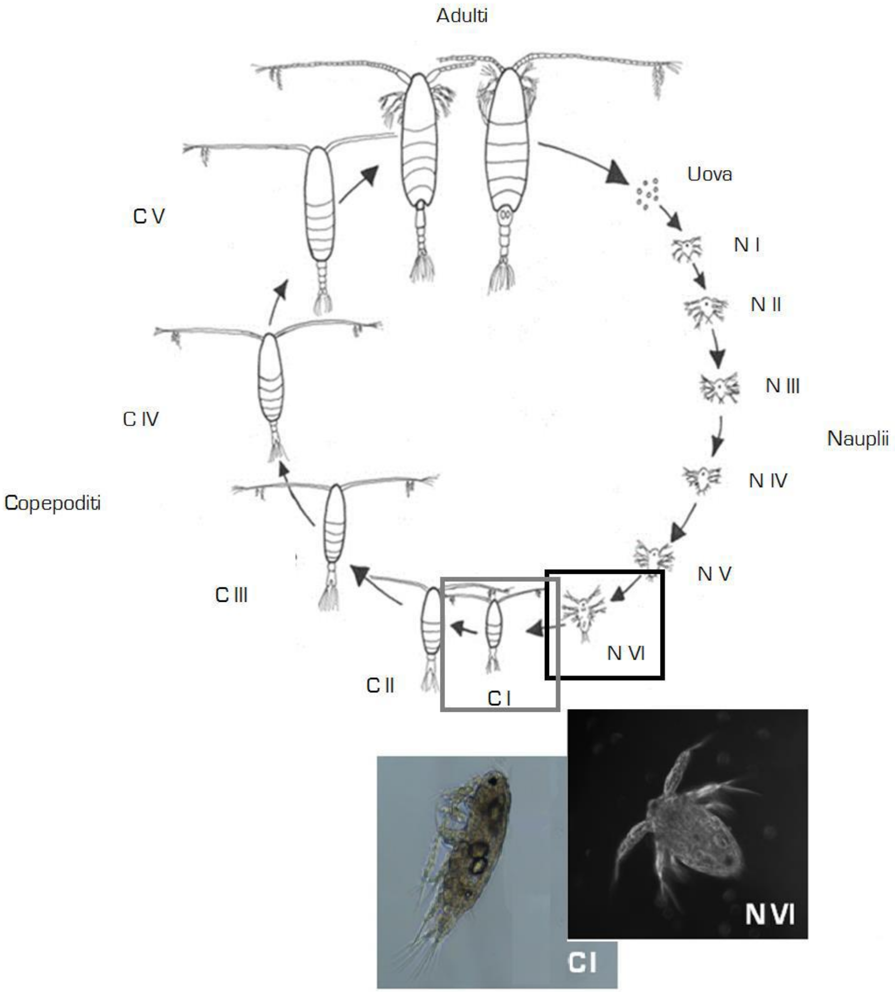
Thank you for your attention

Introduction

Acartia tonsa: life cycle overview

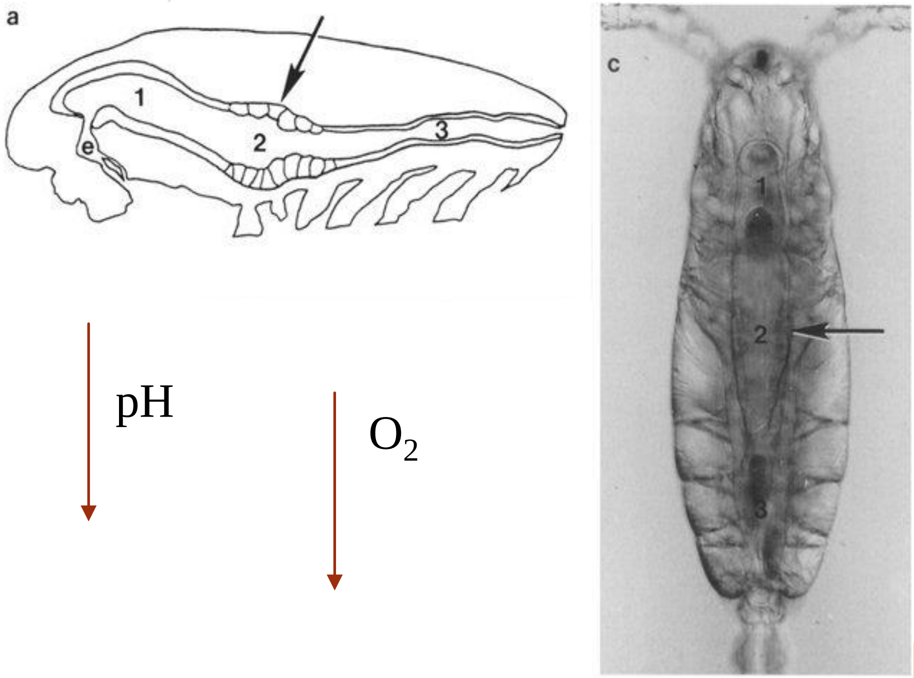
✓ Adults stage 12 ÷ 20 giorni (20°C)
✓ Continous production of eggs
✓ Easy maintenance of the eggs at 4°C

Laboratory cultures



A.tonsa: Core microbiome

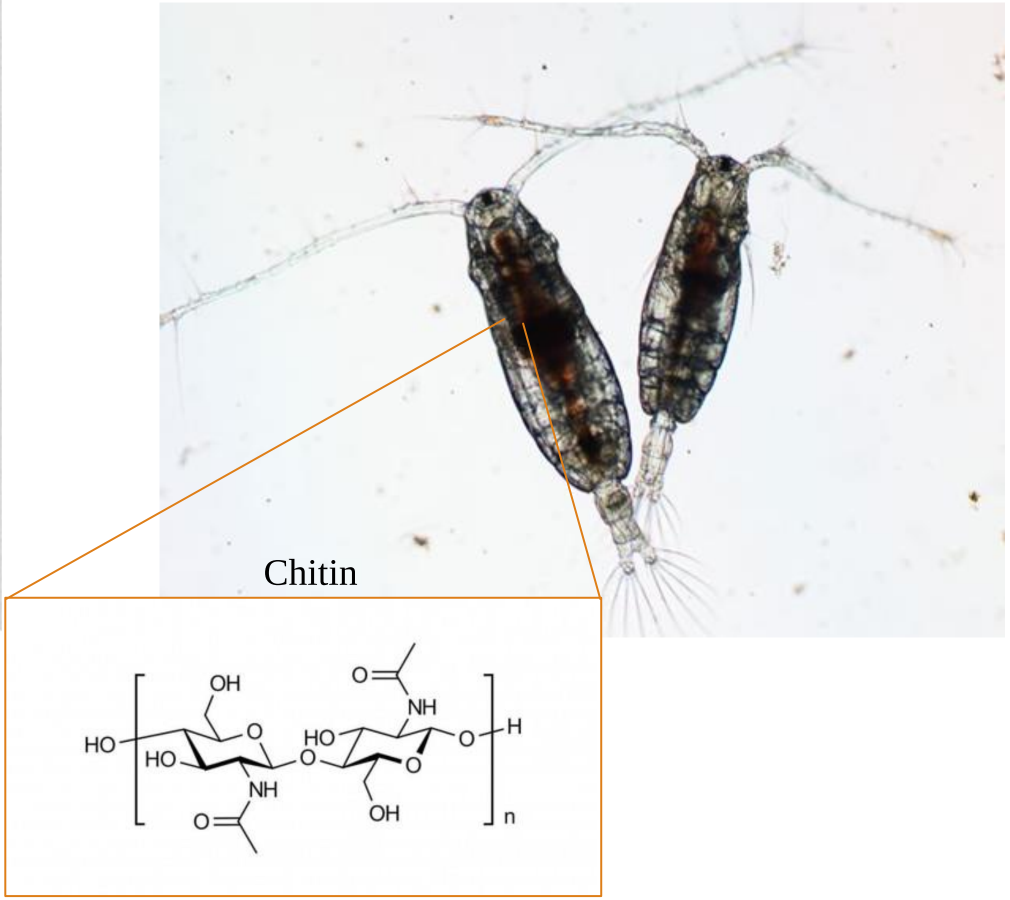
Intestine



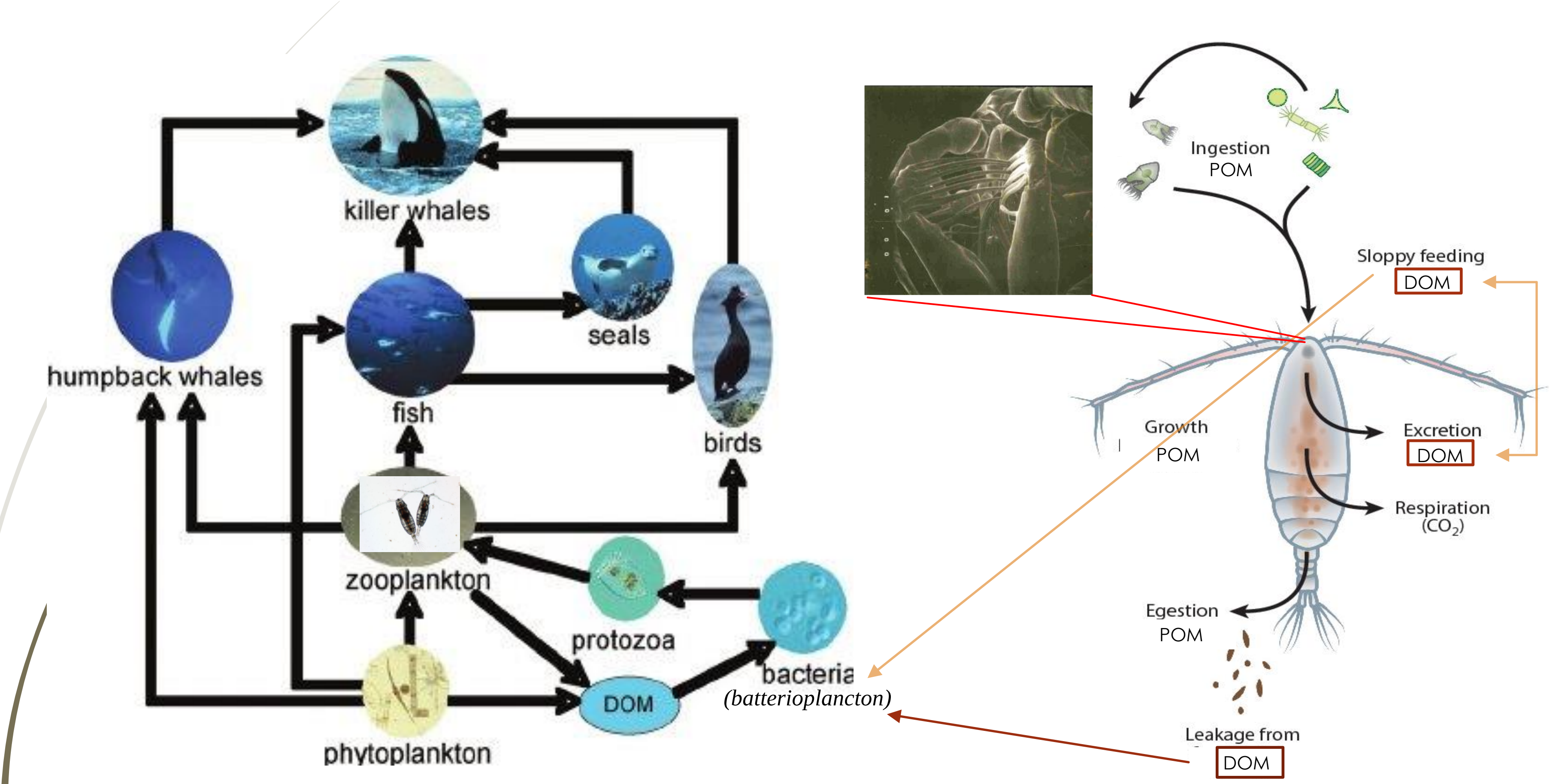
pH

O_2

Exoskeleton



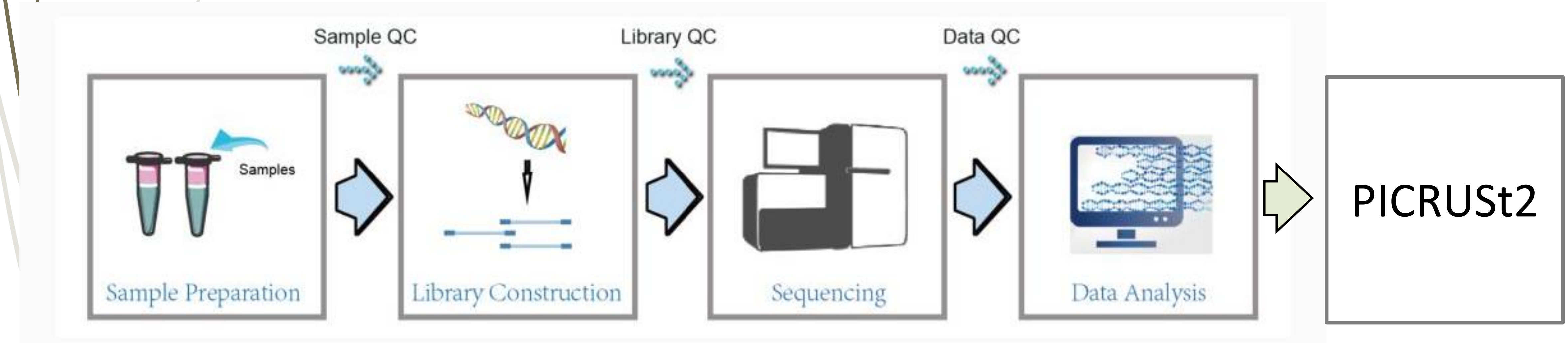
Marine feeding chain position and POM and DOM realease during feeding of A.tonsa



Metods

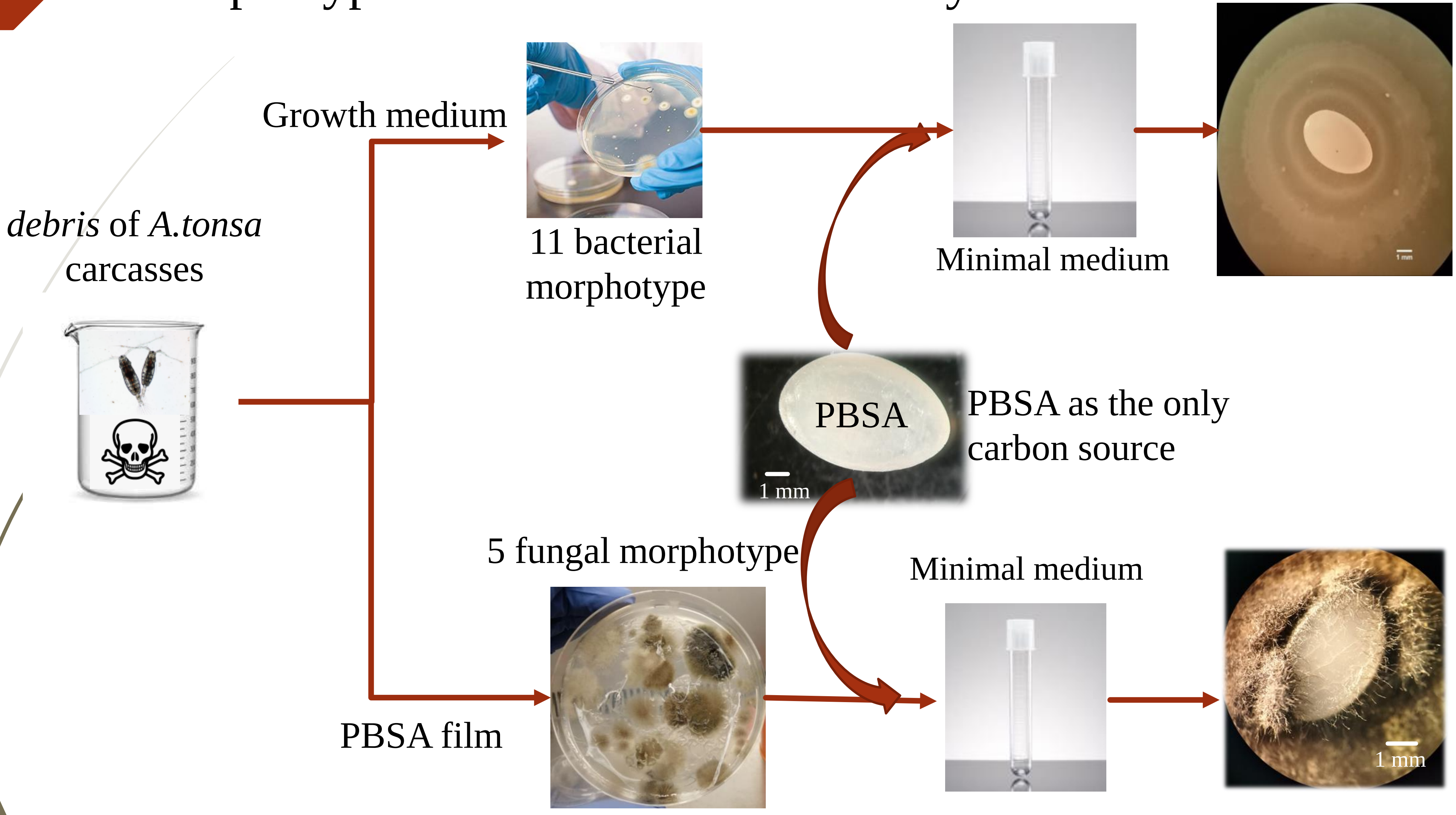
Culture-independent approach: *metabarcoding* rDNA 16S and *Predictive functional profiling*

- Metagenomic DNA extraction
- Metabarcoding of V4-V5 16S rDNA
- Reads analysis→ ASV
- *Predictive functional profiling*



PICRUSt2

Culture-dependent approach: isolation of bacterial and fungal morphotypes that use PBSA as the only carbon source



debris of *A.tonsa* carcasses

Growth medium

11 bacterial morphotype

Minimal medium

PBSA as the only carbon source

5 fungal morphotype

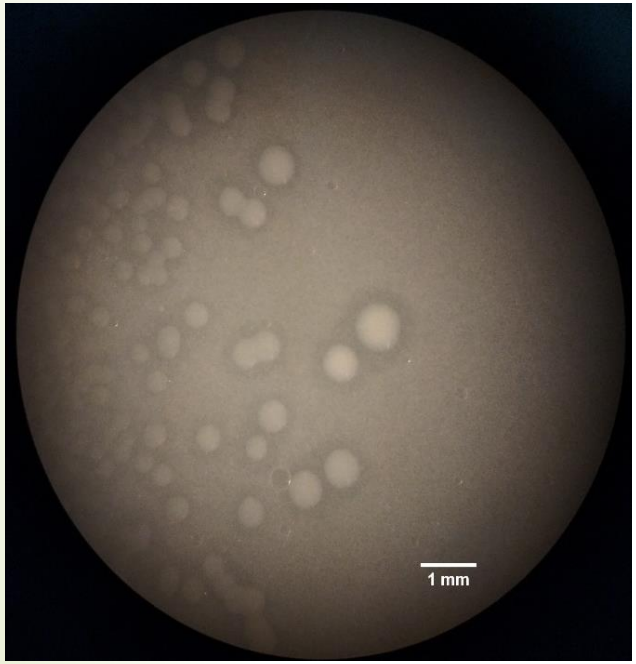
PBSA film

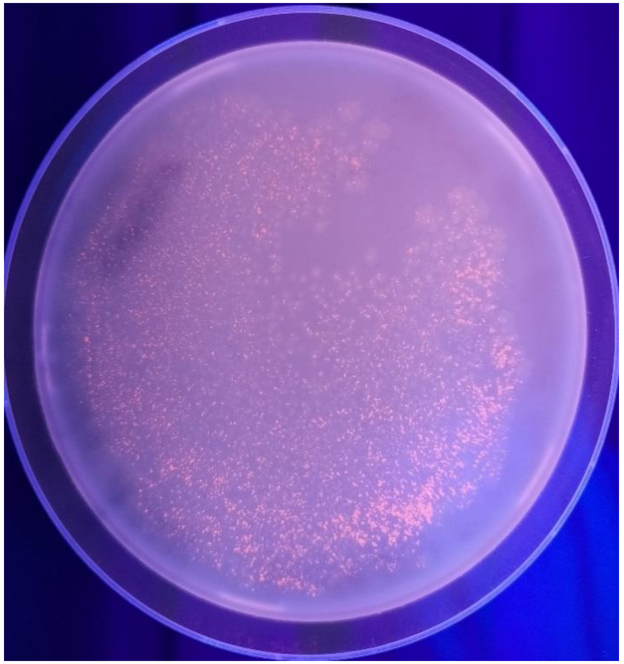
Minimal medium

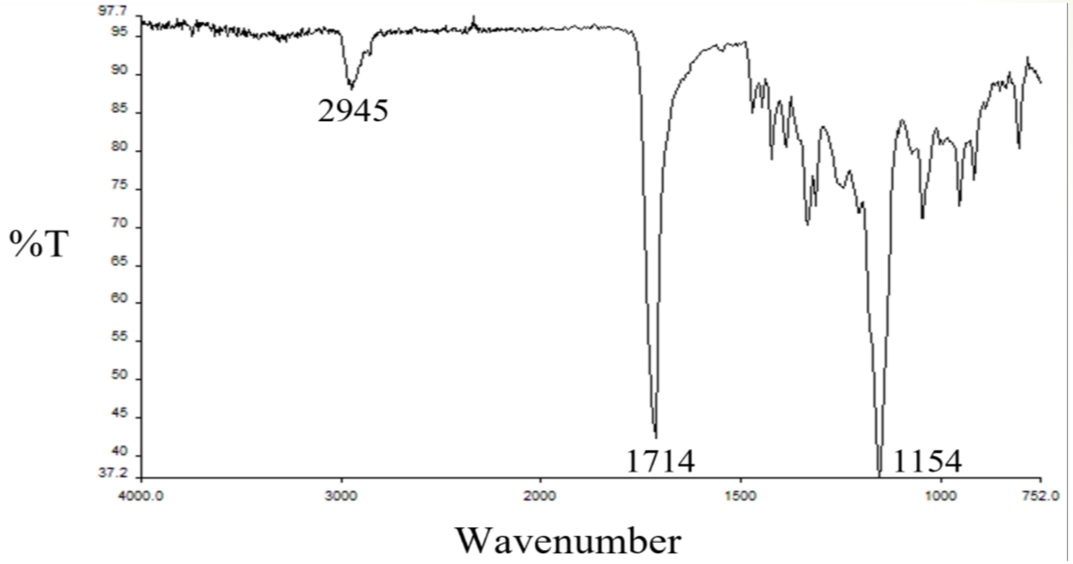
Culture-dependent approach: Screening of carboxylic-ester hydrolase and lipase activities of isolates and FITR-ATR analysis on inoculated PBSA

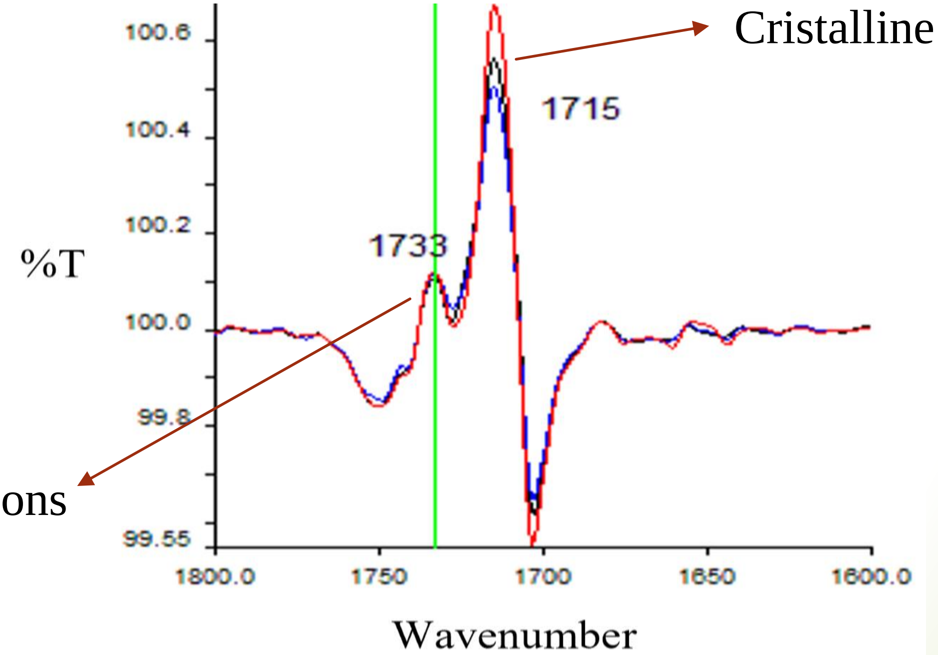
Pre-screening of carboxylic-ester hydrolase activities

Screening of lipase activities





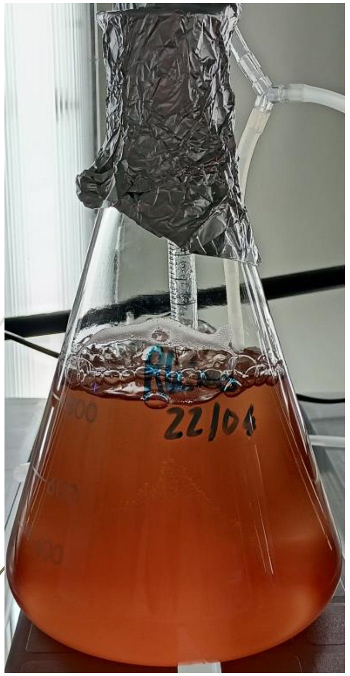




Cristalline regions

Amorphous regions

Rhinomonas reticulata cultures



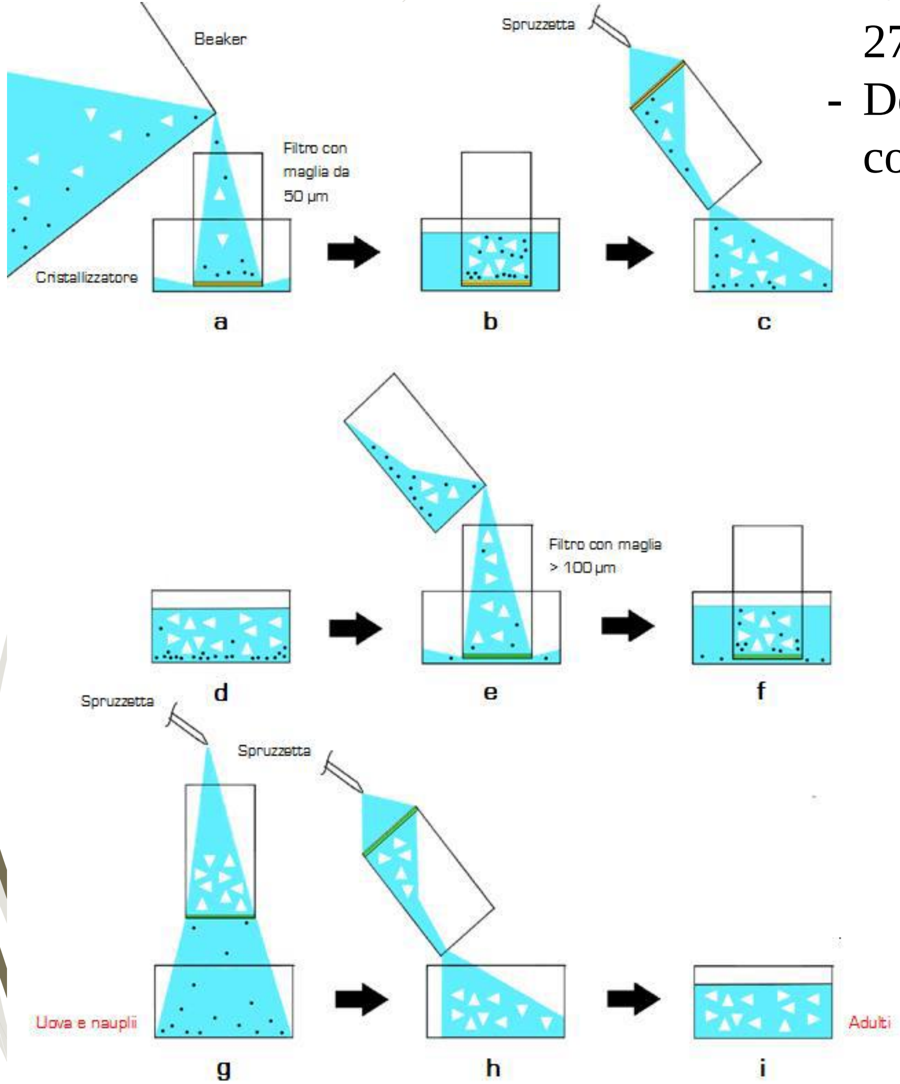
- ✓ Filtered seawater (0,45 μm)
- ✓ Sterilization cycle with Sodium Hypochlorite and Sodium Thiosulphate
- ✓ Salinity (30 psu)
- ✓ Photoperiod: 14 h;10 light;dark
- ✓ Temperature= 20°C

Culture medium f2 (Guillard et al 1975)

	Salts	Quantity (mg)	Final concentration to 1,0 l of culture medium (μM)
Macronutrients	$NaNO_3$	75	883
	$NaH_2PO_4*H_2O$	5	36,3
Micronutrients	Na_2EDTA^+	4,36	11,7
	$FeCl_3*6H_2O^+$	3,15	11,7
	$CuSO_4*5H_2O$	0,01	0,04
	$ZnSO_4*7H_2O$	0,022	0,08
	$CoCl_2*6H_2O$	0,01	0,05
	$MnCl_2*4H_2O$	0,18	0,9
	$Na_2MoO_4*2H_2O$	0,006	0,03
Vitamins	Tiamin*HCl	0,1	/
	Biotin	$5*10^{-4}$	/
	B_{12} vitamin	$5*10^{-4}$	/
Up to 1,0 l with the filtered seawater (0,22 μm			

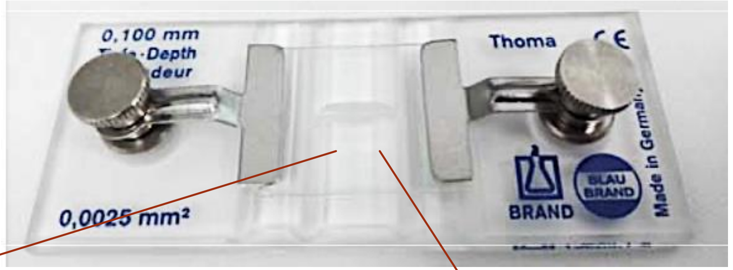
Methods: *Acartia tonsa* culture maintainance

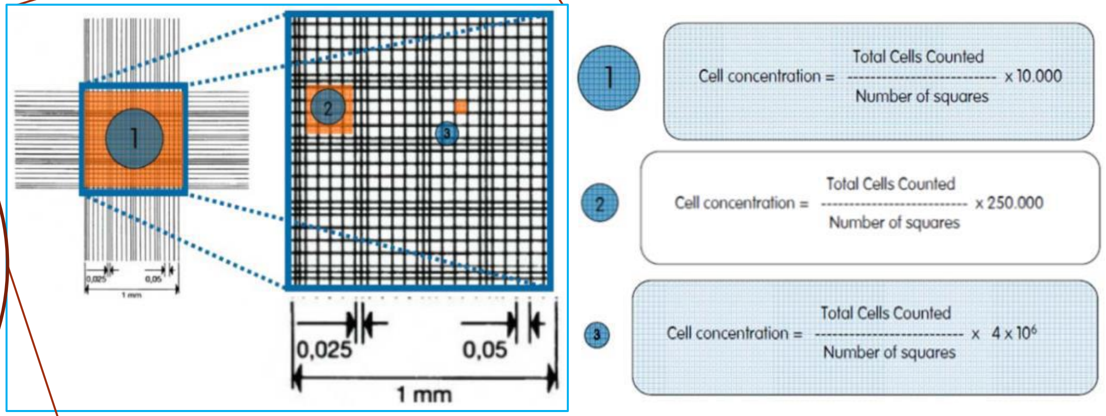
Filtration of the Egg and Naupliar stages



- Density of *Rhinomonas reticulata* cultures= 27000 cells/ml
- Density of *A.tonsa* <1000 copepod/l of culture

Monitoring the density of *Rhinomonas reticulata* cultures



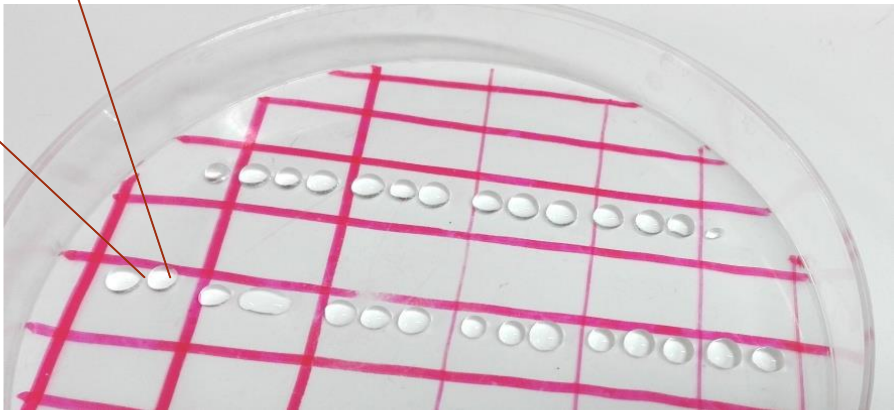


① Cell concentration = $\frac{\text{Total Cells Counted}}{\text{Number of squares}} \times 10.000$

② Cell concentration = $\frac{\text{Total Cells Counted}}{\text{Number of squares}} \times 250.000$

③ Cell concentration = $\frac{\text{Total Cells Counted}}{\text{Number of squares}} \times 4 \times 10^6$

Monitoring the developmental stages of *A.tonsa*



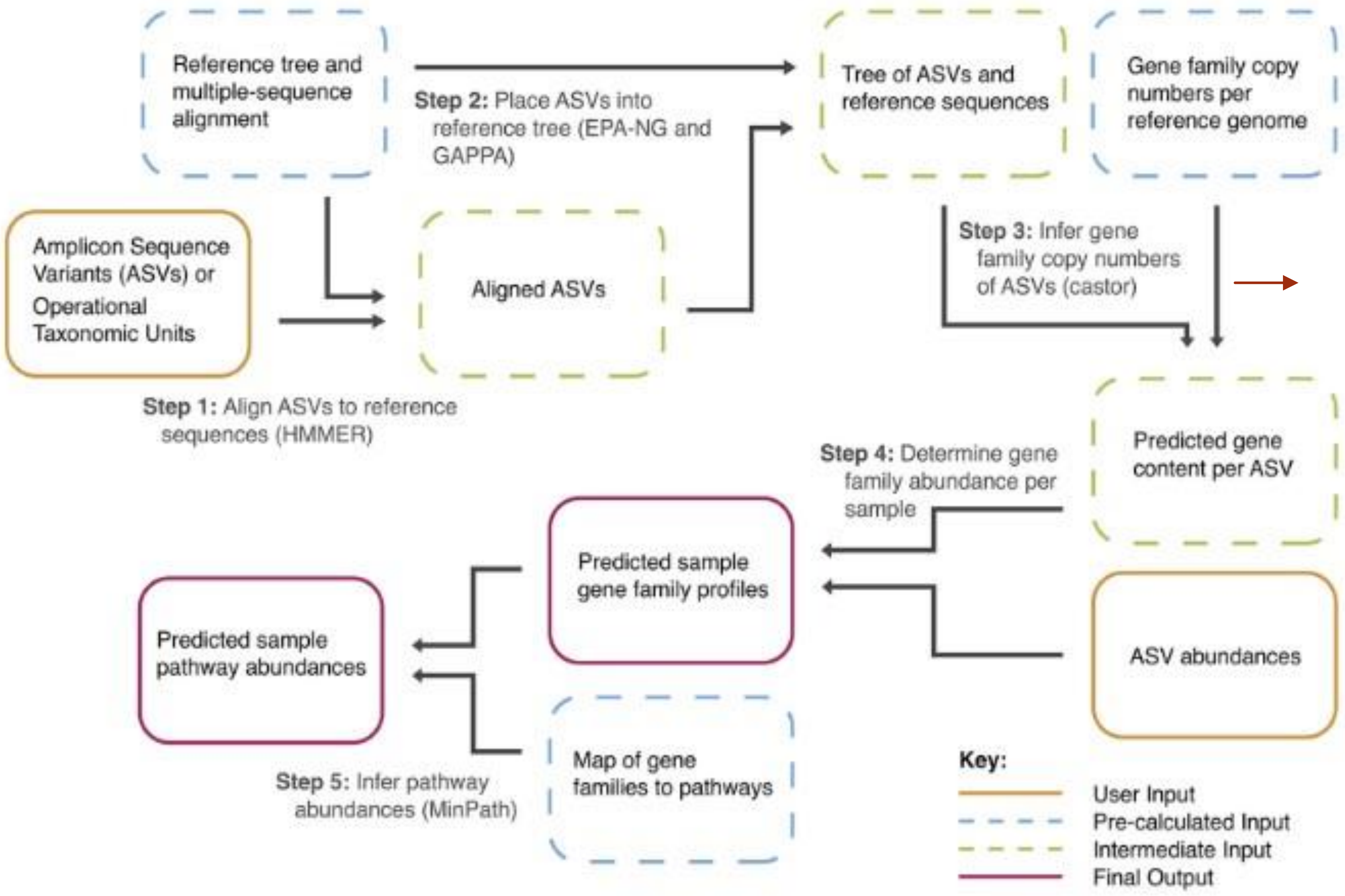
Metagenomics analysis

Raw sequences processed with Trimmomatic and FastQC to remove sample barcodes and evaluate the goodness of the sequence

Denoising with DADA2, reads assembling, chimera check for both Forward and Reverse sequences

Taxonomic assignment with RDP classifier and Greengenes codes for bacterial taxa.

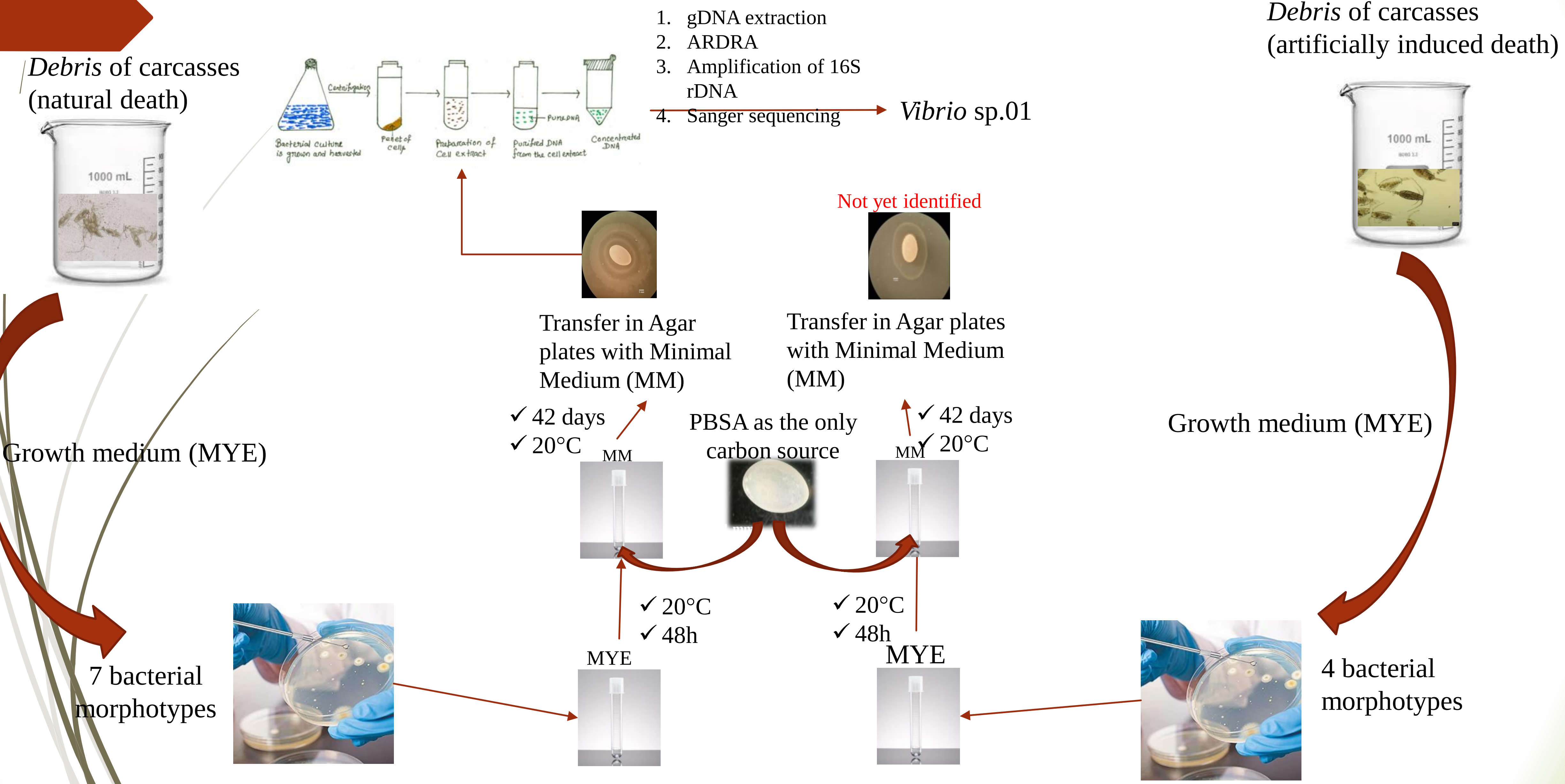
Predictive functional metagenomic profiling→PICRUSt2



→ Nearest Sequenced Taxon Index (NSTI)

«it is possible to predict the gene content of a non-annotated genome of which the 16S rDNA sequence is known starting from the gene content of an annotated genome with the shortest phylogenetic distance»

Culture-dependent approach: Isolation and identification of bacterial morphotypes from *debris* of *A.tonsa* carcasses



Debris of carcasses (natural death)

Growth medium (MYE)

7 bacterial morphotypes

MYE

✓ 20°C
✓ 48h

MM

✓ 42 days
✓ 20°C

Transfer in Agar plates with Minimal Medium (MM)

1. gDNA extraction
2. ARDRA
3. Amplification of 16S rDNA
4. Sanger sequencing

Vibrio sp.01

PBSA as the only carbon source

Debris of carcasses (artificially induced death)

Growth medium (MYE)

4 bacterial morphotypes

MYE

✓ 20°C
✓ 48h

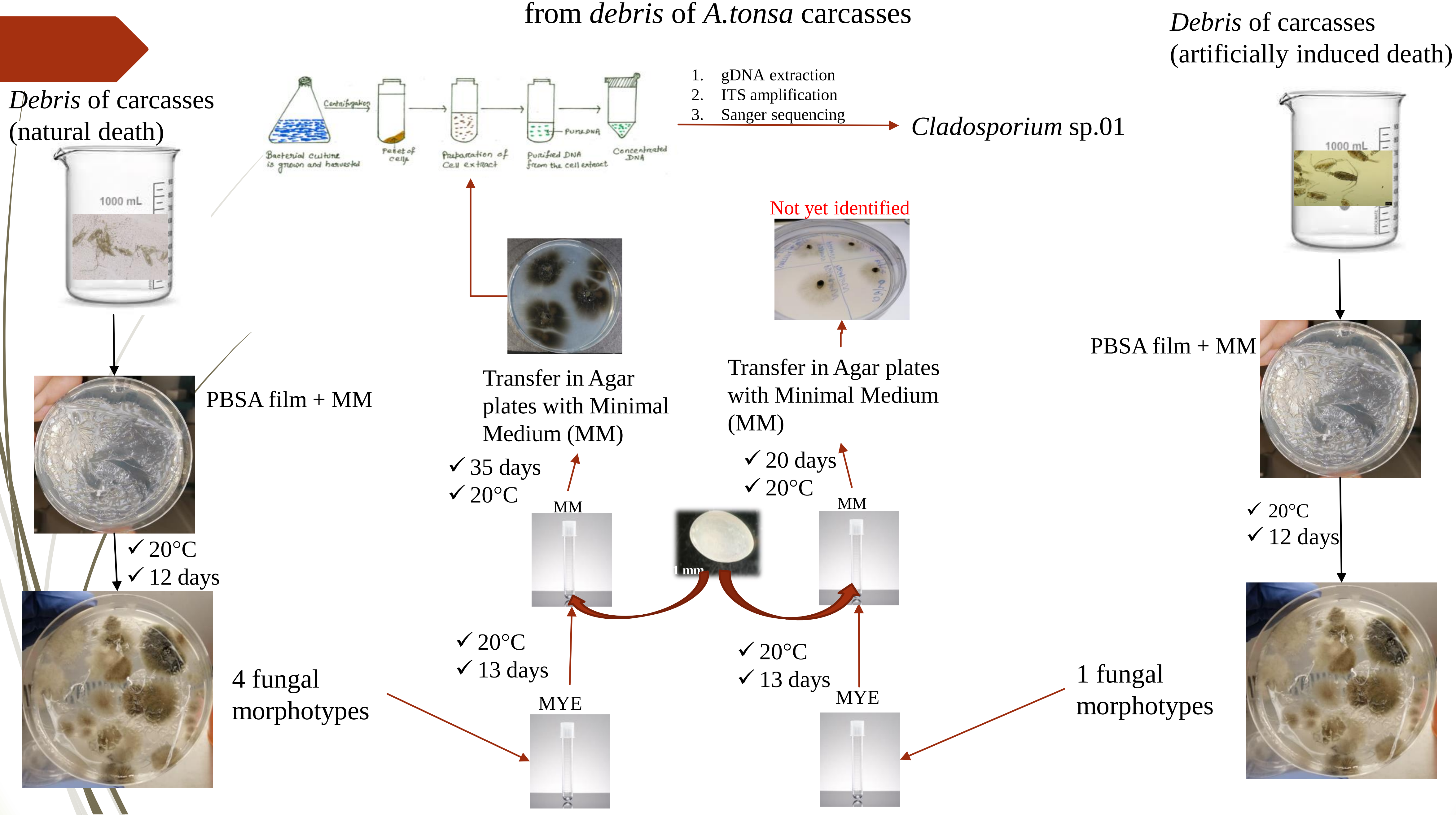
MM

✓ 42 days
✓ 20°C

Transfer in Agar plates with Minimal Medium (MM)

Not yet identified

Culture-dependent approach: Isolation and identification of fungal morphotypes from *debris* of *A.tonsa* carcasses



Debris of carcasses (natural death)

PBSA film + MM

- ✓ 20°C
- ✓ 12 days

4 fungal morphotypes

MYE

- ✓ 20°C
- ✓ 13 days

MM

- ✓ 35 days
- ✓ 20°C

Transfer in Agar plates with Minimal Medium (MM)

1. gDNA extraction
2. ITS amplification
3. Sanger sequencing

Cladosporium sp.01

Not yet identified

Transfer in Agar plates with Minimal Medium (MM)

- ✓ 20 days
- ✓ 20°C

MM

- ✓ 20°C
- ✓ 13 days

MYE

Debris of carcasses (artificially induced death)

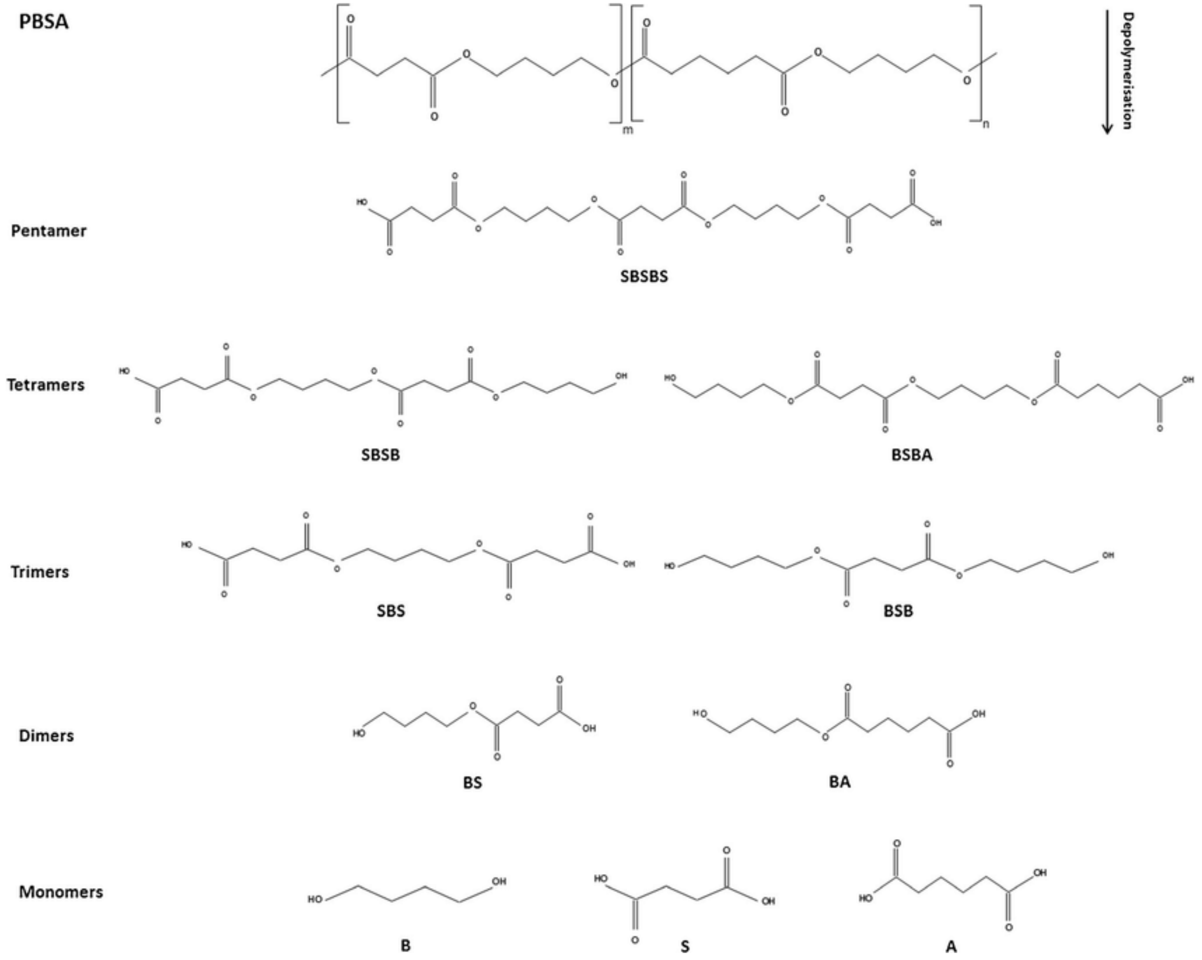
PBSA film + MM

- ✓ 20°C
- ✓ 12 days

1 fungal morphotypes

PBSA degradative mechanism

- α-β fold domain→ catalytic triad → Ser-Glu-His
- Lid domain→ hydrophobic amminoacid residues→ substrate adesion→ conformational change→ exposure of the catalytic triad to the substrate



Modified from (*Shah et. al 2014*).

Results

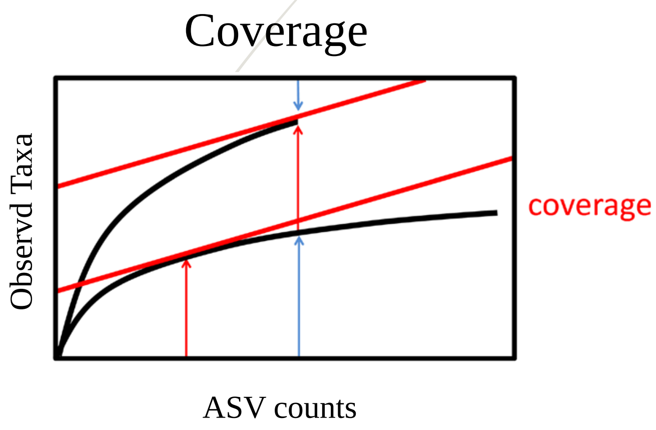
α-Diversity: Coverage e Hill index

Hill index

General equation → $^{q}D = \left(\sum_{i=1}^{S} p_i^{q}\right)^{\frac{1}{(1-q)}}$

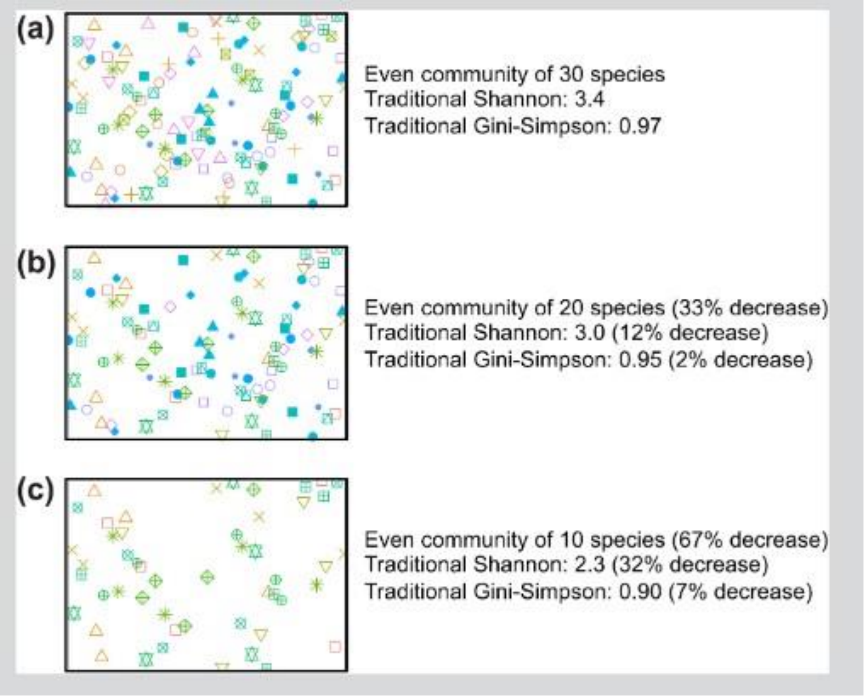
Hill-Shannon → $\lim_{q \to 1} {}^{q}D$ → $^{1}D = exp\boxed{\left(-\sum_{i=1}^{S} p_i \log p_i\right)}$ Shannon-Weiner

Hill-Shannon → q=2 → $^{2}D = 1/\boxed{\sum_{i=1}^{S} p_i^{2}}$ Simpson



modificato da (Roswell et al., 2021).

«the samples are normalized for the same degree of completeness and not for size»



Even community of 30 species
Traditional Shannon: 3.4
Traditional Gini-Simpson: 0.97

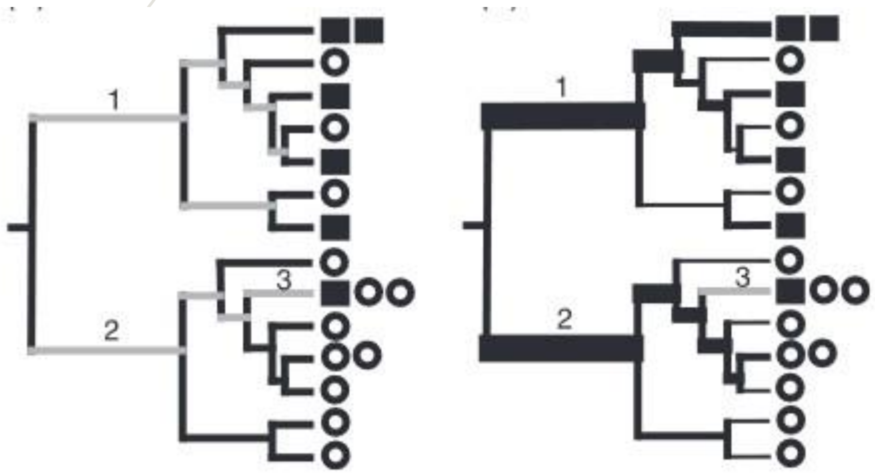
Even community of 20 species (33% decrease)
Traditional Shannon: 3.0 (12% decrease)
Traditional Gini-Simpson: 0.95 (2% decrease)

Even community of 10 species (67% decrease)
Traditional Shannon: 2.3 (32% decrease)
Traditional Gini-Simpson: 0.90 (7% decrease)

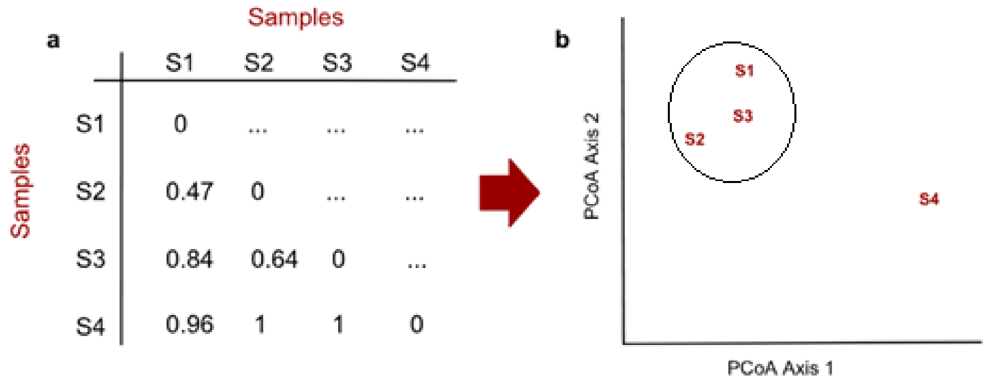
modified from (Roswell et al., 2021).

β-Diversity: Weighted UniFrac

UniFrac Phylogenetic distance



PCoA: Principal Coordinate Analysis



Samples	S1	S2	S3	S4
S1	0	...	...	...
S2	0.47	0	...	...
S3	0.84	0.64	0	...
S4	0.96	1	1	0

Unweighted UniFrac

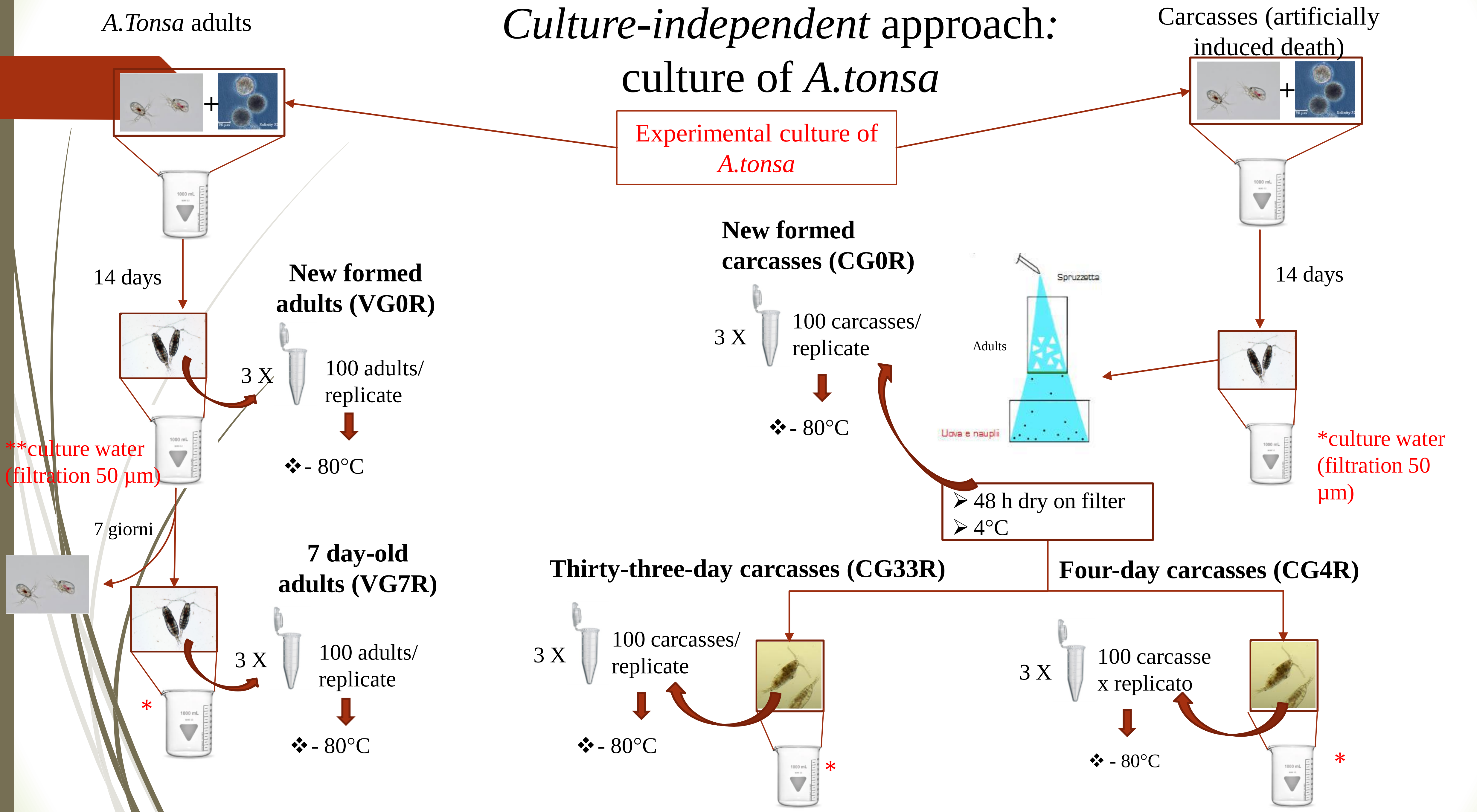
$$u = \sum_{i}^{n} b_i \left| \frac{A_i}{A_{tot}} - \frac{B_i}{B_{tot}} \right|$$

$$D = \sum_{j}^{n} d_j \left| \frac{A_j}{A_{tot}} + \frac{B_j}{B_{tot}} \right|$$

$$WeightedUniFrac_{AB} = \frac{u_{AB}}{D_{AB}}$$

b_i = lunghezza ramo i
A_i e B_i = numero di sequenze nel ramo i per le comunità A e B
A_{tot} e B_{tot} = numero totale di sequenze per le comunità A e B
d_j = distanza della sequenza j dalle radici dell'albero filogenetico
A_j e B_j = conteggi della sequenza j nelle comunità A e B

Culture-independent approach: culture of *A.tonsa*



Experimental culture of *A.tonsa*

A.Tonsa adults

14 days

New formed adults (VG0R)

3 X

100 adults/ replicate

❖ - 80°C

**culture water (filtration 50 µm)

7 giorni

7 day-old adults (VG7R)

3 X

100 adults/ replicate

❖ - 80°C

*

Carcasses (artificially induced death)

14 days

*culture water (filtration 50 µm)

New formed carcasses (CG0R)

3 X

100 carcasses/ replicate

❖ - 80°C

- ➢ 48 h dry on filter
- ➢ 4°C

Thirty-three-day carcasses (CG33R)

3 X

100 carcasses/ replicate

❖ - 80°C

*

Four-day carcasses (CG4R)

3 X

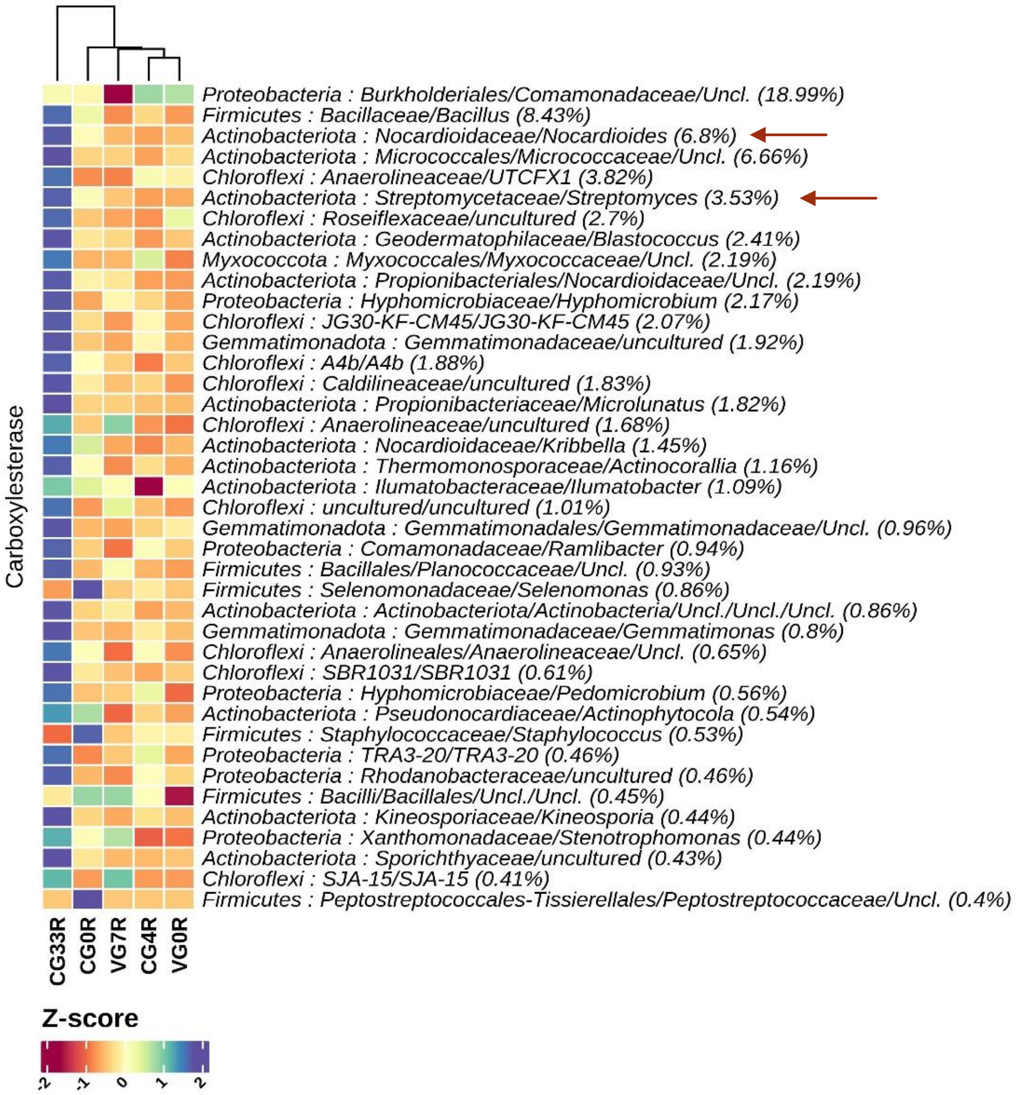
100 carcasse x replicato

❖ - 80°C

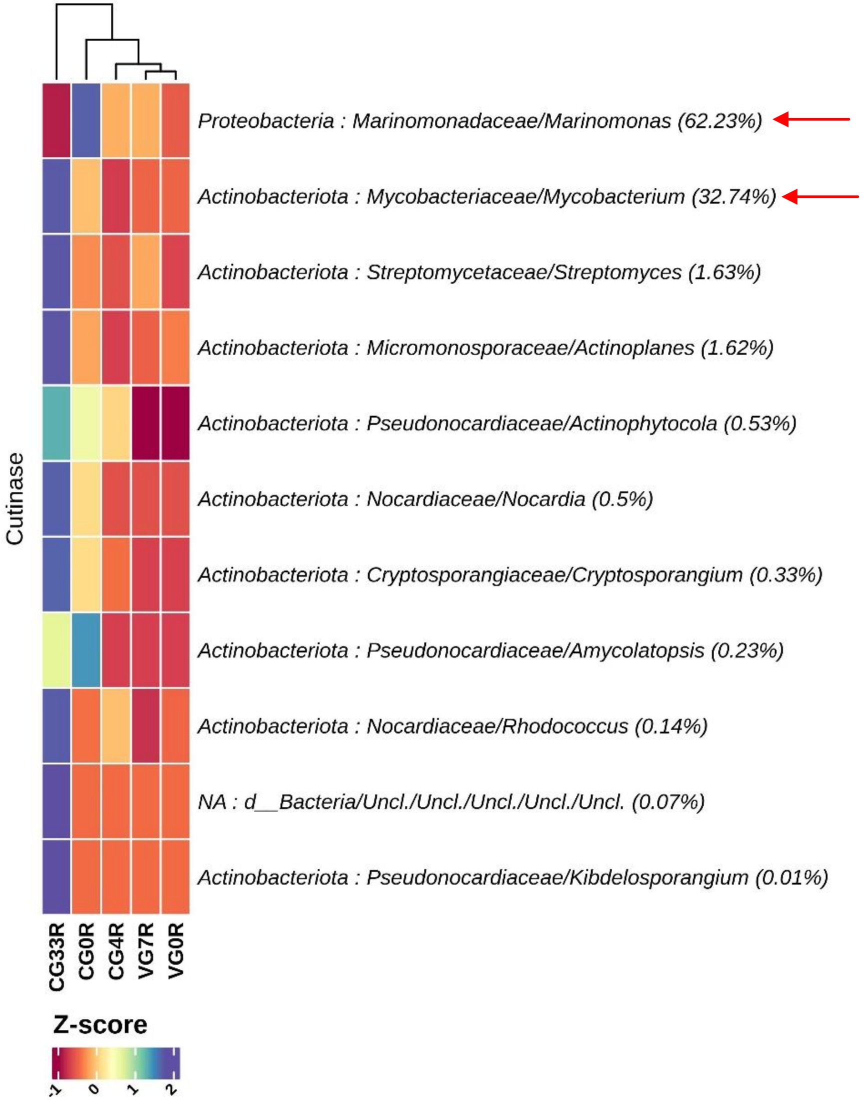
*

Results of *culture-independent* approach:*Predictive functional profile* of bacterial communities

Carbxylesterase (EC 3.1.1.1)

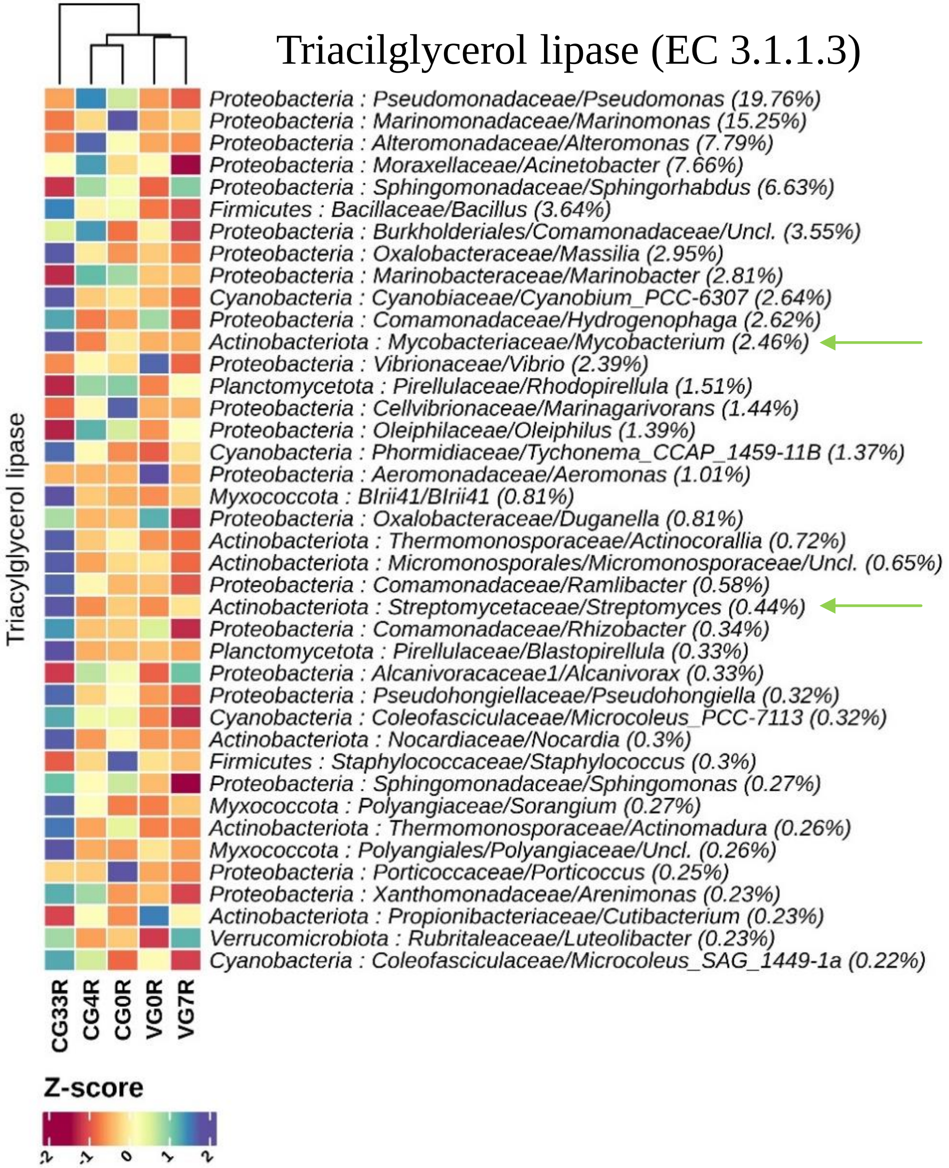


Cutinase (EC 3.1.1.74)

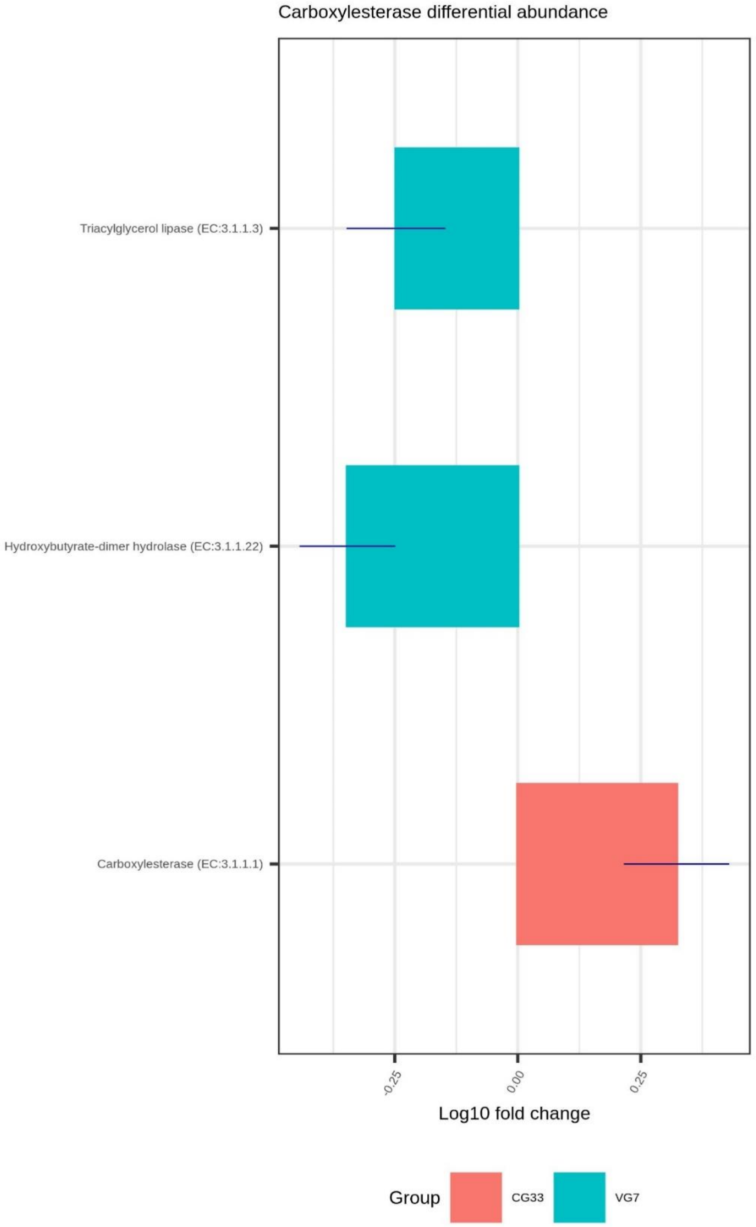


Results of *culture-independent* approach:*Predictive functional profile* of bacterial communities

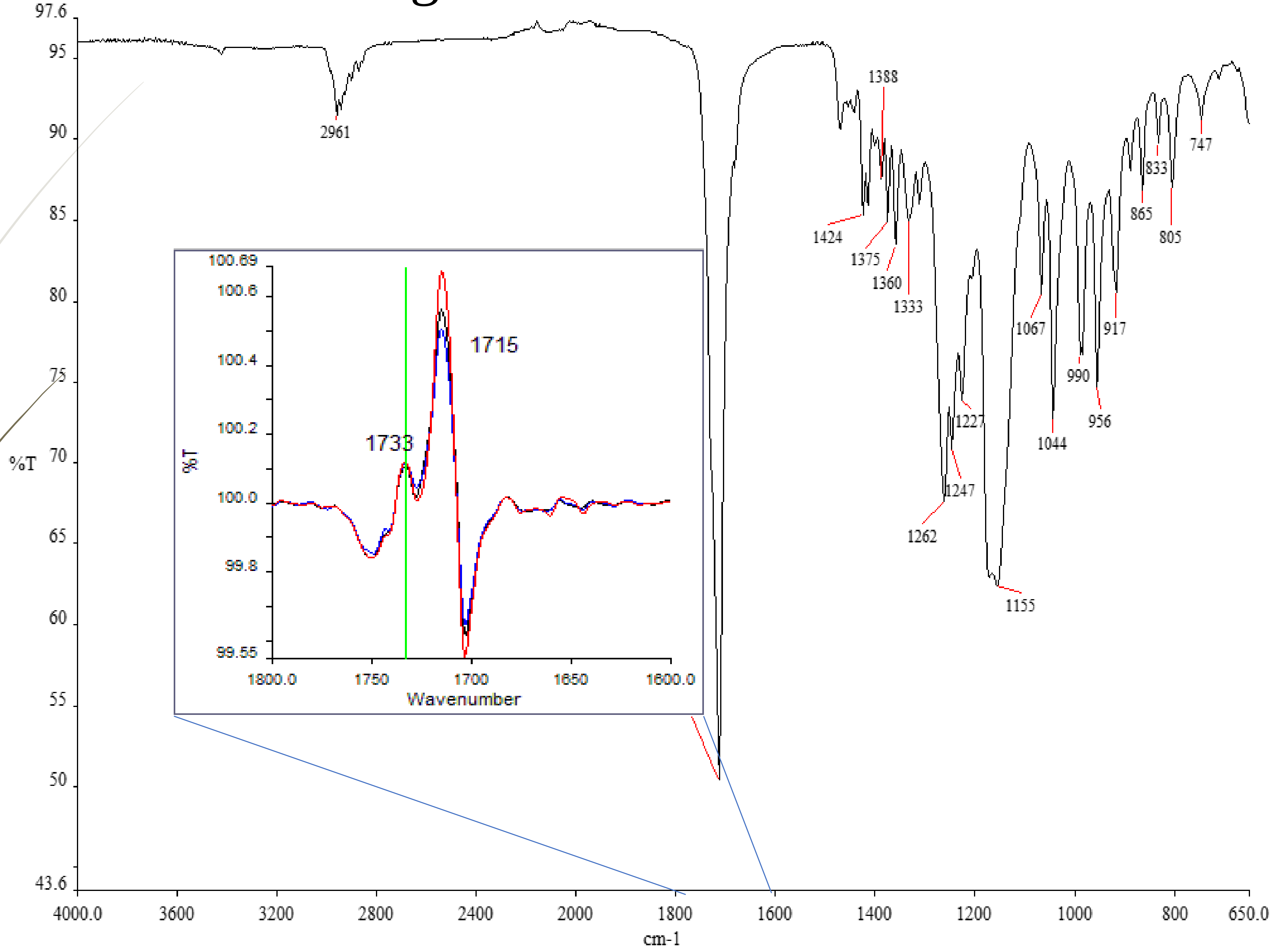
Triacilglycerol lipase (EC 3.1.1.3)



Results of *culture-independent* approach: unstratified ANCOM-BC with carboxyl-ester hydrolase activities of bacterial communities

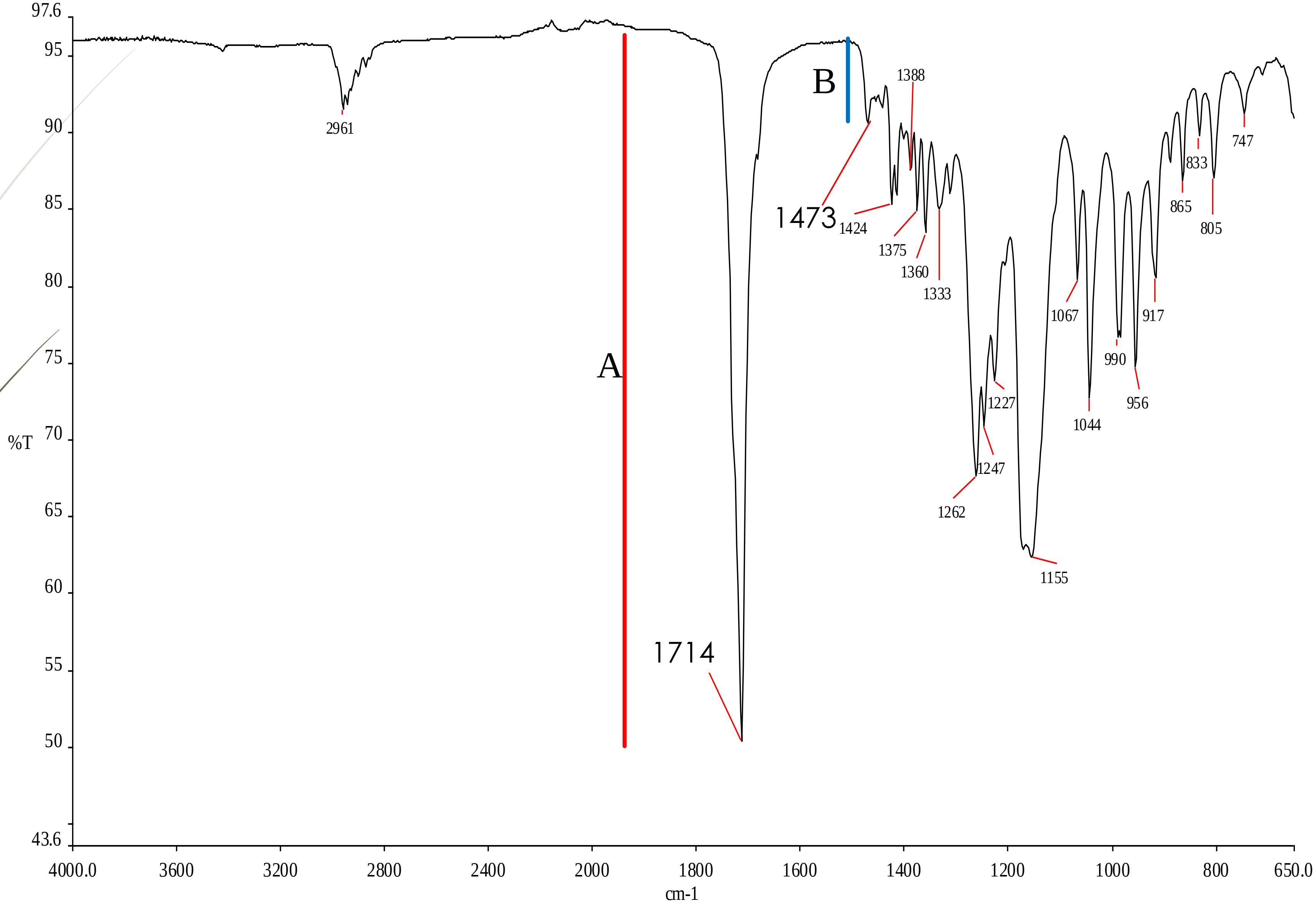


IR spectrum of the superficial regions of PBSA granule and second derivative

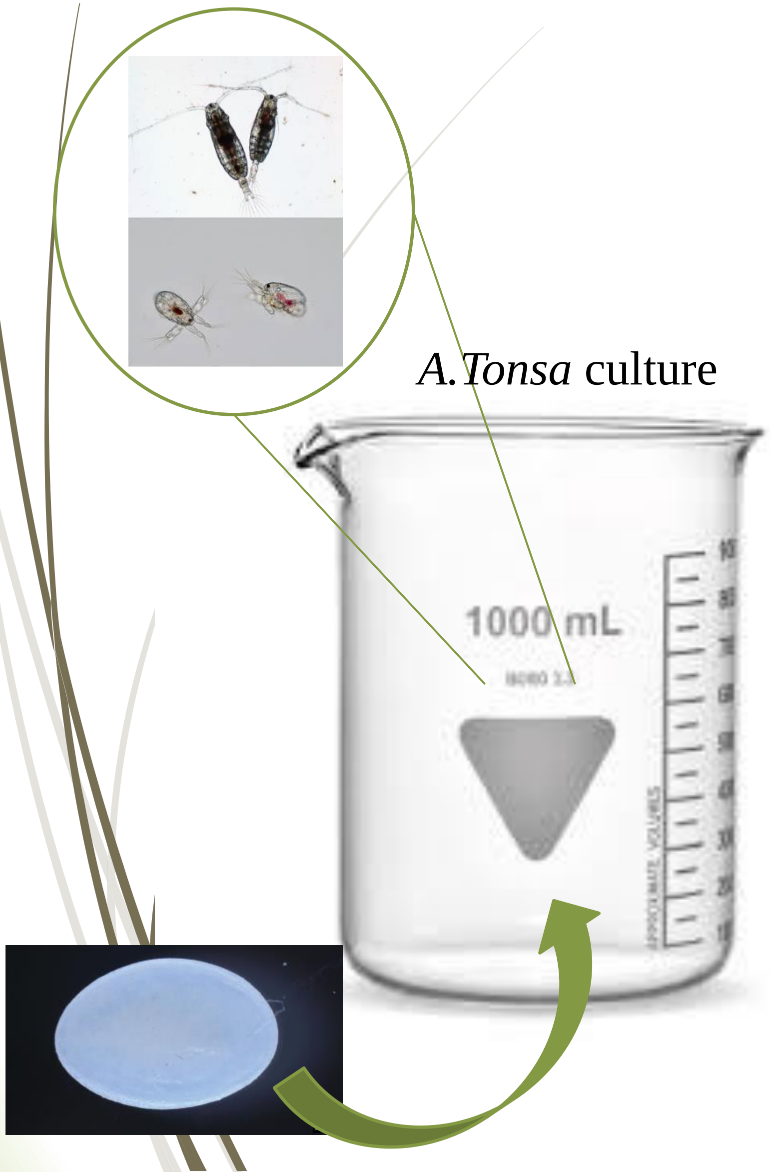


Carbonyl index= A/B

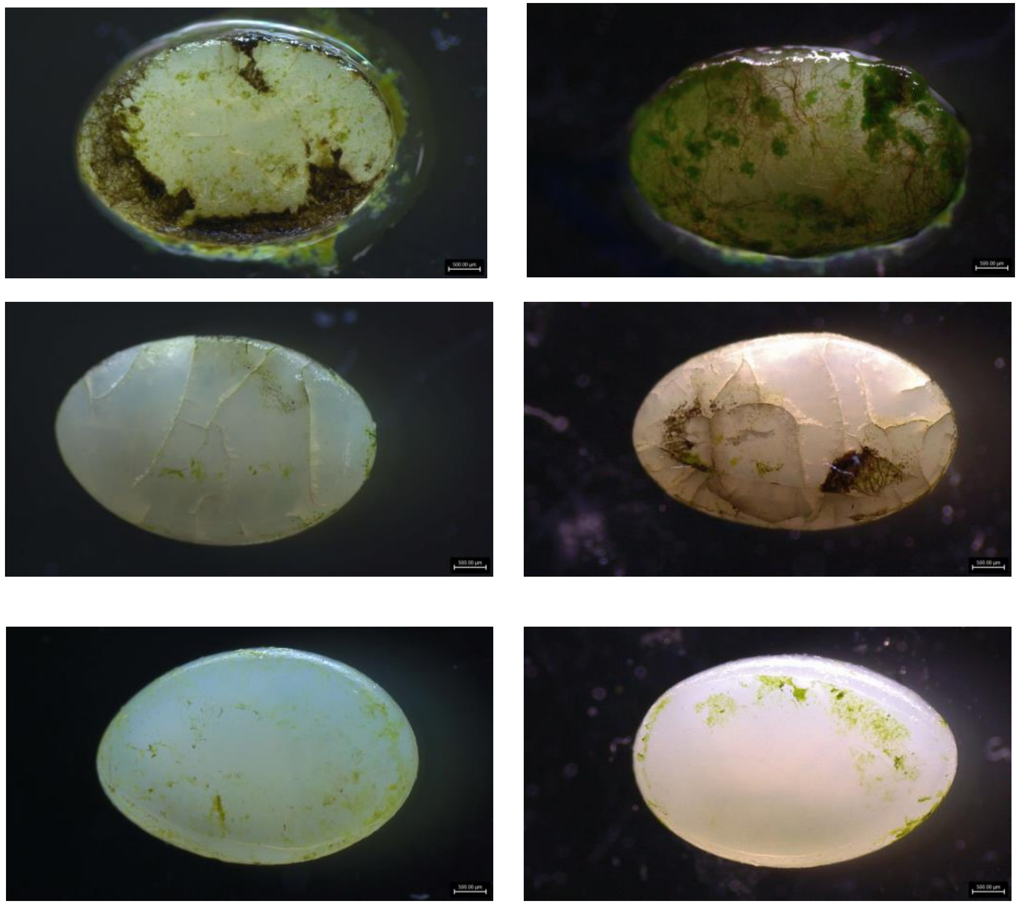
(carbonyl index= Peak intensity at 1715 cm^{-1}/ Peak intensity at 1473 cm^{-1})



In progress: PBSA degradation→ *in vivo* experiments



A.Tonsa culture



30 days

60 days

Inoculated with Cladosporium sp.01 (with fungal biomass)

Inoculated with Cladosporium sp.01 (without fungal biomass)

Uninoculated PBSA