



厭氧微生物特性研究

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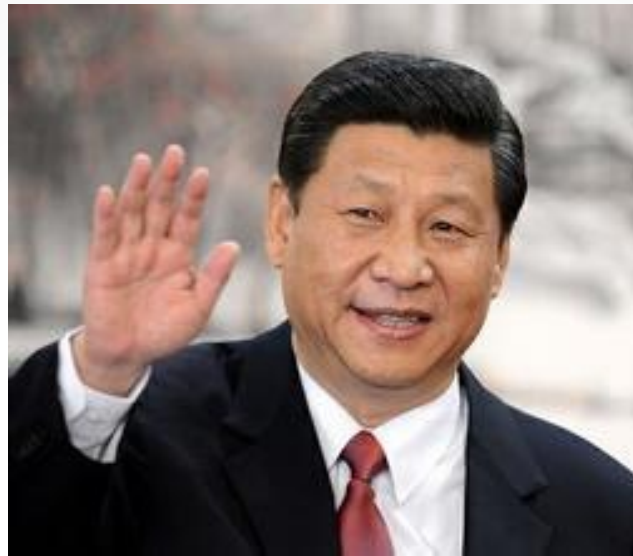
南京大学环境学院客座教授







1923, HKU Main Bld



The first biogas reactor at Shanxi Province of China back to 1970s
<http://www.ibtimes.co.uk/xi-jinping-profile-story-who-400967>

Video

For seven years, Xi lived in a cave-house dug into the hillside in Shaanxi province, one of China's poorest regions.

But following the perfect communist hero narrative, the forced internal exile among the people forged the character of China's future leader.

Xi reportedly spent his nights in his cave reading books on Marxism, chemistry and mathematics. He learnt so much that he was able to build the region's first tank for biogas from pigs' manure.

"When I arrived at the Yellow Earth [Shaanxi] aged 15, I was anxious and confused. When I left the Yellow Earth at 22, my life goals were firm and I was filled with confidence," Xi wrote in 1998.

Degradation Pathway of Phenol under Methanogenic Condition

Phenol



Clostridium

Benzoate



Syntrophus

Acetate, H₂, CO₂



Methanogens

CH₄

A) *Phenol to benzoate*

Degradation of phenol via carboxylation to benzoate by *Clostridium* (renamed to *Sedimentibacter hydroxybenzoicus*).

B) *Benzoate to acetate and H₂/CO₂*

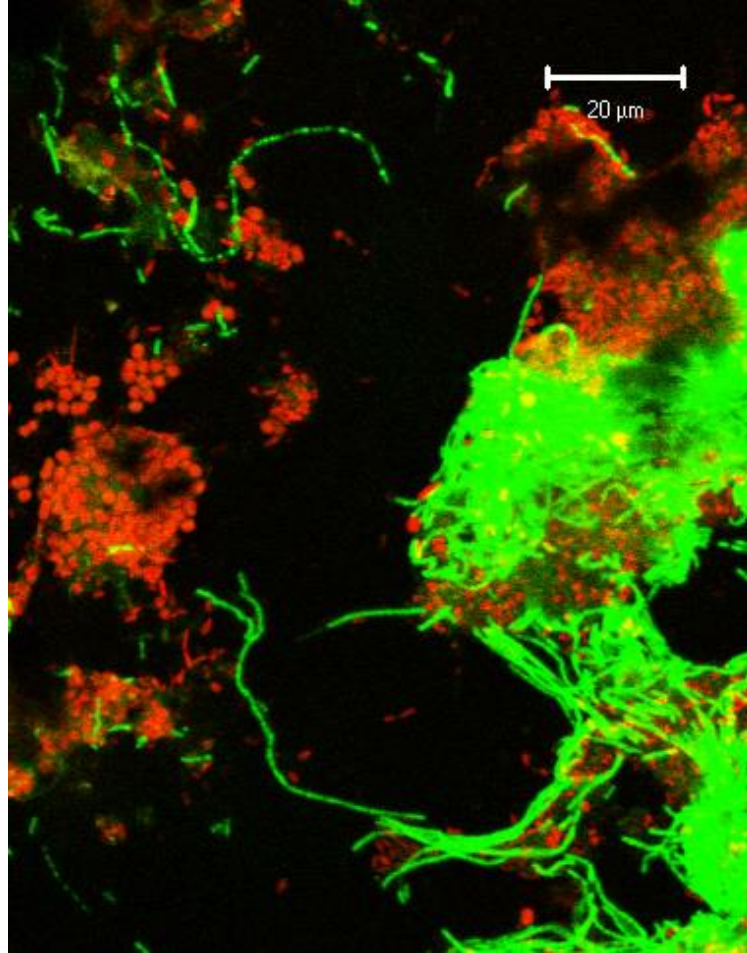
Syntrophus species then further degraded benzoate to acetate and H₂/CO₂ for final methanogenesis.

C) *Acetate and H₂/CO₂ to methane*

The methanogens.

Phenol-degrading Anaerobic Granule Sludge

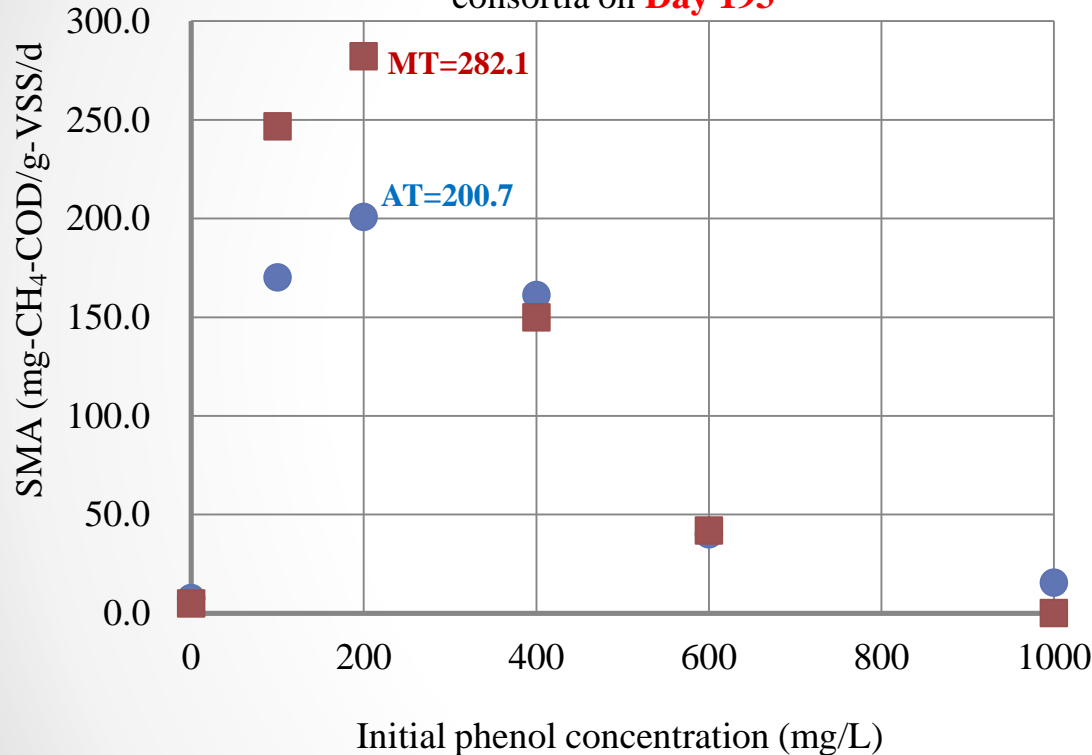
Red: *Bacteria* cells; **Green:** *Archaea* cells (methanogen)



Zhang, T et al. 2005. Microbial characteristics of a methanogenic phenol-degrading sludge. *Wat Sci Technol.* 52, 73-78

Specific methanogenic activities of phenol-degrading consortia

Specific methanogenic activity (SMA) of MT and AT consortia on **Day 193**



A summary of SMA of different phenol-degrading methanogenic consortia

T (°C)	Max. phenol con. (mg.L ⁻¹)	Max. SMA	Reference
26	1260	190	Zhang et al., 2005
AT (20)	875	201	Ju and Zhang, 2014
37	1260	240	Fang et al., 1996
37	500	480	Chen et al., 2008
37	634	371	Chen et al., 2009
MT (37)	1000	282	Ju and Zhang, 2014

*SMA: mg. CH₄-COD.g.VSS⁻¹.d⁻¹

- Mesophilic (37°C, MT) and ambient (20°C, AT) phenol-degrading methanogenic consortia enriched in anaerobic semi-continuous batch reactors

An observation about the history of computing hardware: the price of dense integrated circuit halve every 24 months.

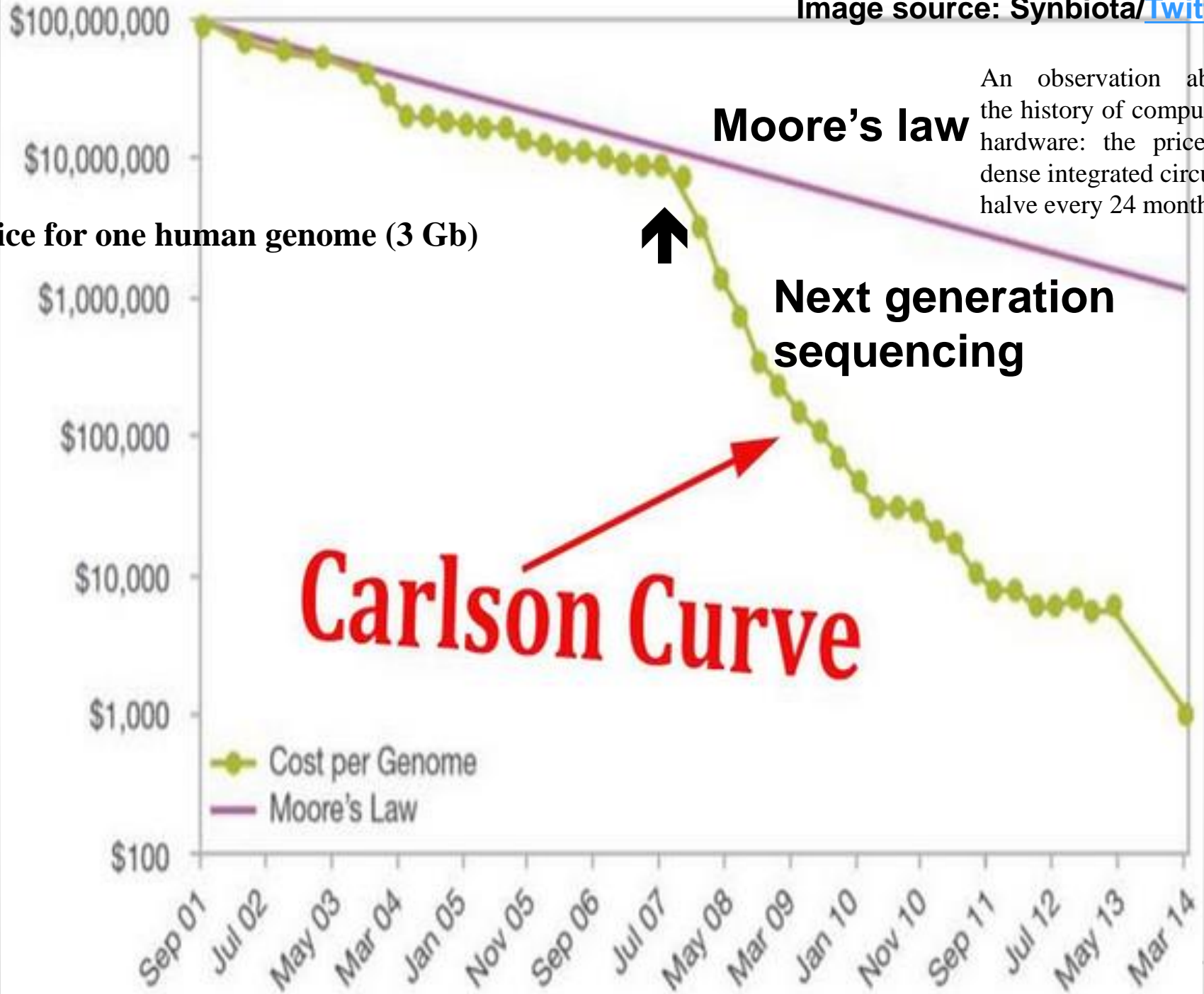
Price for one human genome (3 Gb)

Moore's law

Next generation sequencing

Carlson Curve

● Cost per Genome
— Moore's Law





(I) Illumina HiSeq



(II) Roche GS Junior system



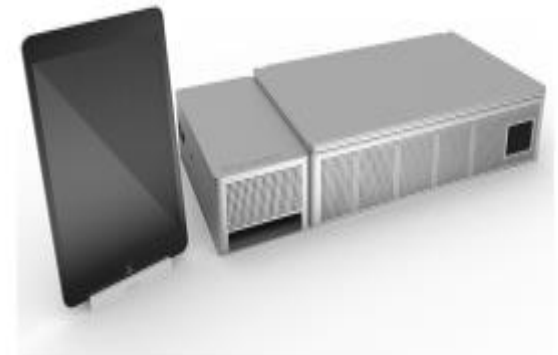
(III) Ion Torrent PGM system



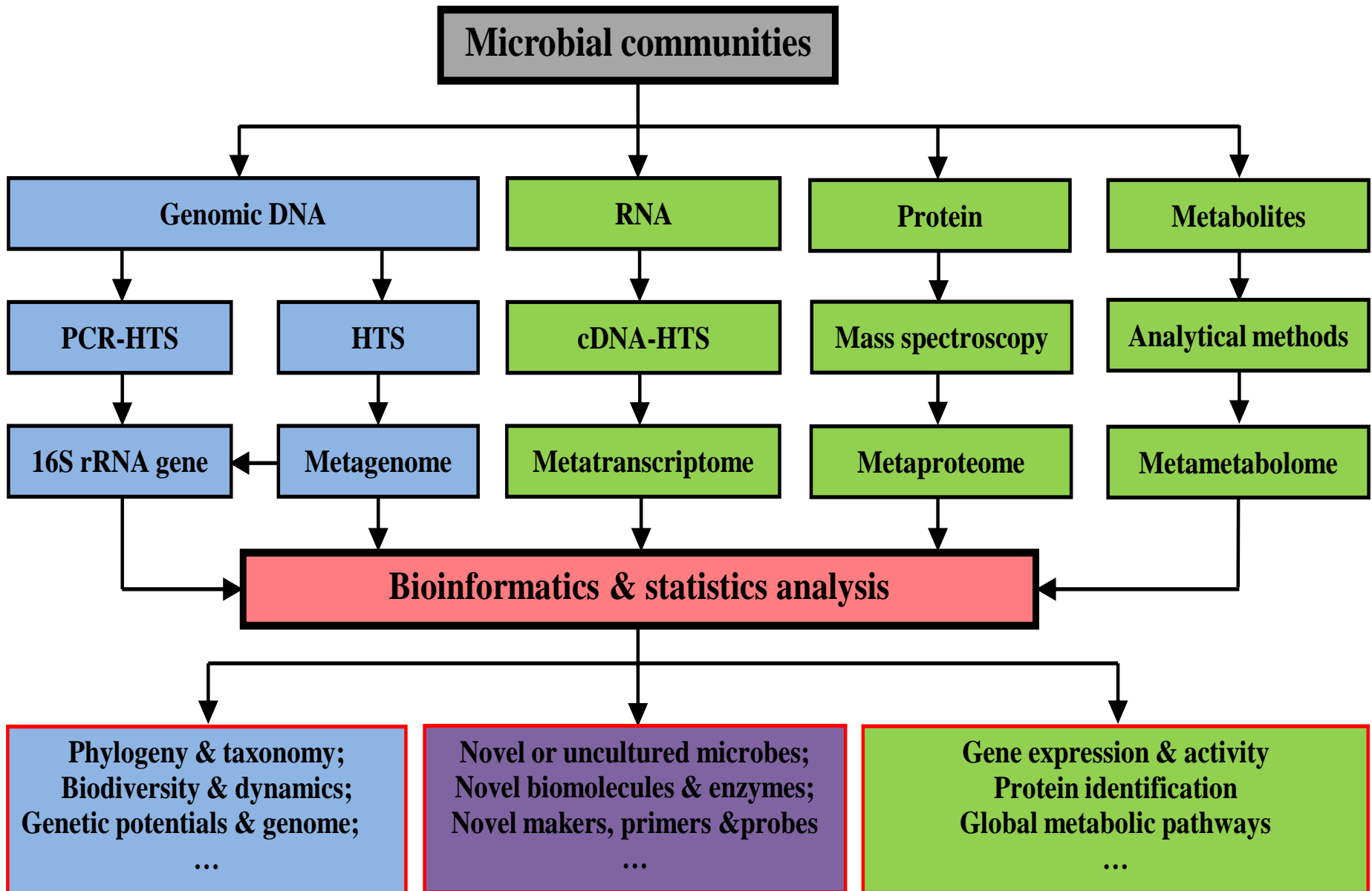
(IV) PacBio RS



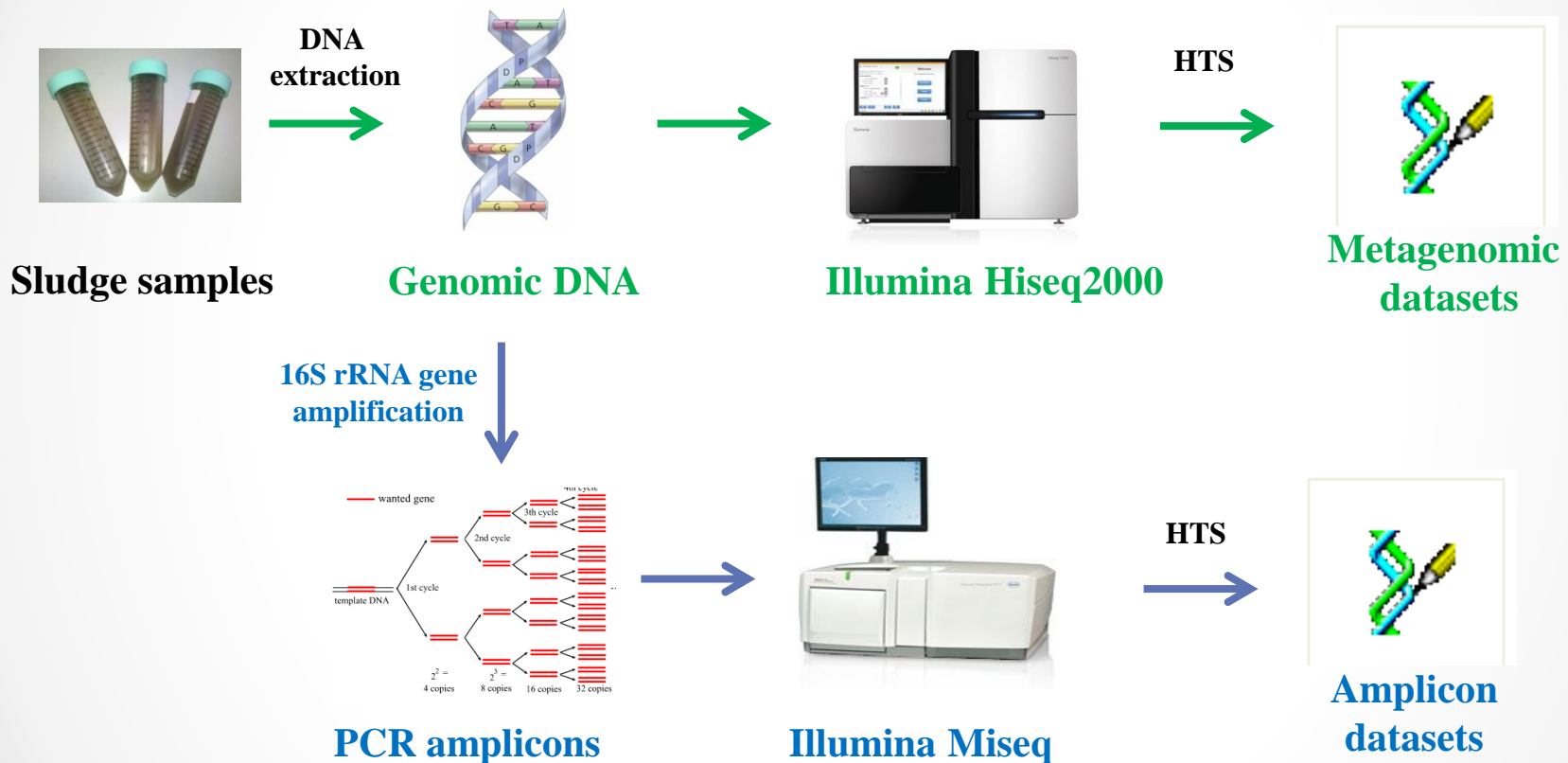
(V) Oxford Nanopore MinION™



(VI) Oxford Nanopore PromethION



Next-generation sequencing of metagenomic DNAs and 16S rRNA gene



DNA extraction: FastDNA[®] Spin kit for Soil (MP Biomedicals);

Amplification of V3-V4 regions of 16S rRNA gene :

- forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3');
- reverse primers: R1 (5'-TACCRGGGTHCTAATCC-3'), R2 (5'-TACCAGAGTATCTAATTC-3'), R3 (5'-CTACDSRGGTMTCTAATC-3') and R4 (5'-TACNVGGGTATCTAATCC-3')

16S Analysis Pipeline

(I) Data Pretreatment

Sequences (.fna)
Quality Scores (.qual)
Flowgram Files (.sff.txt)

De-multiplexing &
Quality Control

Denoise of Amplicons
Data (e.g., Denoiser)

Chimera Filtering (e.g.
ChimeraSlayer)

(II) Construction of OTU Table

OTUs Picking

Representative
Sequence Selection

Representative
Sequence Alignment

Taxonomy Assignment

Phylogenetic Tree
Construction

Construction of OTU
Abundance Table

(III) Data Diversity Analysis & Visualization

Construction of OTUs
Network

Alpha Diversity &
Rarefaction Analysis

Beta Diversity Analysis

Multivariate Analysis
(e.g., PCoA, NMDS)

Data Visualization
(e.g., bar, pie, heatmap)

(IV) OTU Occupancy & Co-occurrence Analysis

OTU Incidence &
Abundance Matrixes

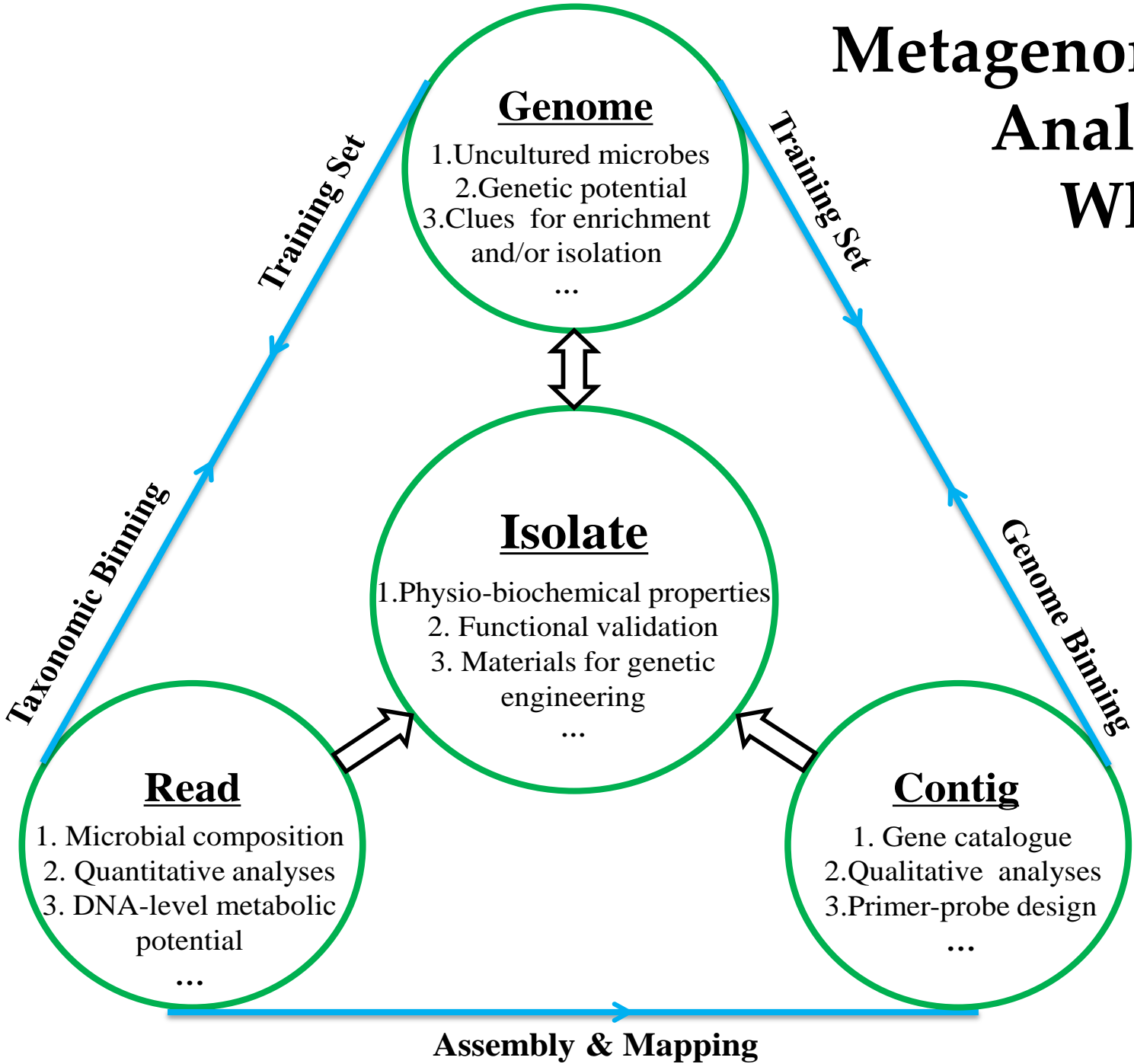
Checkerboard Score
(C-score) Test

Generalist vs. Specialist;
Persistent vs. Transient

Similarity or Regression
Based Network Analysis

Microbial Interactions
& Assembly Patterns

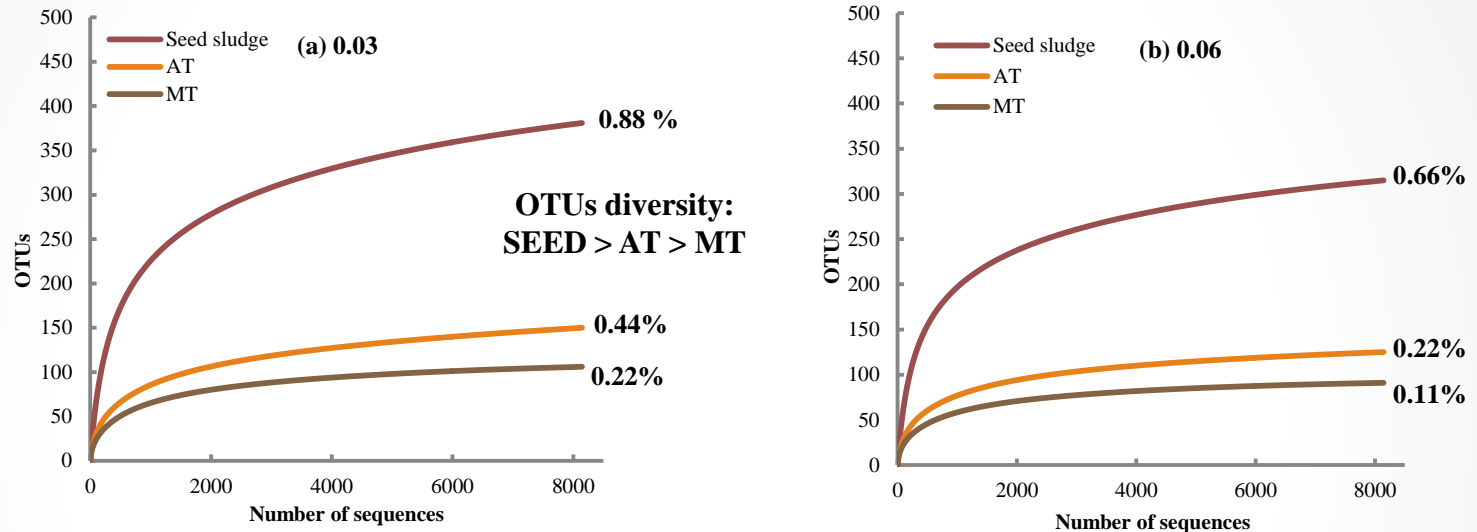
Metagenomic Analysis Wheel



Compositions of Bacteria and Archaea

Samples	Archaea	Bacteria
Mesophilic cellulose-converting consortium (~200 days)	9.0%	90.0%
Thermophilic cellulose converting consortium (~120 days)	11.0%	83.4%
Thermophilic cellulose converting consortium (~545 days)	6.2%	91.2%
Thermophilic beer-lees-converting consortium (45 days)	4.3%	95.5%
Thermophilic beer-lees-converting consortium (75days)	7.3%	92.6%
Thermophilic beer-lees-converting consortium (110 days)	6.0%	92.5%
Phenol-degrading consortium (ambient)	8.8%	91.2%
Phenol-degrading consortium (mesophilic)	12.7%	87.3%
Full-scale ADS (ST)	2.9%	95.0%
Full-scale ADS (ST)	4.3%	83.9%
Full-scale ADS (ST)	2.0%	97.5%
Full-scale ADS (SWH)	2.8%	86.0%
Full-scale ADS (SWH)	4.6%	91.0%
Full-scale ADS (SWH)	5.2%	94.8%
Full-scale ADS (SWH)	4.5%	95.5%

OTUs diversity of phenol-degrading methanogenic consortia

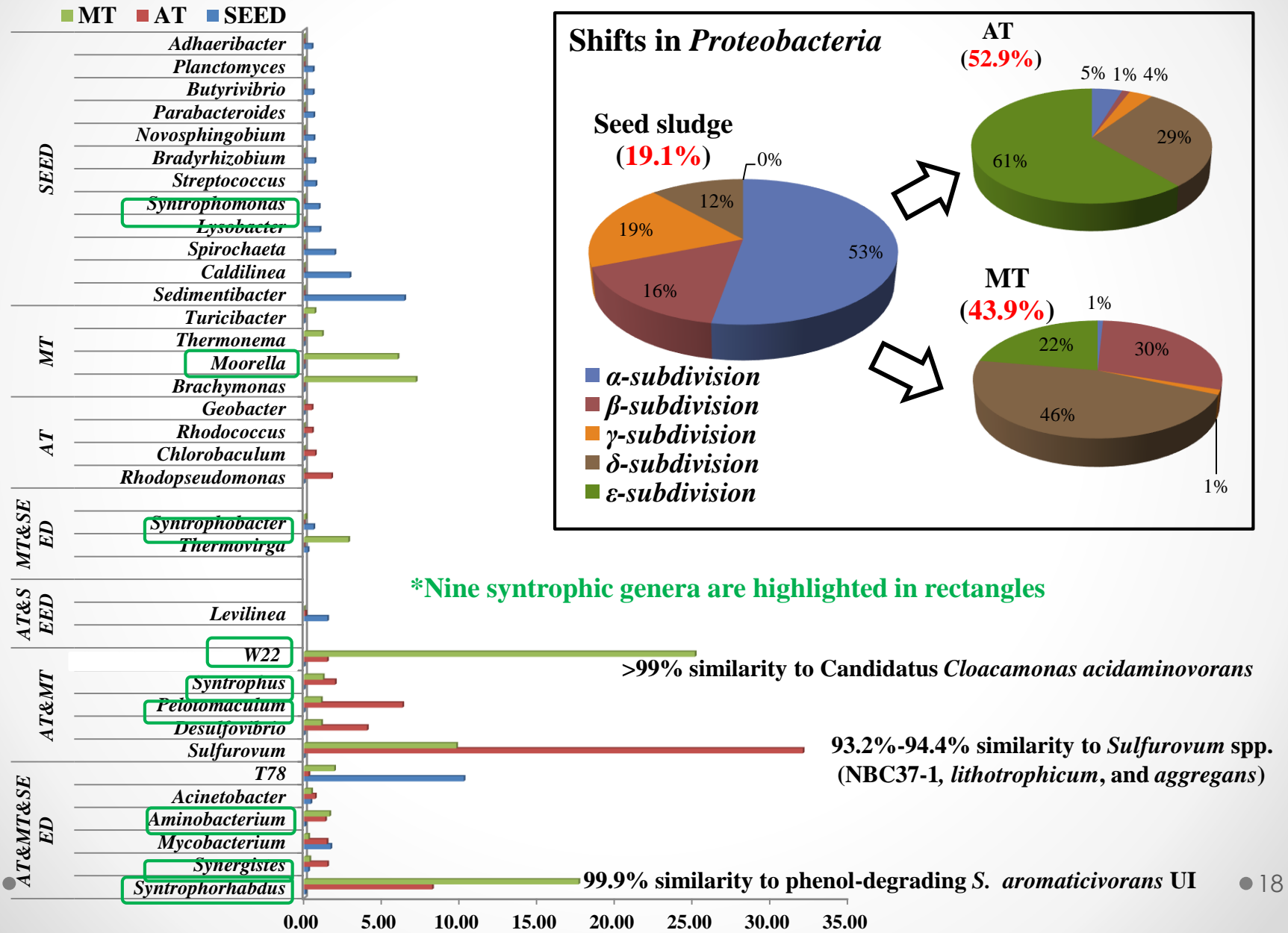


Rarefaction curves of seed, AT and MT samples at cutoff levels of 3% (a) and 6% (b).

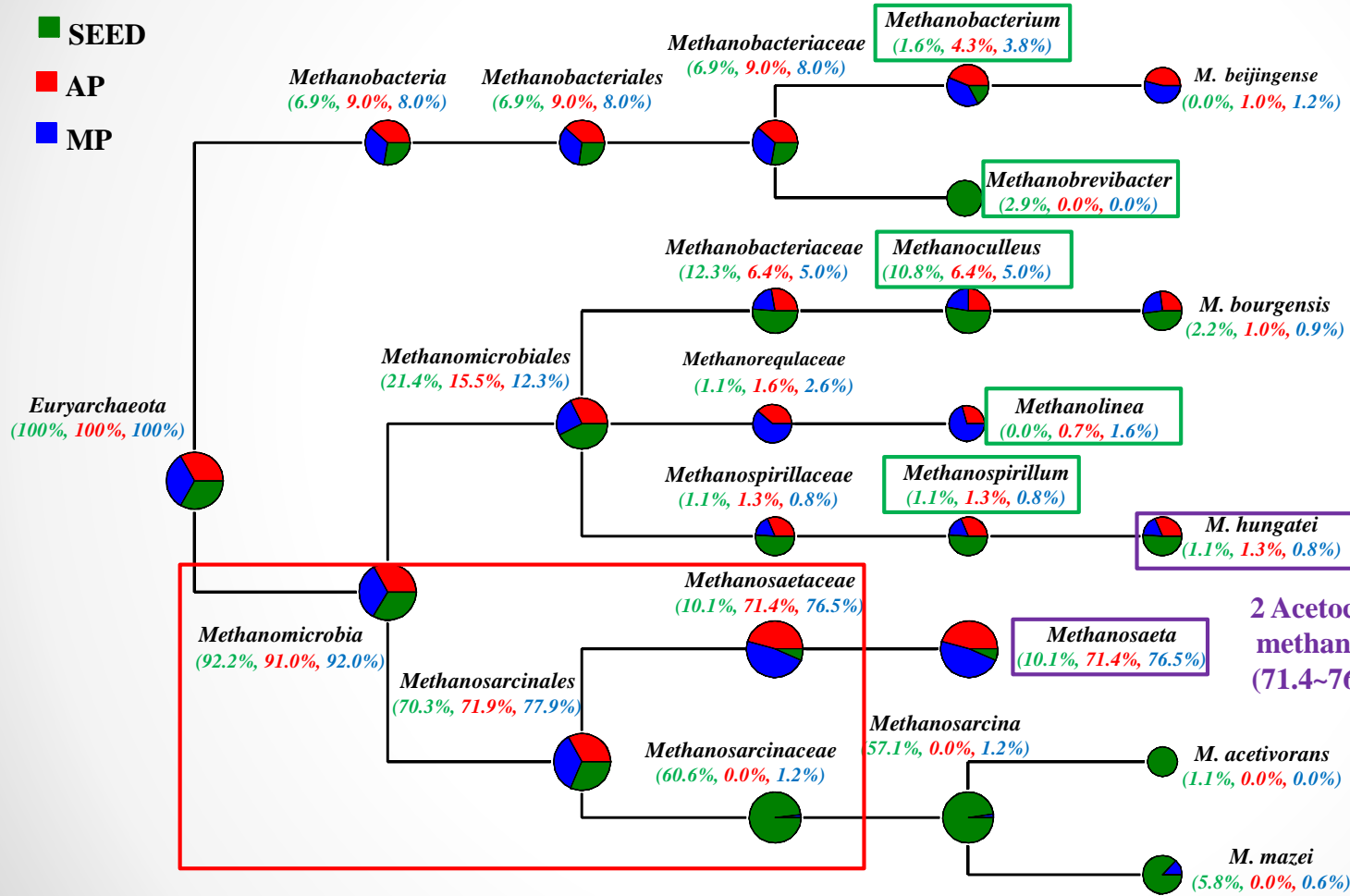
A list of OTUs diversity in phenol-degrading consortia using NGS-based and cloning-based methods

Temperature	Methods	Sequences NO.	OTUs NO.	References
26 °C	Cloning	90	13	Zhang et al., 2005
AT (20 °C)	NGS	8150	150	Ju and Zhang, 2014
37 °C	Cloning	106	20	Chen et al., 2009
37 °C	Cloning	107	21	Chen et al, 2008
37 °C	Cloning	114	6	Levén and Schnürer, 2010
MT (37 °C)	NGS	8150	106	Ju and Zhang, 2014

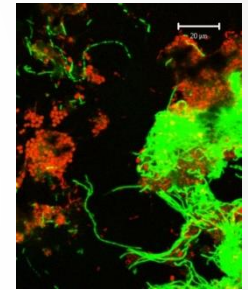
Major shifts in bacterial communities



Major shifts in archaeal communities



5 Hydrogenotrophic methanogens (11.2~15.7%)



Methanosaeta (green)
(Zhang et al., 2005)



Methanosarcina
(Fang et al., 1996)

2 Acetoclastic methanogens (71.4~76.5%)

A major shift of methanogens from *Methanosarcina* to *Methanosaeta*

Definition of Coverage



Length of genomic segment (contig): L

Number of reads/sequences used to assemble the contig: n

Average length of each read : l

Definition: **Coverage** $C = n \times l / L$

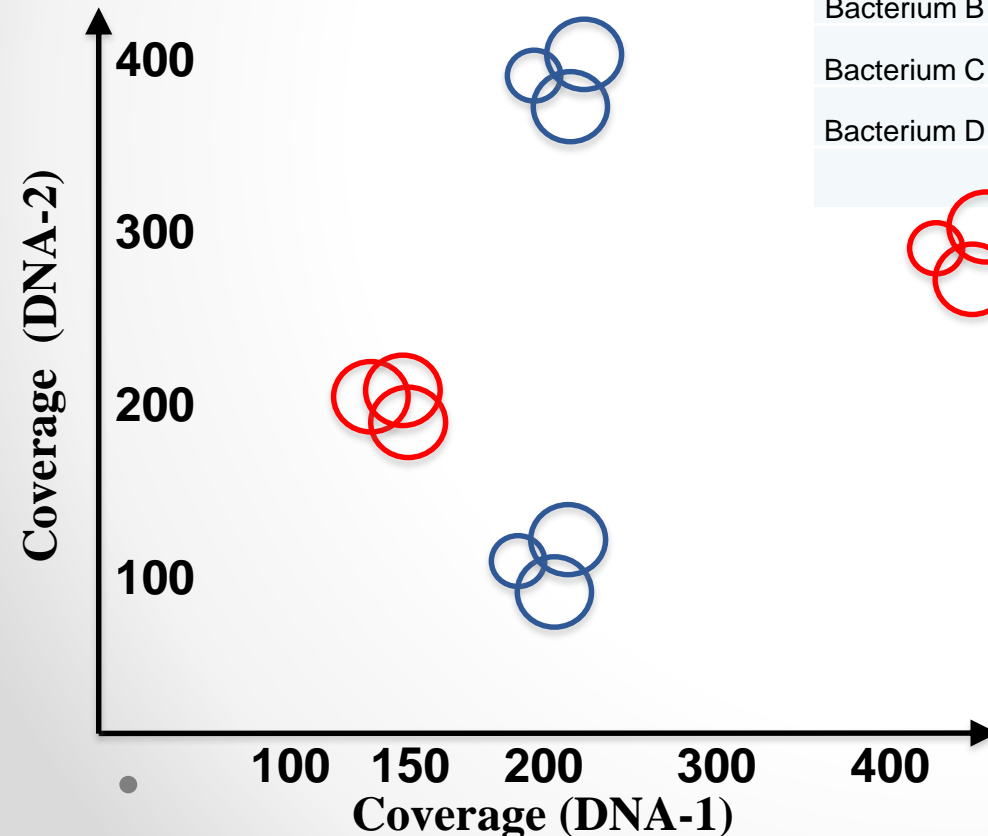
For those genomic segments from the same bacteria species, their coverage should be exactly the same (*theoretically*) or very close/similar (*in reality*) to each other.

Conversely, those contigs/genomic segments having similar coverage in a sample are **probably (high probability, but not 100% necessarily)** from the same species. This could be used as a criteria to pick the contigs of the same species out from the mixture of all the contigs in a sample.

Sample 1: under mesophilic condition

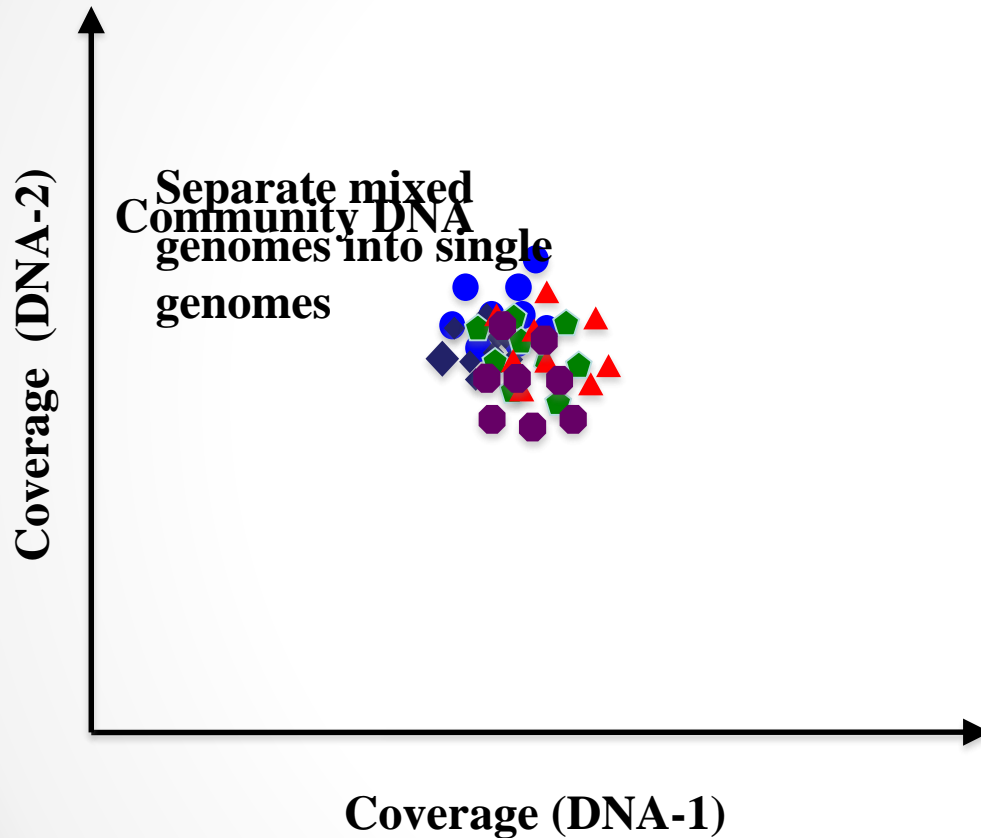
Sample 2: under thermophilic condition

Sample 1	Genome size	Percentage	1000 cells	Size of data	Coverage
Bacterium A	2M	20%	200	400 M	200X
Bacterium B	3M	20%	200	600 M	200X
Bacterium C	4M	15%	150	600 M	150X
Bacterium D	3M	45%	450	1350 M	450X
		100%		2.95 G (total)	
Sample 2	Genome size	Percentage	1000 cells	Size of data	Coverage
Bacterium A	2M	10%	100	200 M	100X
Bacterium B	3M	40%	400	1200 M	400X
Bacterium C	4M	20%	200	800 M	200X
Bacterium D	3M	30%	300	900 M	300X
		100%		3.1 G (total)	



Using some scripts (a few lines of commands of a computer program, like Python, R, etc.) to pick out these contigs with the same/similar coverage in the two samples and define them as a bin (draft genome of a bacterium).

Bacterial Genome Binning

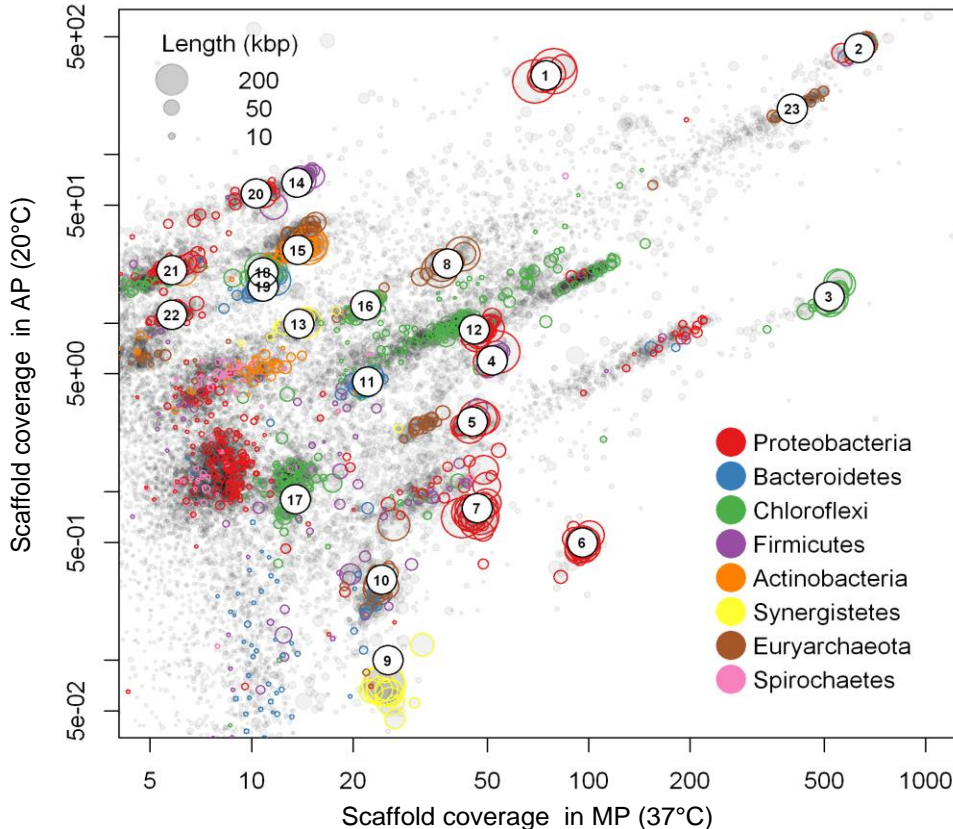


Important criteria for genomic binning:

1. Coverage (depending on percentage of a species in the mixture)
2. Tetra-nucleotide frequency (depending on the DNA sequences of a species)
3. GC content
4. Codon composition
5. Taxonomy

Albertsen *et al.*, 2013. *Nature Biotechnology* 31, 533–538

Differential coverage binning for reconstruction of 23 prokaryotic genomes



Parameter statistics	MT	AT
Enrichment temperature (°C)	37	20
Metagenome size (Gbp)	11.0	8.6
Number of paired-end reads (million)	110	86
Average length of read (bp)	100	100
Total assembly size (Mbp) ¹	172	209
N50 (bp) ²	6519	6612
Max contig size (Kbp)	42	98
Reads mapped to MT scaffolds	79%	50%
Co-assembly of shot-gun paired-end reads with library insert lengths of 180 and 800 bps		

¹Only scaffolds ≥ 1000 bp were considered.

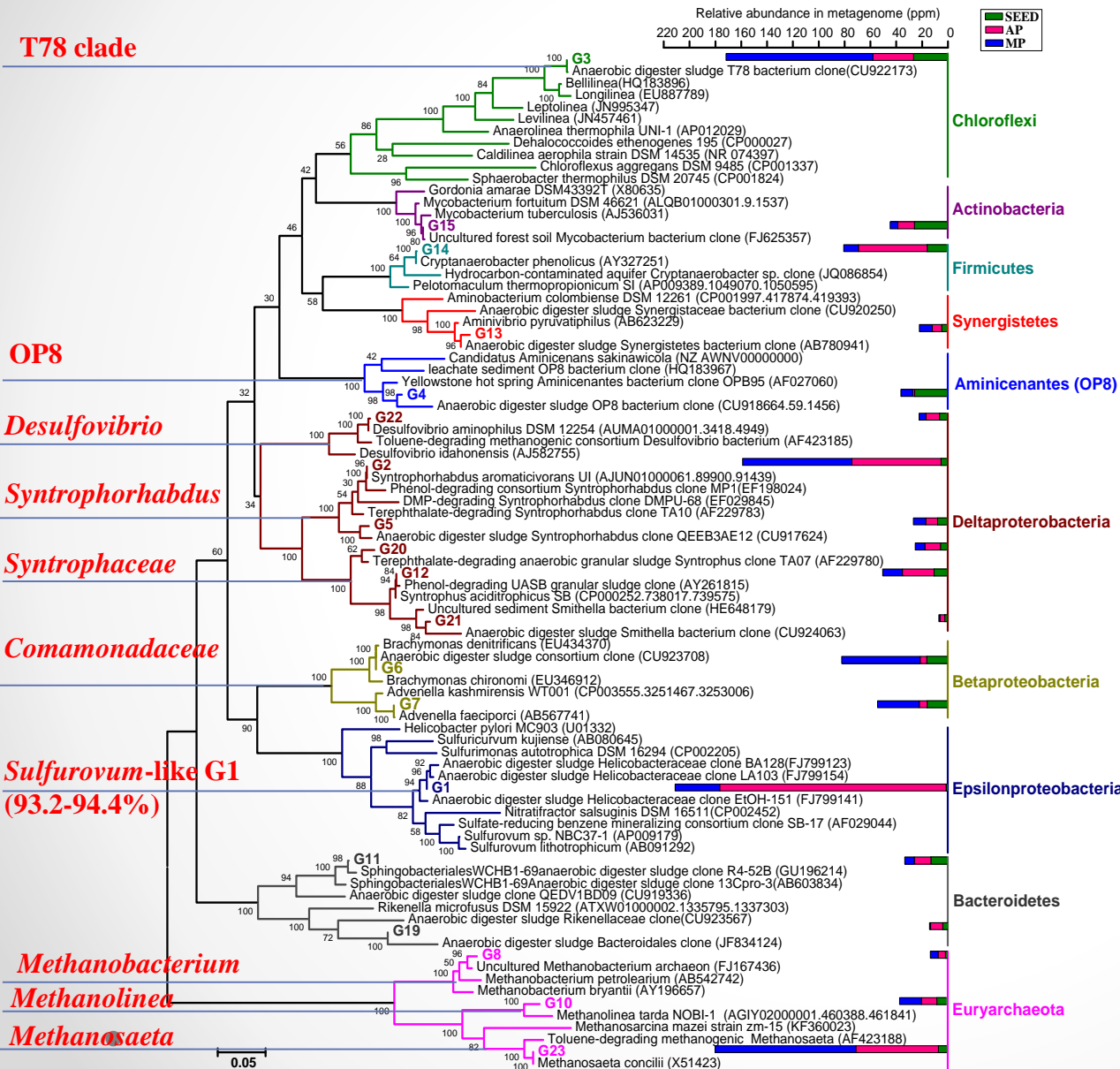
²Calculated by mapping reads to scaffold ≥ 1000 bp using CLC workbench 6.0.

(Reference of the binning approach: Albertsen et al., 2013; NBT)

Proteobacteria: G1, G2, G5, G6, G7, G12, G14, G20, G21 and G22 (**10 genomes**); **Chloroflexi:** G3, G16, G17 and G18 (**4 genomes**); **Synergistetes:** G9 and G13 ; Bacteroidetes: G11 and G19; **Actinobacteria:** G15; **OP8:** G4
Euryarchaeota: G8, G10 and G23 (**3 genomes**);

Maximum-likelihood phylogenetic tree

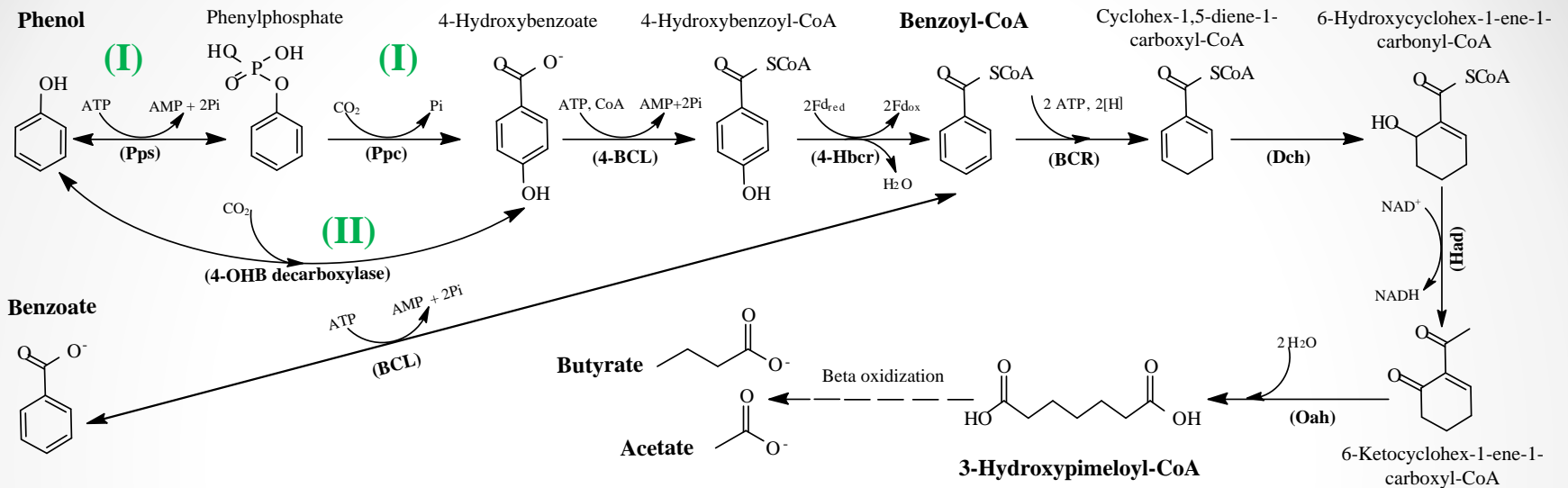
(for 19 genomes which have 16S rRNA gene, > 1200 bp. Obtained by de novo assembly, PE tracking and Emirge)



Genome size and completeness

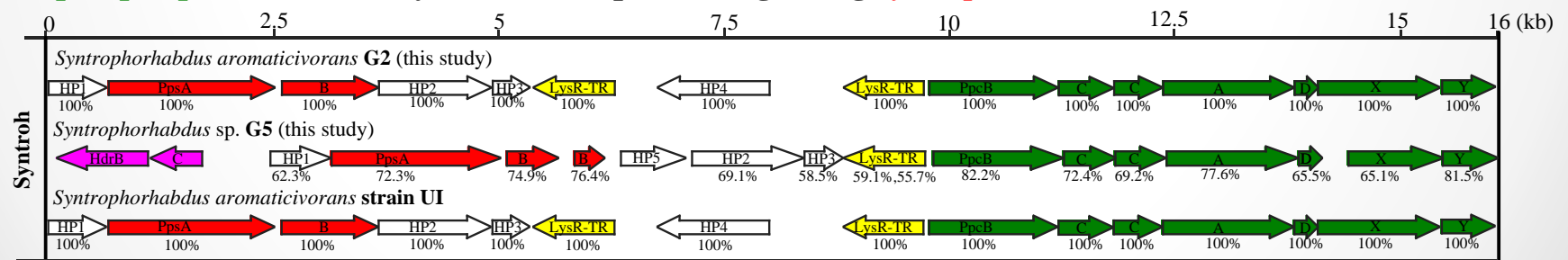
	S(M)	N	GC(%)	C(%)	R(%)
G1	1.78	28	37.0	99.1	0.9
G2	2.90	133	51.9	92.5	1.9
G3	1.85	24	46.8	88.8	1.9
G4	2.29	33	45.7	91.6	1.9
G5	3.17	77	44.0	87.9	0.9
G6	2.57	44	64.6	84.1	3.7
G7	2.91	28	49.8	95.3	3.7
G8	1.72	35	37.9	88.6	0.0
G9	1.90	17	56.1	90.7	3.7
G10	2.21	56	57.3	88.6	0.0
G11	2.40	84	39.7	91.6	0.9
G12	2.43	65	51.8	88.8	1.9
G13	2.48	93	60.0	86.0	1.9
G14	2.97	127	48.2	85.0	0.0
G15	4.91	159	68.6	89.7	0.0
G16	1.88	103	52.2	84.1	0.9
G17	2.16	130	52.5	86.9	0.9
G18	2.13	38	50.8	92.5	1.9
G19	2.70	75	49.6	89.7	0.9
G20	2.35	132	55.7	73.8	1.9
G21	2.42	106	55.3	92.5	0.9
G22	2.64	240	66.8	86.0	0.0
G23	2.08	220	52.8	60.0	2.9

Estimated by 107 ESCGs and 35 COGs
 S(M): genome size; N: contigs number;
 C%: completeness; R%: contamination



(I) Phosphorylation-carboxylation pathway (by **G2** and **G5**); (II) carboxylation pathway (**G14**)

Pps-Ppc operon encoded by **G2**, **G5** and phenol-degrading *Syntrophorhabdus aromaticivorans* **UI**



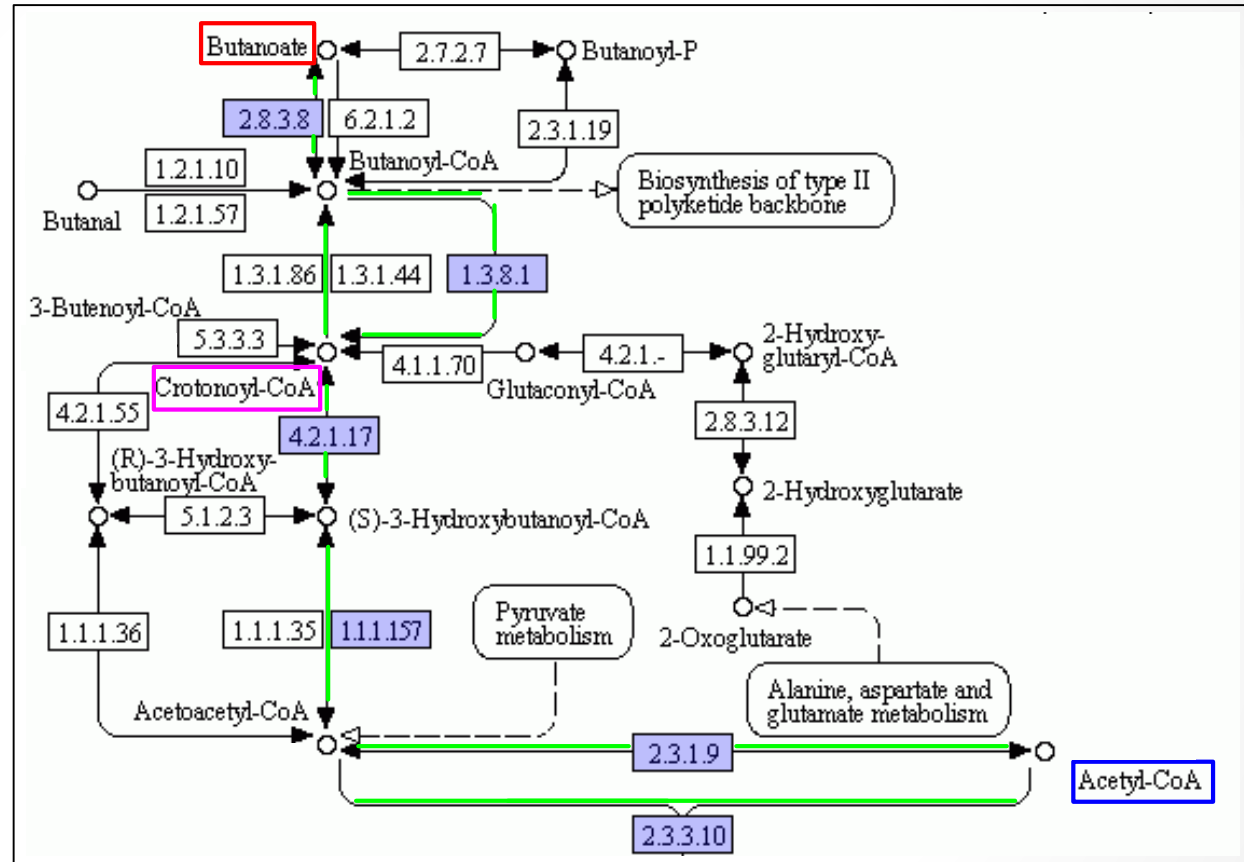
DDH: G2 vs. UI: 79.2% (same species); G5 vs. UI: 12.9% (different species)

ANI: G2 vs. UI: 99.8% (same species); G5 vs. UI: 75.6% (different species)

First genomic insights into T78 clade

G3: uncultured *Chloroflexi* T78 clade sp.

Butyrate oxidation
(via crotonyl-CoA)



Relatives: *Bellilinea* sp. clone De3218 (95.2%); *Longilinea* sp. clone 48IIISN (95.0%) and *Anaerolinea thermophila* UNI-1 (87.2%)

Potential proteins for electron transfer and energy conservation

Electron transfer flavoproteins (2600084386-87);

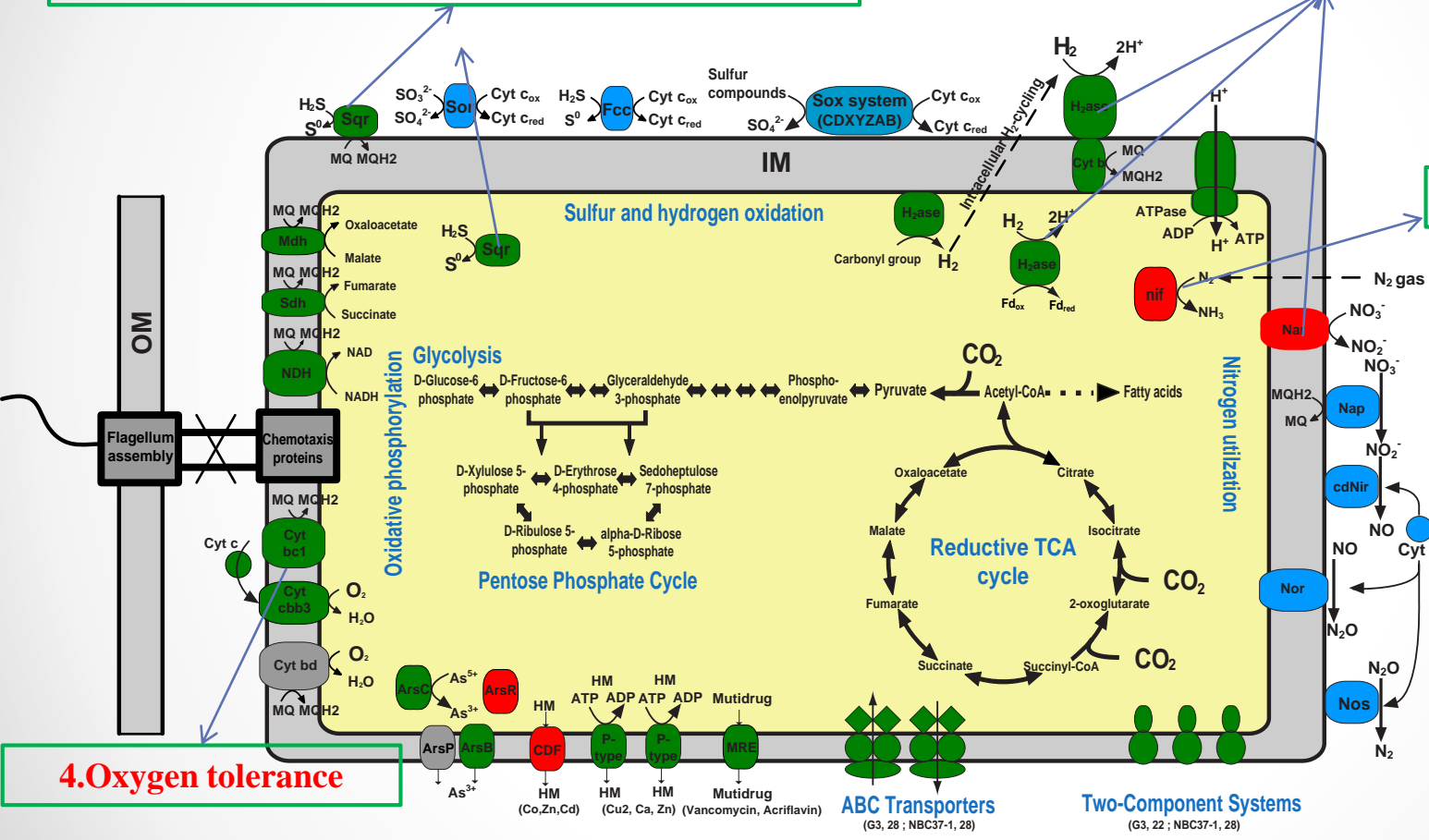
NAD(P)-dependent iron-only hydrogenases (2600085382-85) and NAD(P) transhydrogenases

Uncultured ϵ -Proteobacterium G1: versatile energy metabolism (S/H₂/N)

1. Sulfide oxidation

2. Hydrogen oxidation

3. Nitrogen fixation



G1

- 1.78 Mb
- >99% completeness
- Not isolated yet.
- No close isolated relatives

4. Oxygen tolerance

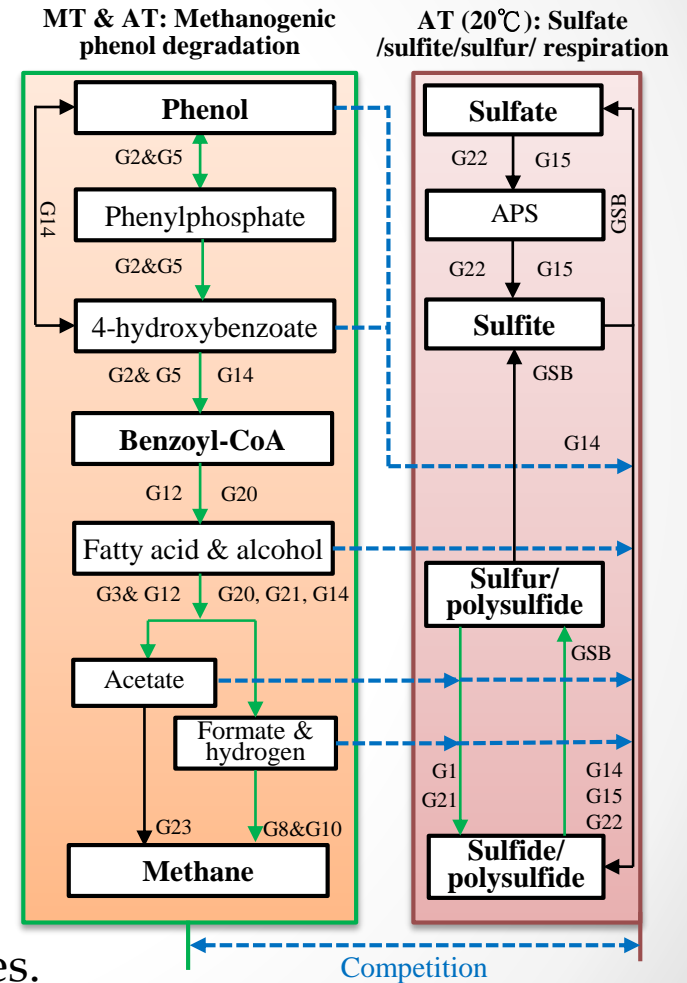
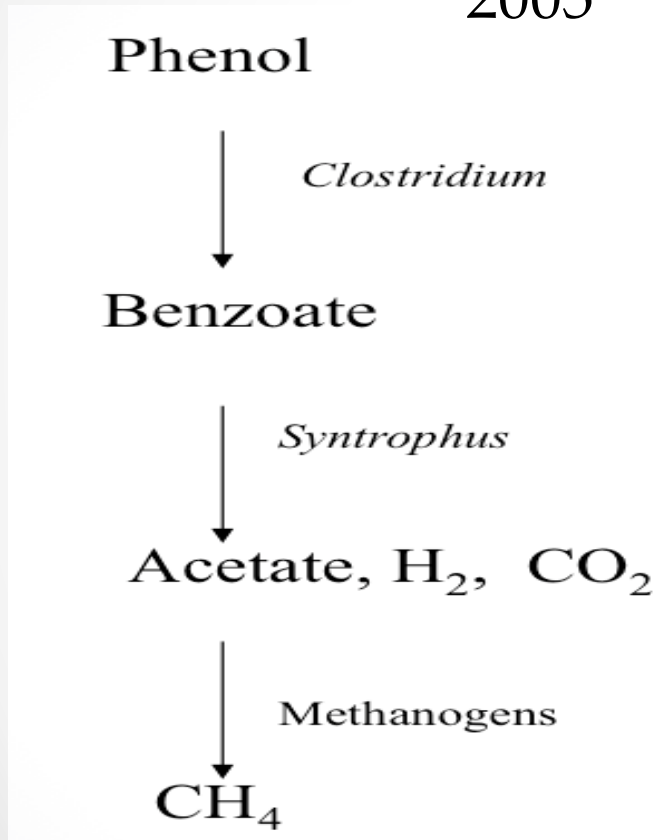
5. Antibiotics, arsenate and metal resistance

Give some hints/directions for isolation.

Proteins unfound in G1
Proteins unfound in NBC 37-1
Proteins unfound in both

Proposed microbial syntrophic and competitive relationships in phenol-degrading consortia

2005 → 2015



We know more about the microbial communities.

But, how will these knowledges contribute to better performance of the reactors?

Consultancy Anaerobic Digestion Projects

- Co-digestion of food waste and combined sludge
(Stage I to Stage III)
- Anaerobic digestion of CEPT sludge

Co-digestion of food waste with sewage sludge

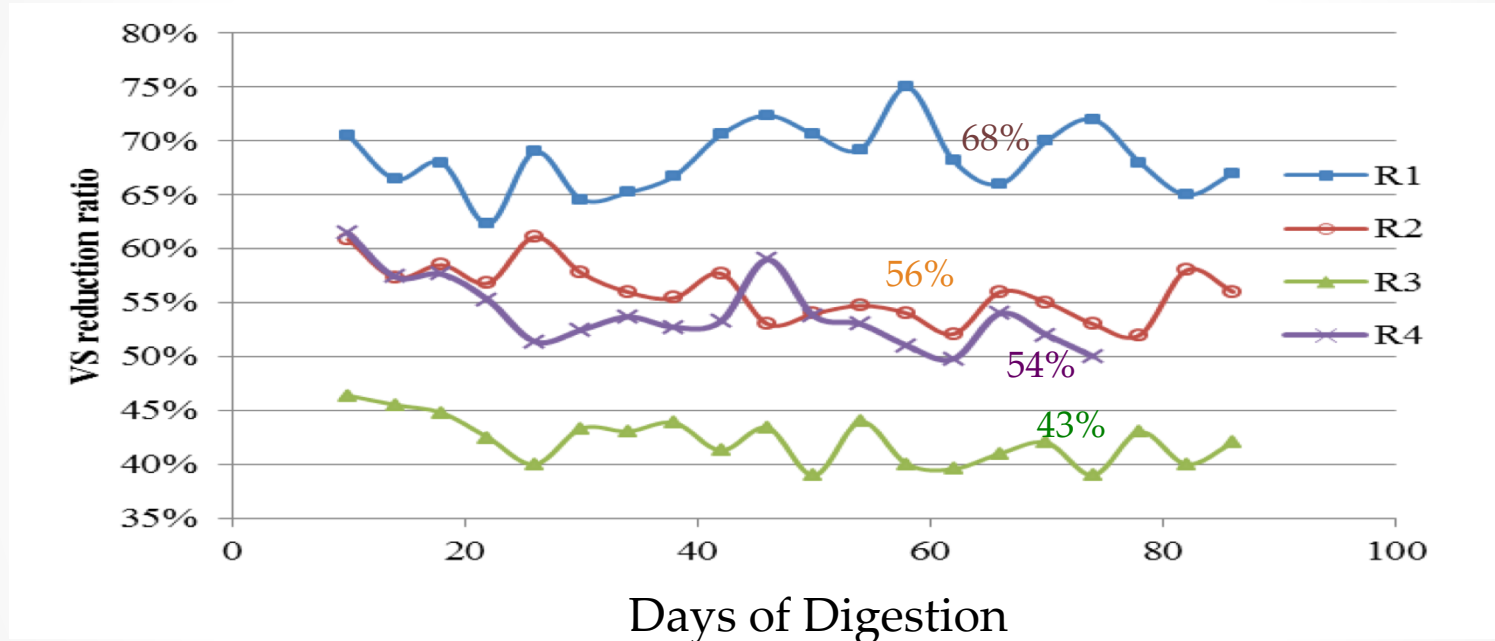
VS reduction ratio in the four reactors

HRT 25 d---R1: FW/FSS=5:5;

R2: FW/FSS=2:8;

R3: FW/FSS=0:10;

HRT 15 d---R4: FW/FSS=2:8



- ✧ Consistent with the results obtained in previous batch tests, a higher FW/FSS ratio corresponded with a higher VS reduction ratio.
- ✧ The two HRTs of 15 days and 25 days did not show obvious impact on the VSR.

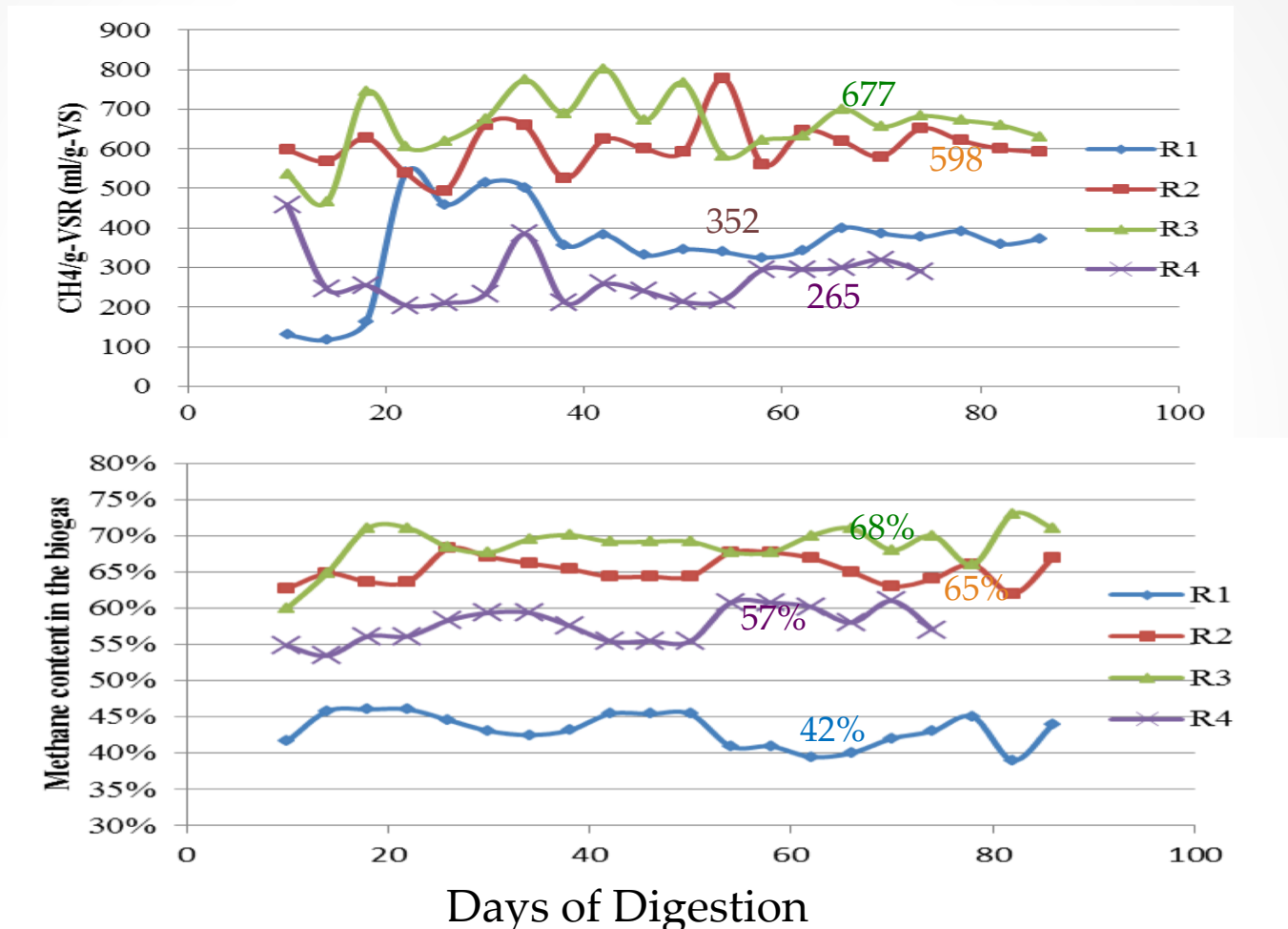
CH₄/VSR and CH₄ content

HRT 25 d---R1: FW/FSS=5:5;

R2: FW/FSS=2:8;

R3: FW/FSS=0:10;

HRT 15 d---R4: FW/FSS=2:8



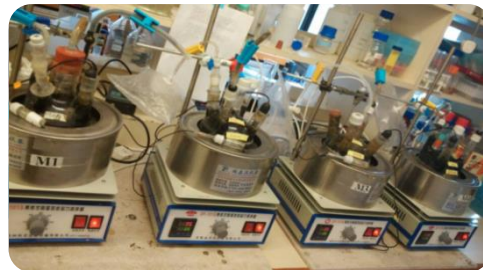
- ✧ A **higher FW/FSS ratio** corresponded with **lower methane content** detected in the biogas generated, and a **longer HRT** had a **larger total volume** of biogas generated

Anaerobic digestion of CEPT sludge and the microbial ecology

- Process optimization for anaerobic digestion of **Chemical Enhanced Primary Treatment (CEPT)** sludge.



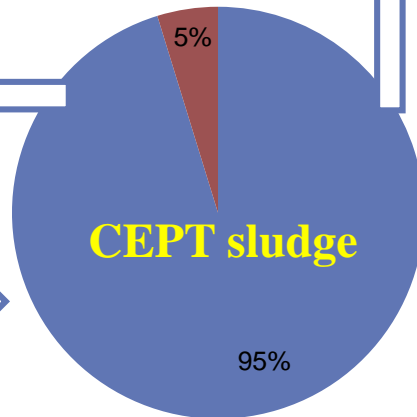
Two 8-L digesters (R1 & R2)



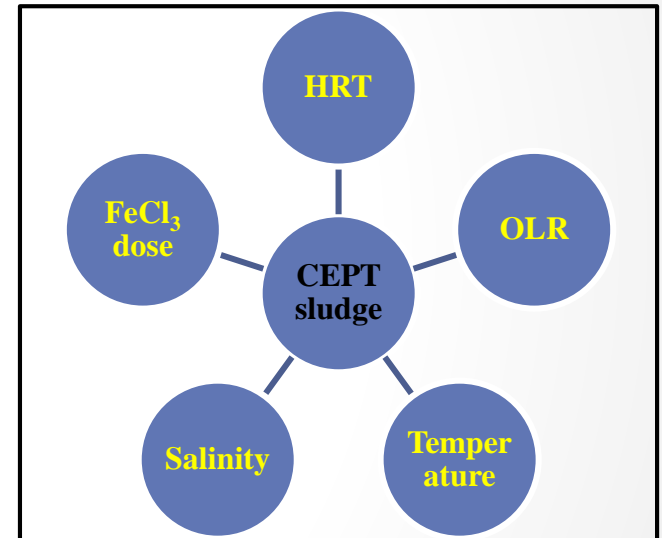
Four 0.8-L digesters (M1-M4)



Coagulants and polymer used for CEPT process



■ SCI-STW (600t) ■ Other-STWs (30t)



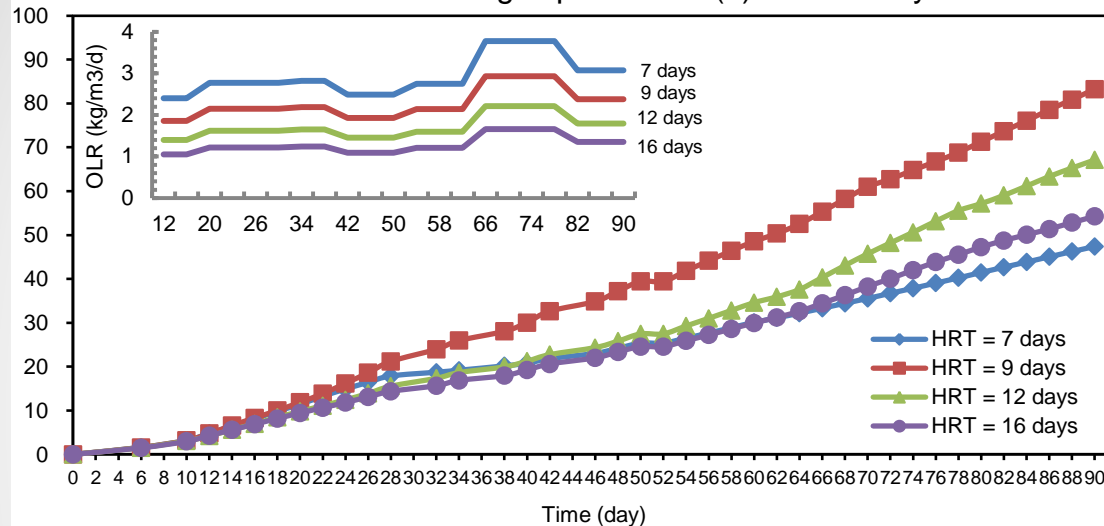
Key operational parameters examined

Feed frequency: every 2 days; FeCl₃ dose; NaHCO₃ supply to control pH above 6.80; **Equal SRT and HRT**

- **75% of 2.2 million m³ sewage daily** disposed at **Stonecutters Island (SCI) STW using CEPT process** (2001-now), yielding **600 wet tons (per day)** of CEPT sludge.

Effect of SRT/HRT and co-varied OLR on methanogenic digestion of CEPT sludge

Accumulative biogas production (L) over 90 days

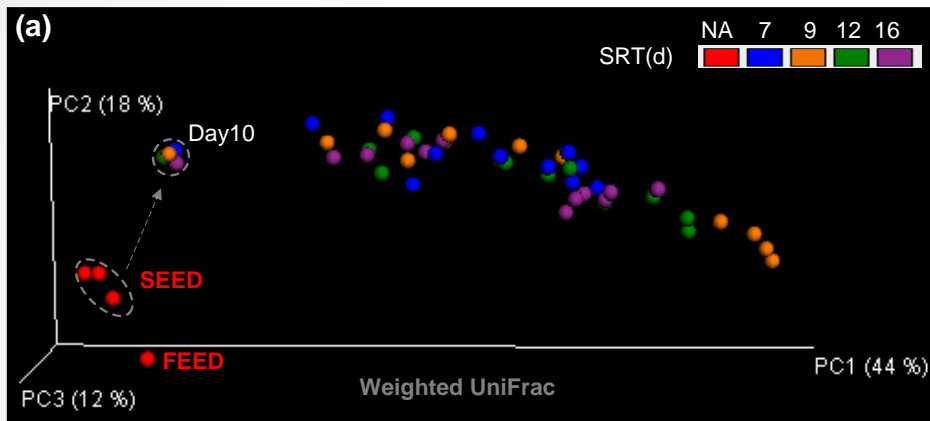


Performance (average value)	HRT (day)			
	7	9	12	16
SMP (m ³ /kg-VS)	0.31	0.84	0.83	0.97
SBP (m ³ /kg-VS)	0.45	1.24	1.25	1.46
V _{gas} (L/d)	0.56	1.16	0.94	0.73
VSR (g/d)	1.29	0.98	0.75	0.51
VSR%	53	52	53	48
CH ₄ (%)	68.0	68.0	66.3	66.5
Effluent pH	6.92	6.98	7.08	7.12
No. of measure	16	16	16	16

*Other conditions: FeCl₃ dose =1.3 mL/L-sludge; T = 35°C; Feed sludge source: SCI STW CEPT sludge; Operating time: 90 days.

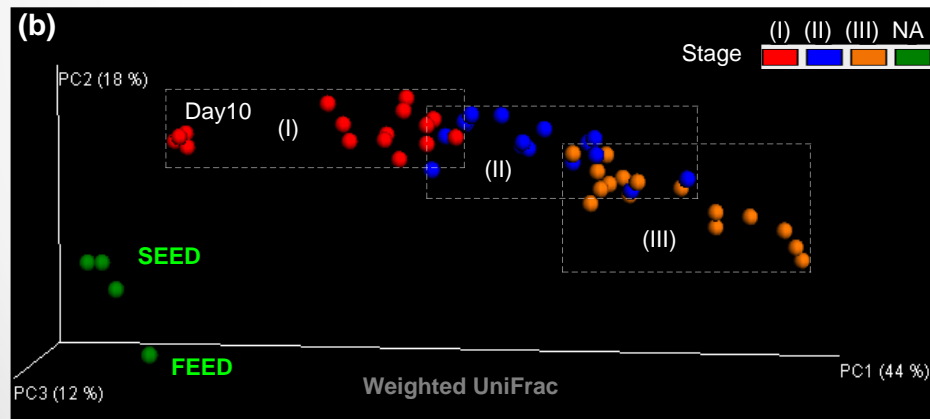
- **Shortening of HRT from 16 to 12 or 9 days** contributed to **significant increases (P-value <0.05) in biogas production by 29-59%** (from 0.73 L/d), **VSR by 39-92%** (from 0.51 g/d), accompanied with **slight decreases in SMP by 14%** (from 0.97), **SBP by 14-15%** (from 1.46), and **an increase in alkalinity supply** (from 0 to 0.83-1.44 mL/d NaHCO₃ solution).
- **Further decrease of HRT from 9 to 7 days** led to **sharp drops in SMP (by 63%), SBP (by 64%), and V_{gas} (by 48% L/d)**, and **dramatic increase in both alkalinity consumption by 255% and VFAs accumulation by 211%** (from 72.8 mg/L).
- **Therefore, a HRT of 9-12 days is recommend for design of CEPT sludge digesters in Hong Kong**

Deterministic factors affect microbial community structure much more than SRT/HRT



3D-PCoA (left figures), Procrustes Analysis (of PCs) and BIO-ENV Analysis congruously support :

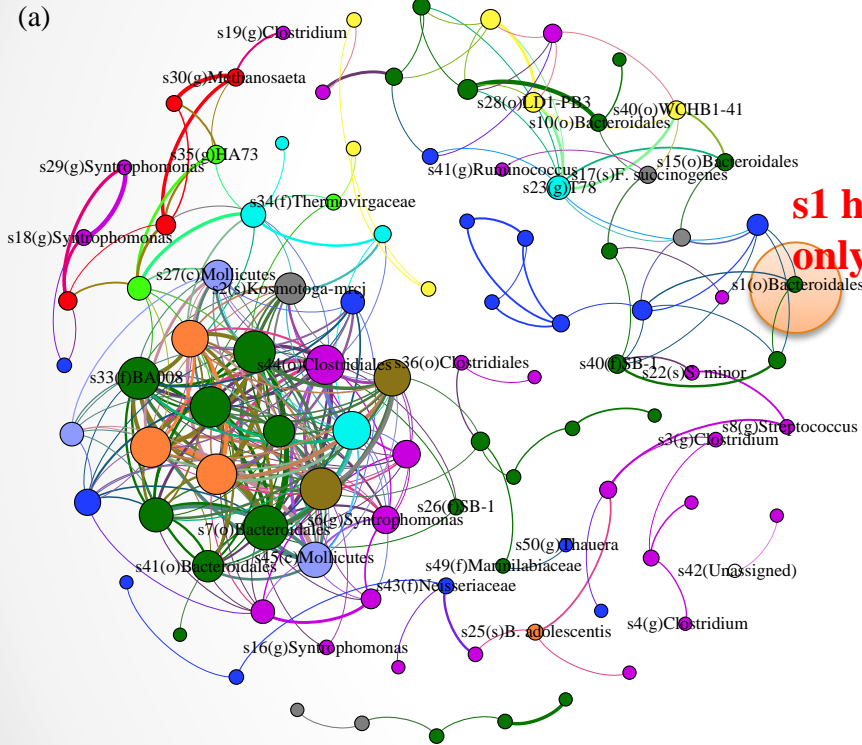
1. Despite of **the difference in HRTs** (16, 12, 9 and 7 days), microbial communities in the four digesters were **highly synchronised** and dynamic over 90 days.
2. *Bacteroidales* (42.8% in average), *Clostridiales* (23.0%) and *Kosmotoga mrcj* (8.6%) dominated in all 48 digester samples



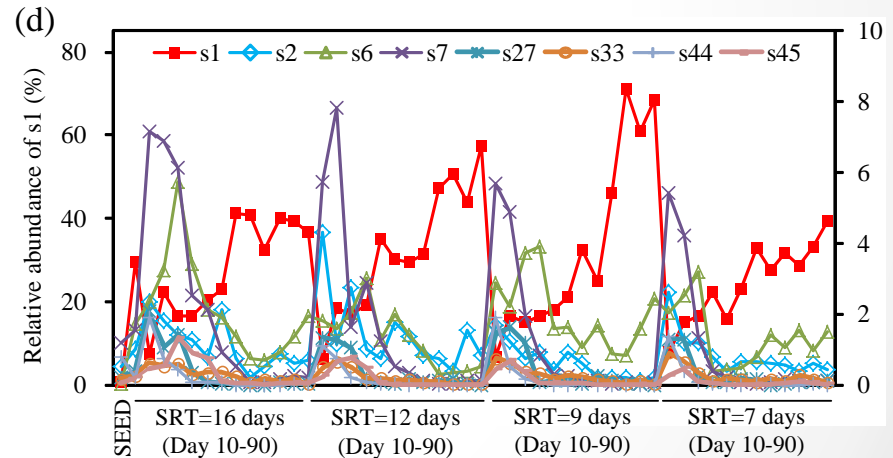
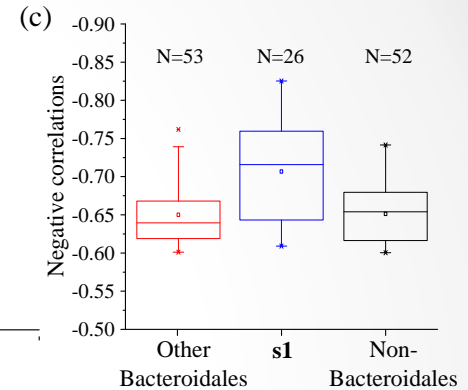
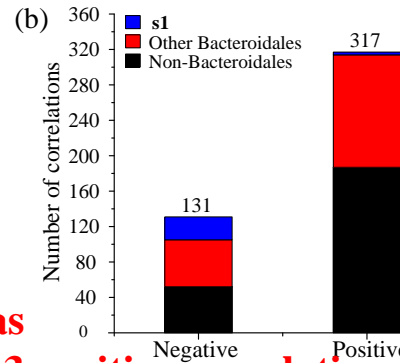
Microbial dynamics in four methanogenic digesters with different SRTs. **Stage I: Day 10-34; Stage II: Day 42-66; Stage III: Day 74-90.**

This implicates **deterministic factors**, such as substrate **nature (CEPT) and availability and species interactions (e.g., competition)**, dominate over operational parameters (**SRT and co-varied OLR**) in shaping microbial dynamics during **startup and initial steady operational periods**.

Strong competitive roles of *Bacteroidales* populations in CEPT sludge digesters



Left panel: **positive correlations**
(Spearman's rho > 0.6, P-value < 0.01)



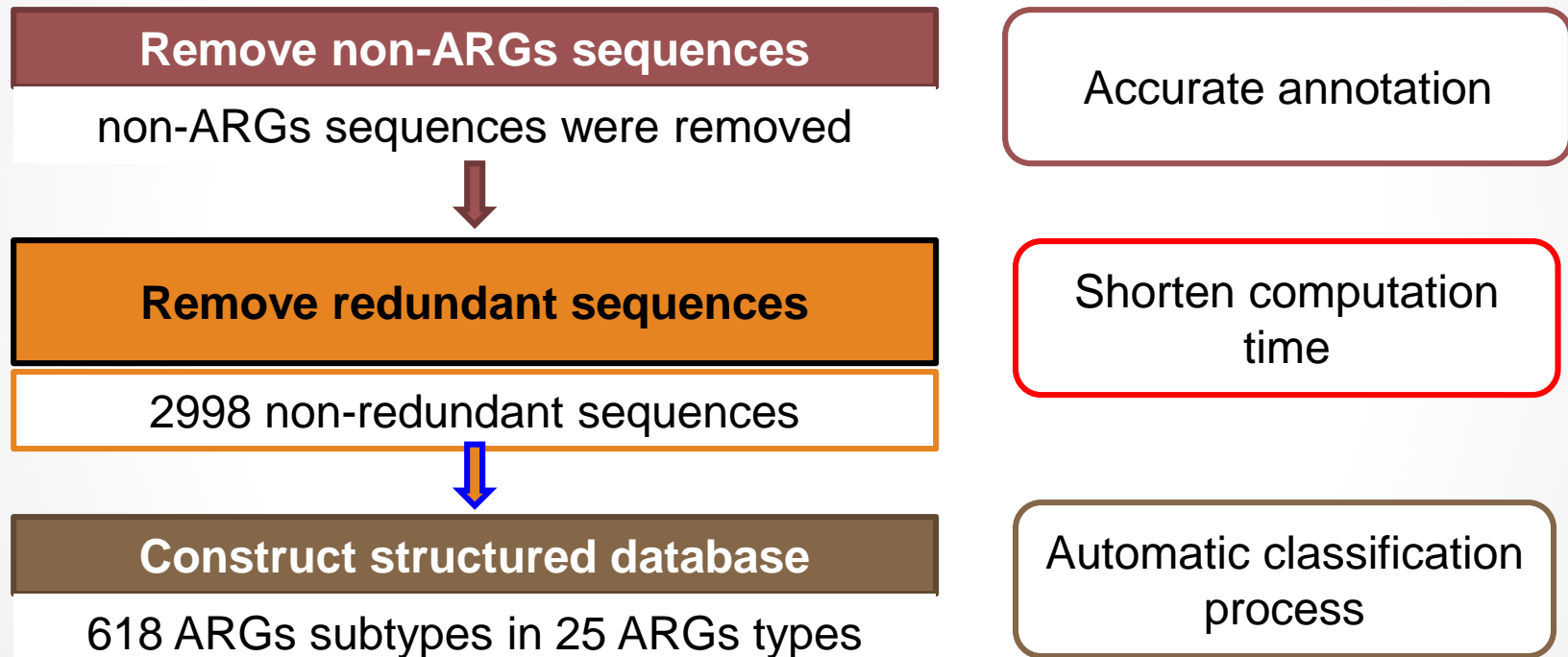
Right panels: **negative correlations**
(Spearman's rho < -0.6, P-value < 0.01)

- Over 60% of negative correlations (i.e., 131 co-exclusion instances) involved the dominant uncultured *Bacteroidales* species s1 (20%, GreenGenes taxonomy ID: 837605) or other *Bacteroidales* species-OTUs (40%).

Optimization of ARDB Database

ARDB-Antibiotic Resistance Genes Database (Liu and Pop, 2009)

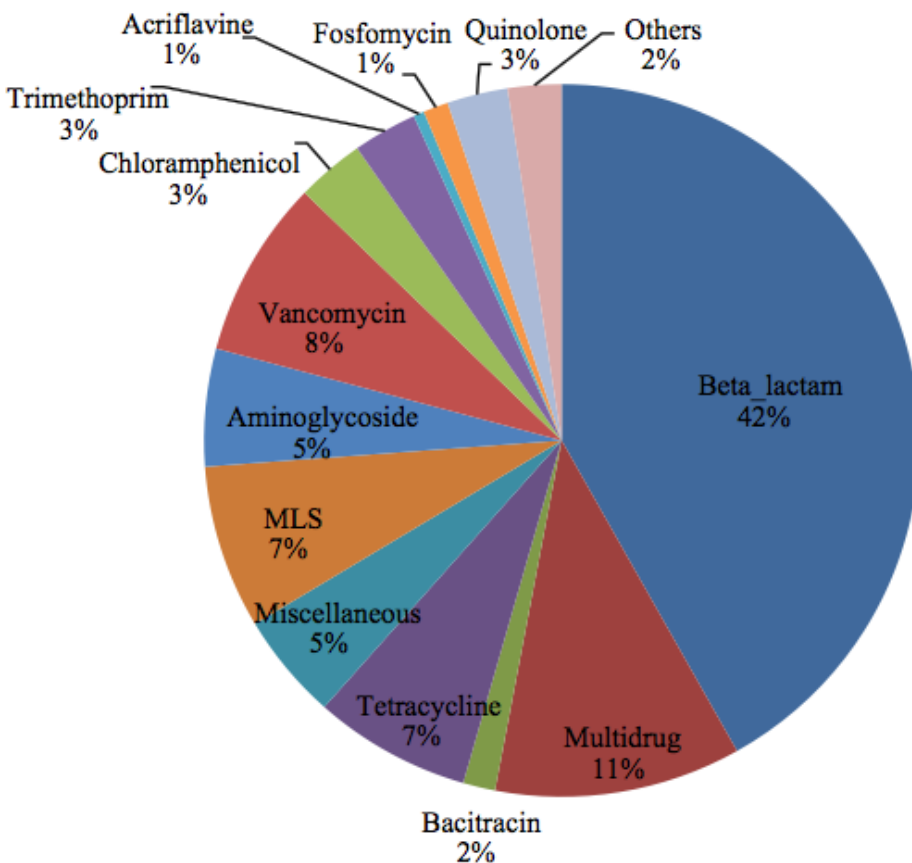
<http://ardb.cbcb.umd.edu/>



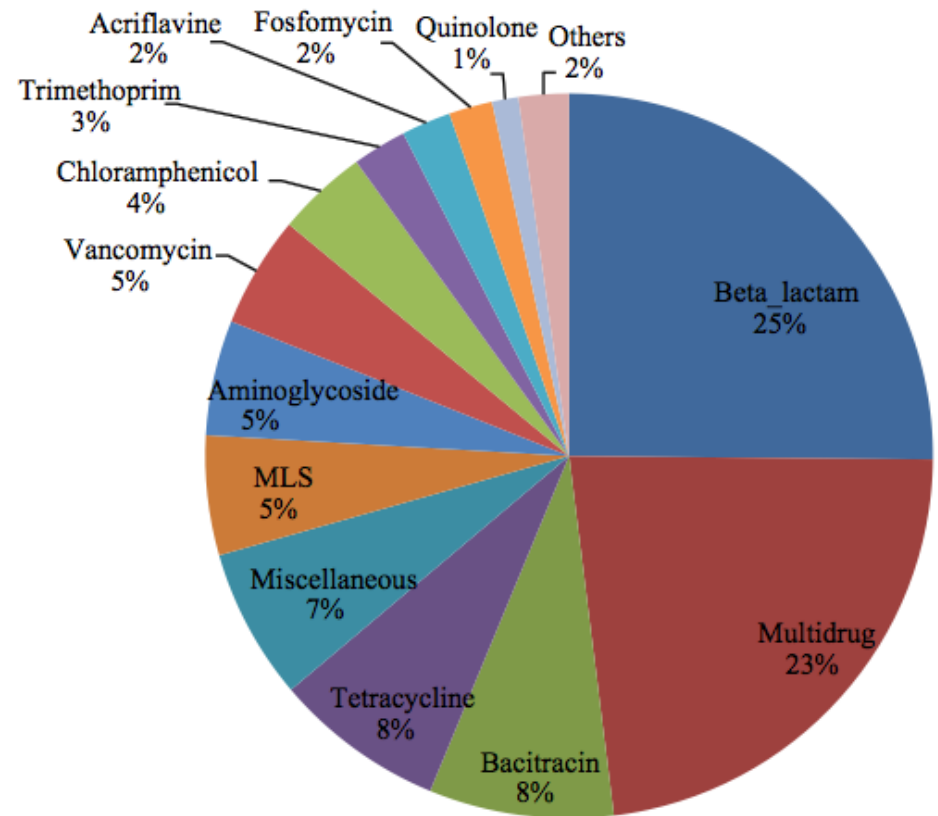
Yang Y, Li B, Ju F, Zhang T*. 2013. Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environmental Science and Technology*. 47 (18), 10197–10205.

Non-redundant Structured ARDB

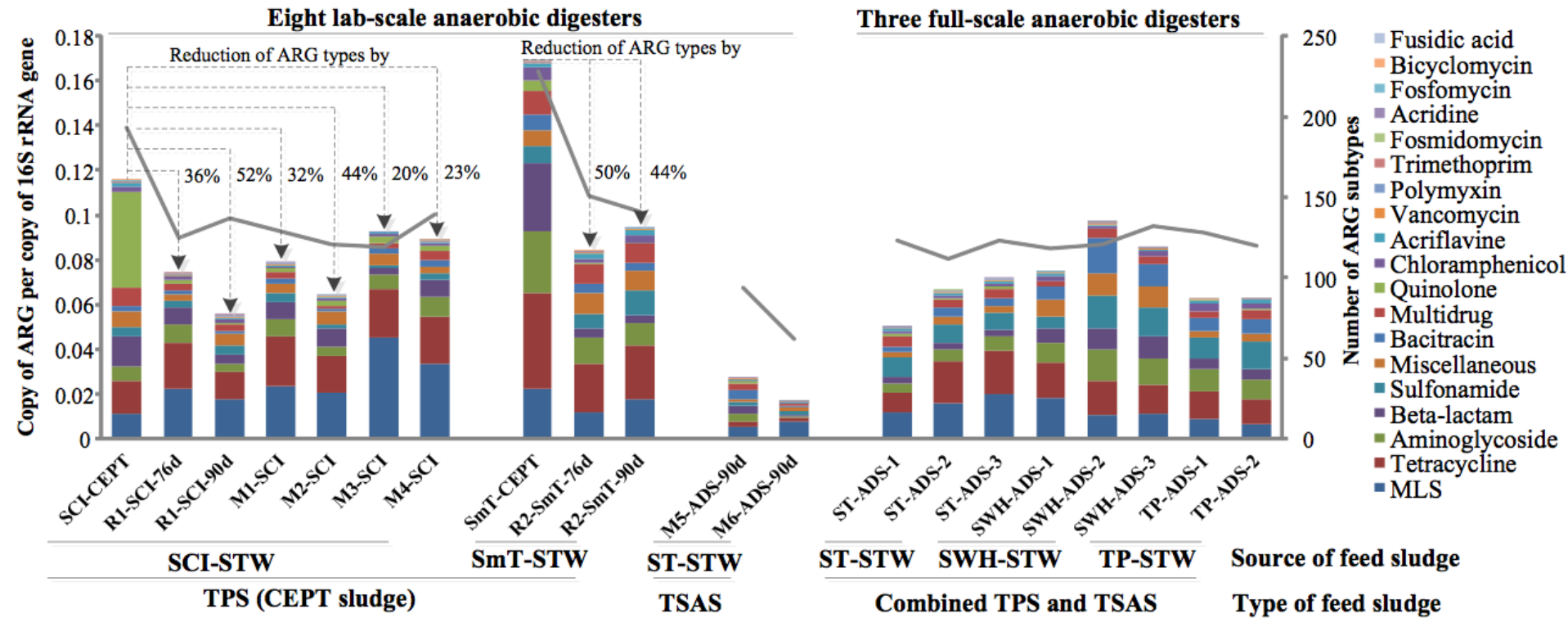
(a) Distribution of 619 ARG subtypes



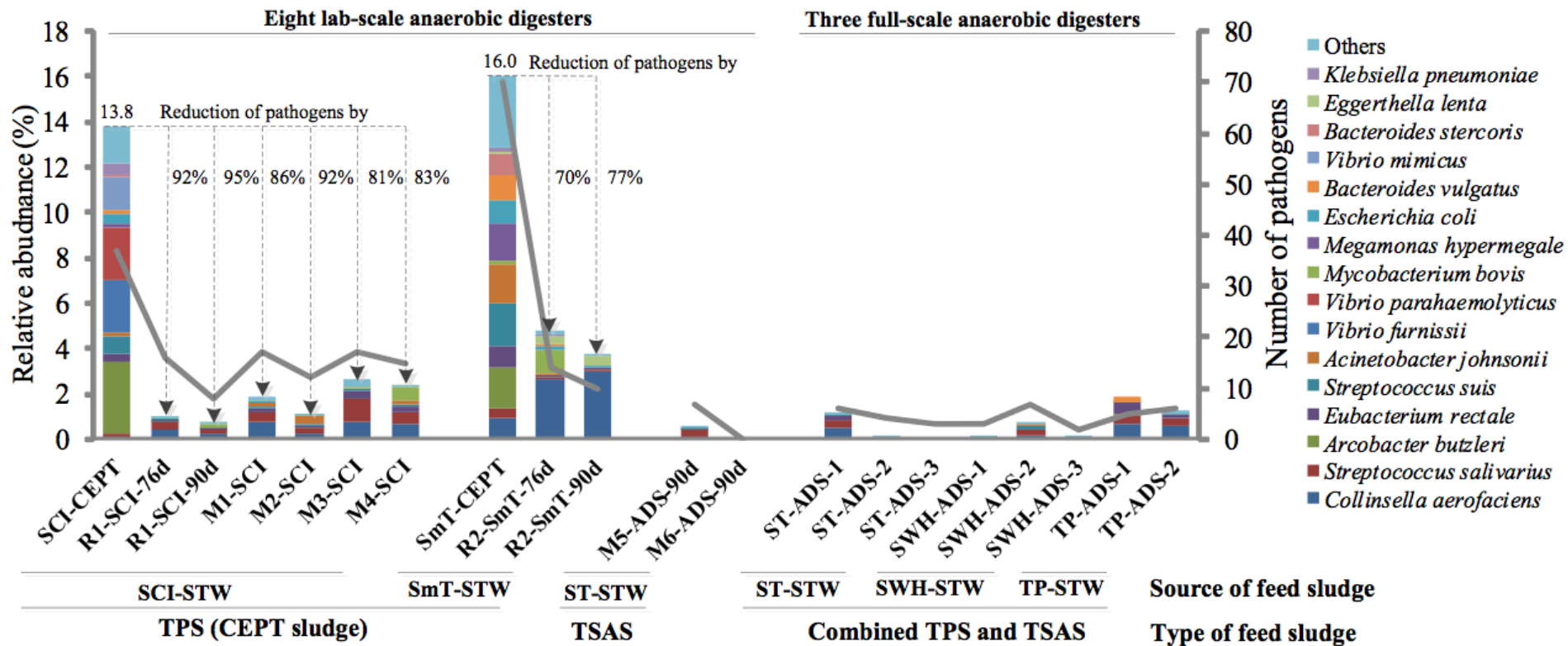
(b) Distribution of 2998 ARG reference sequences



Removal of ARGs during Anaerobic Digestion



Elimination of Pathogen during Anaerobic Digestion



Environmental Biotechnology Laboratory

Department of Civil Engineering

The University of Hong Kong

Grants:

GRF and ITF of Hong Kong

Hong Kong DSD

Group members

(working on
Anaerobic Projects)

- Ju Feng
- Wang Yubo
- Xia Yu
- Huang Danping
- Wang Yulin





**Thank
You**

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