From living cells to stable isotopes:

An interdisciplinary approach for unravelling microbial interactions in ammonia-overloaded anaerobic digesters

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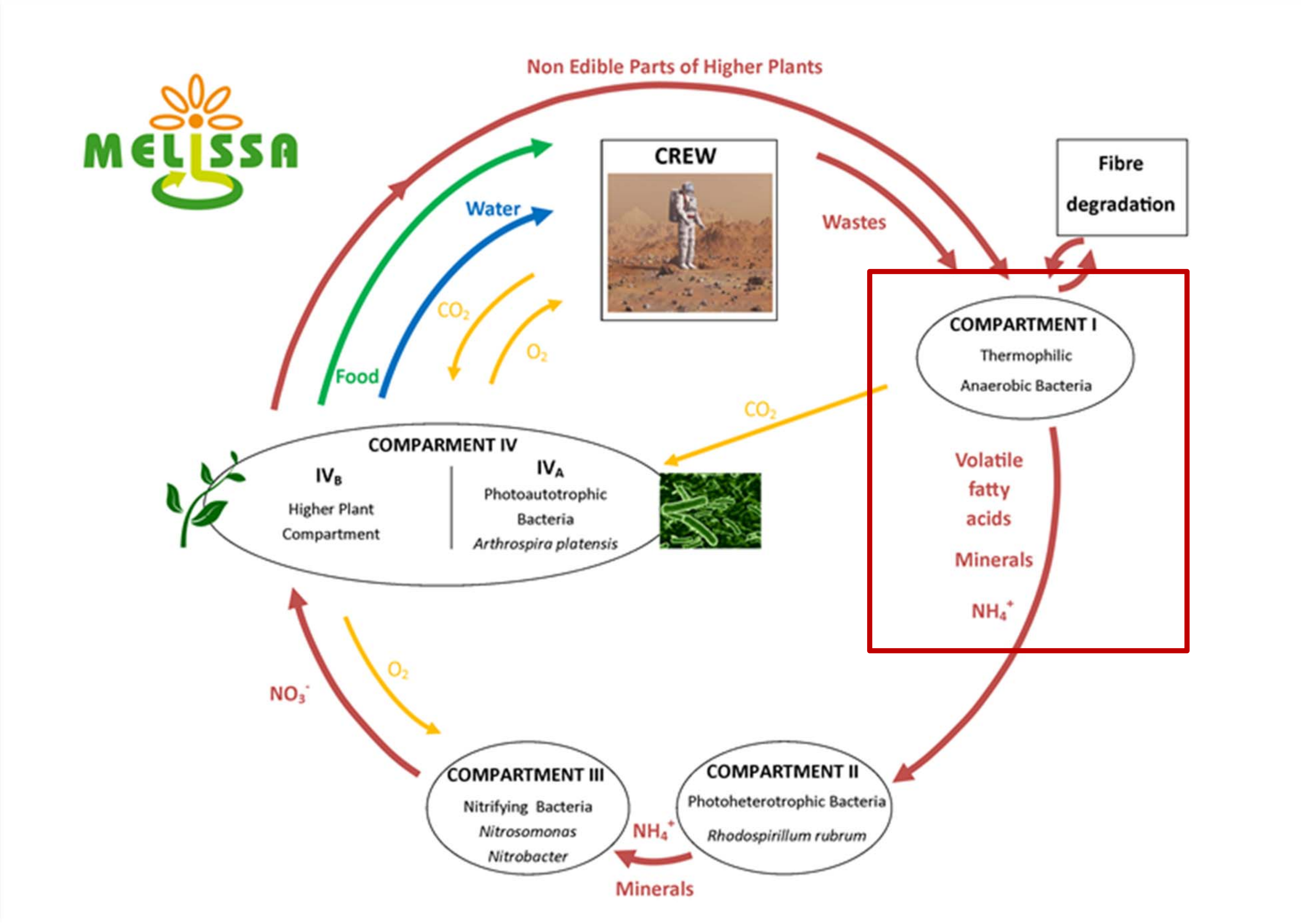
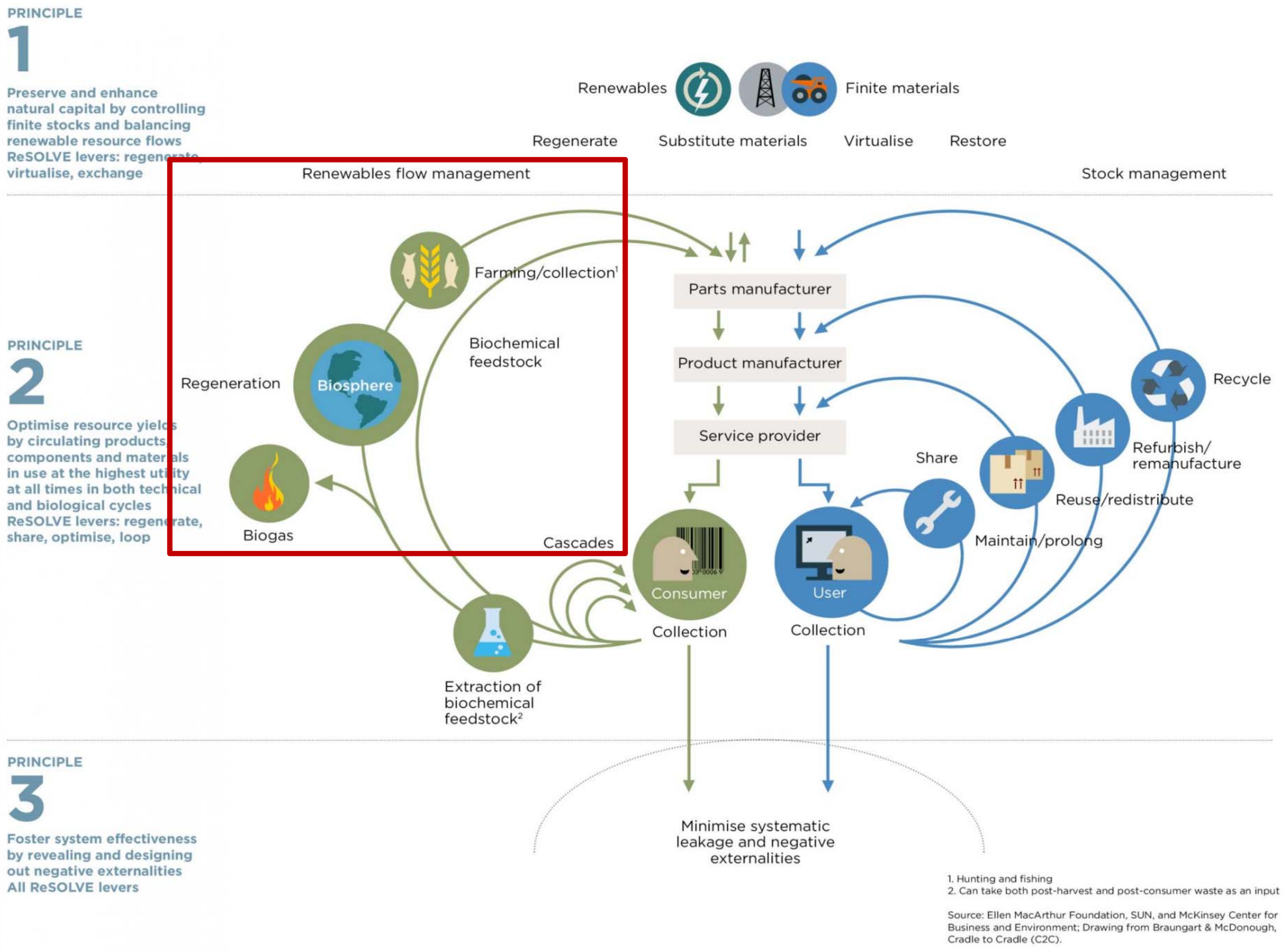
Closing loops in 'Micro Ecological Life Support System Alternative'



Closing loops in circular economy



PRINCIPLE 1

Preserve and enhance natural capital by controlling finite stocks and balancing renewable resource flows
ReSOLVE levers: regenerate, virtualise, exchange

PRINCIPLE 2

Optimise resource yields by circulating products, components and materials in use at the highest utility at all times in both technical and biological cycles
ReSOLVE levers: regenerate, share, optimise, loop

PRINCIPLE 3

Foster system effectiveness by revealing and designing out negative externalities
All ReSOLVE levers

[1] Hunting and fishing
[2] Can take both post-harvest and post-consumer waste as an input

Source: Ellen MacArthur Foundation, SUN, and McKinsey Center for Business and Environment; Drawing from Braungart & McDonough, Cradle to Cradle (C2C).

Nitrogen fate and toxicity in anaerobic digesters

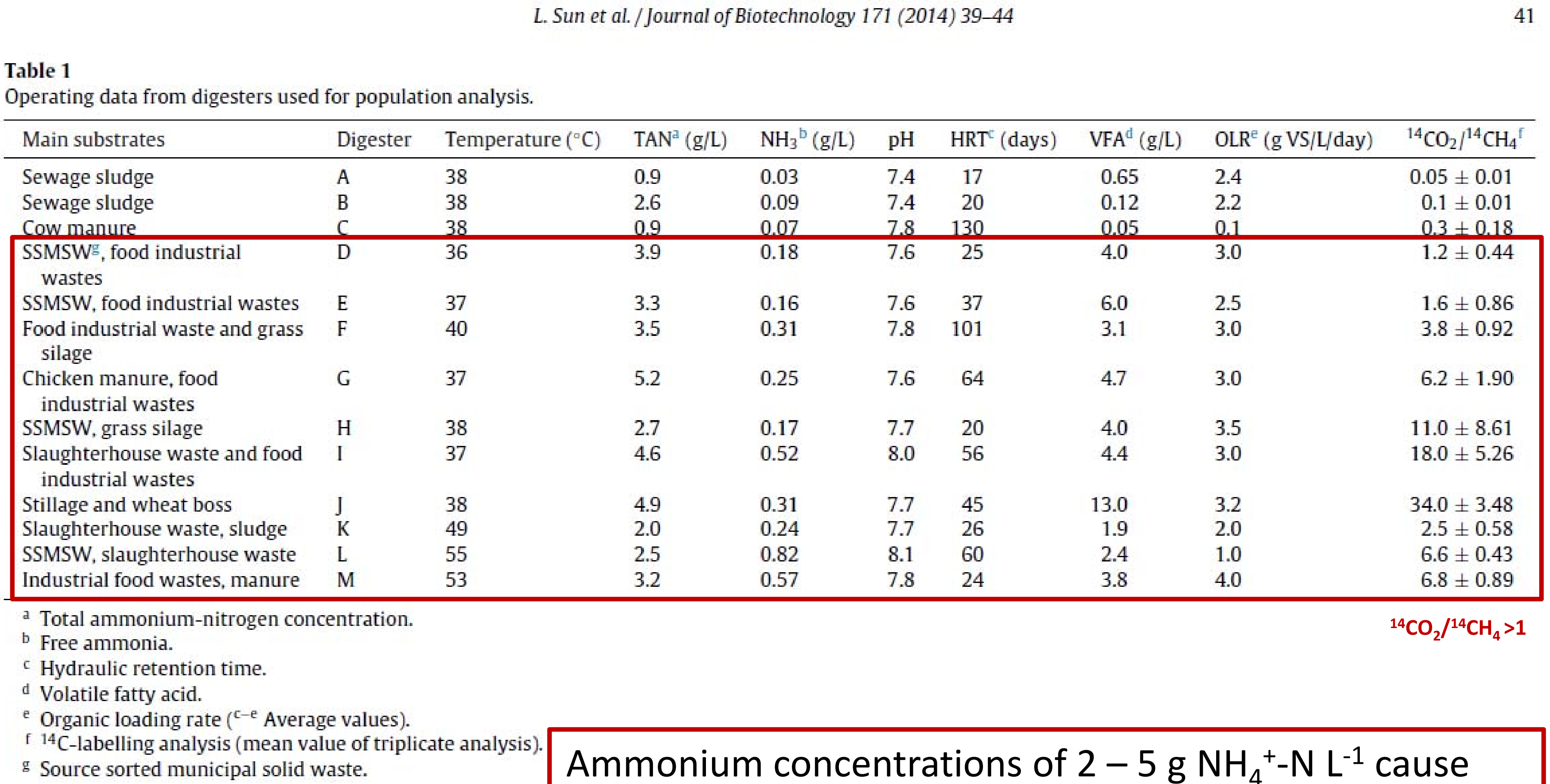


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Table 1
Operating data from digesters used for population analysis.

Main substrates	Digester	Temperature (°C)	TAN[a] (g/L)	NH_3[b] (g/L)	pH	HRT[c] (days)	VFA[d] (g/L)	OLR[e] (g VS/L/day)	$^{14}CO_2/^{14}CH_4$[f]
Sewage sludge	A	38	0.9	0.03	7.4	17	0.65	2.4	0.05 ± 0.01
Sewage sludge	B	38	2.6	0.09	7.4	20	0.12	2.2	0.1 ± 0.01
Cow manure	C	38	0.9	0.07	7.8	130	0.05	0.1	0.3 ± 0.18
SSMSW[g], food industrial wastes	D	36	3.9	0.18	7.6	25	4.0	3.0	1.2 ± 0.44
SSMSW, food industrial wastes	E	37	3.3	0.16	7.6	37	6.0	2.5	1.6 ± 0.86
Food industrial waste and grass silage	F	40	3.5	0.31	7.8	101	3.1	3.0	3.8 ± 0.92
Chicken manure, food industrial wastes	G	37	5.2	0.25	7.6	64	4.7	3.0	6.2 ± 1.90
SSMSW, grass silage	H	38	2.7	0.17	7.7	20	4.0	3.5	11.0 ± 8.61
Slaughterhouse waste and food industrial wastes	I	37	4.6	0.52	8.0	56	4.4	3.0	18.0 ± 5.26
Stillage and wheat boss	J	38	4.9	0.31	7.7	45	13.0	3.2	34.0 ± 3.48
Slaughterhouse waste, sludge	K	49	2.0	0.24	7.7	26	1.9	2.0	2.5 ± 0.58
SSMSW, slaughterhouse waste	L	55	2.5	0.82	8.1	60	2.4	1.0	6.6 ± 0.43
Industrial food wastes, manure	M	53	3.2	0.57	7.8	24	3.8	4.0	6.8 ± 0.89

[a] Total ammonium-nitrogen concentration.
[b] Free ammonia.
[c] Hydraulic retention time.
[d] Volatile fatty acid.
[e] Organic loading rate ([c–e] Average values).
[f] ^{14}C-labelling analysis (mean value of triplicate analysis).
[g] Source sorted municipal solid waste.

$^{14}CO_2/^{14}CH_4>1$

Ammonium concentrations of 2 – 5 g NH_4^+-N L^{-1} cause significant inhibition of methanogenesis, depending on T°, and pH conditions (free ammonia is the toxic species)

(Angelidaki & Ahring 1994, Sung & Liu 2003)

The syntrophic acetate oxydation

SAOB are homoacetogenic bacteria that can reverse the Wood-Ljungdahl pathway, oxidizing acetate to CO_2 and H_2 , which are further metabolized by hydrogenotrophic archaea (SAOA)

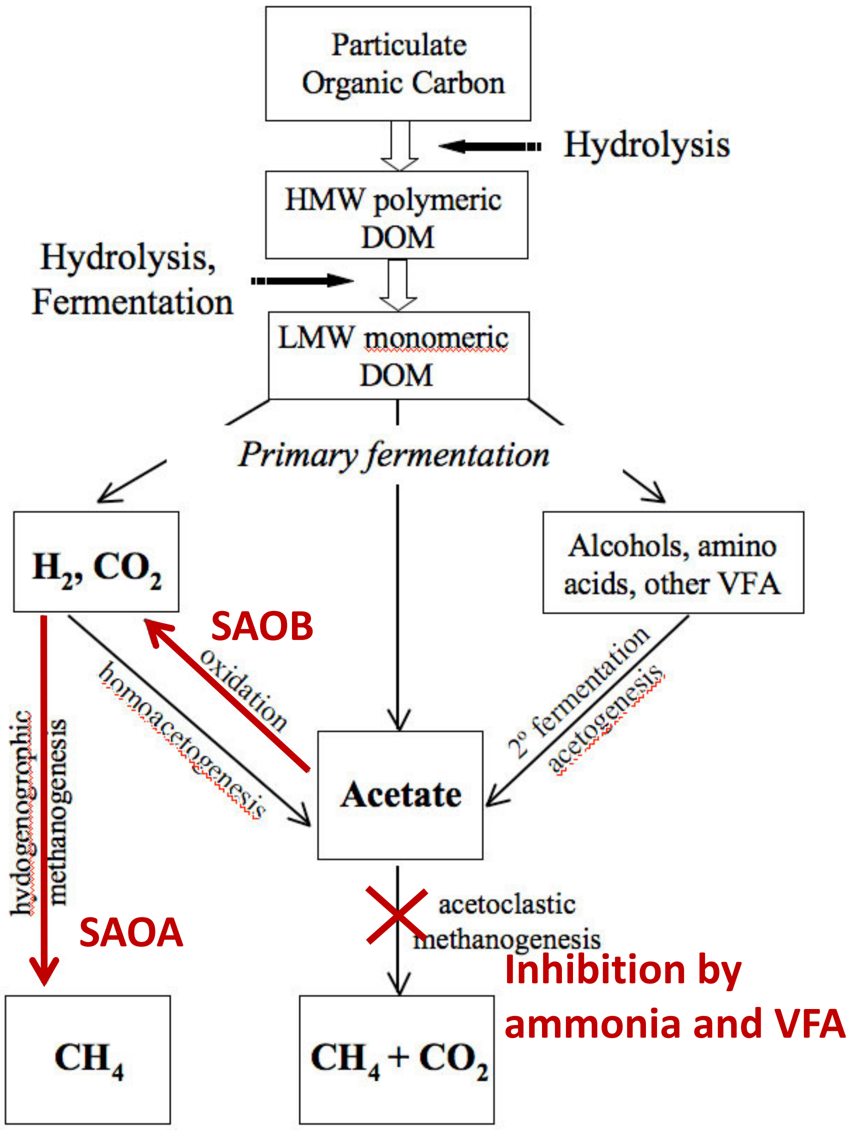
SAOB have relatively low growing rates (doubling times above 40 days)

SAOB are polyphyletic but mainly linked to the *Clostridia* class (phylum *Firmicutes*)

Knowledge on the biodiversity, ecophysiology, and biochemistry of SAOB is limited

SAOB are very important for a stable anaerobic digestion process under high ammonia concentrations

(Müller et al. 2012)



Case study: methanogenic biomass from an agricultural biogas plant

Location: Vilasana, Lleida (Spain)
Reactor type: CSTR
Volume: 1500 m^3 (2x)
HRT: 65 days
TAN: 2 – 4 gTAN L^{-1}
Operation regime: Mesophilic
Treatment capacity: 11.000 m^3 of pig slurry and 4.500 m^3 of organic residues

Batch activity assays

Treatment conditions (x3)

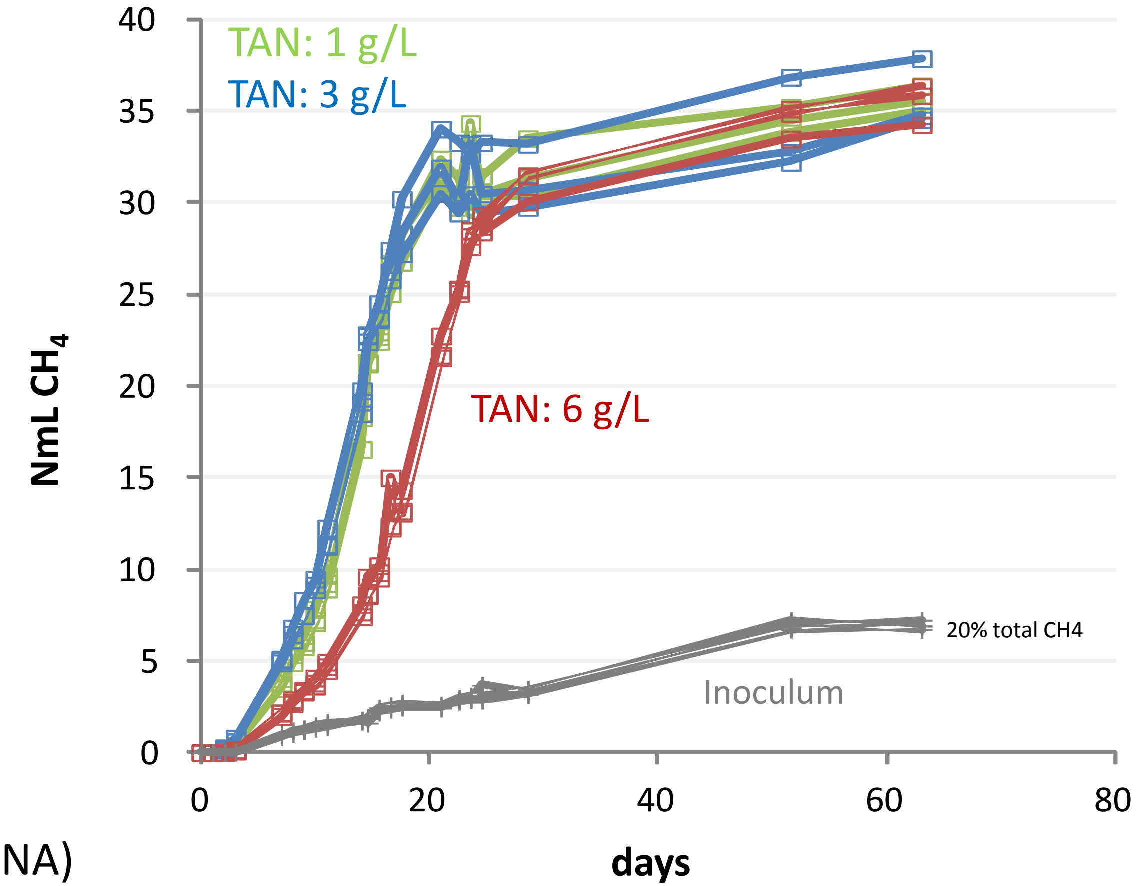
Acetic Acid: 3.5 g Ac L^{-1}

(*Acetic acid : $^{13}CH_3$-COOH)

Inoculum: 12.5 gVSS L^{-1}

Ammonia: 1, 3 and 6 gTAN L^{-1}

Incubation: 65 days at 37°C



Monitored parameters

VFA/COD

CO_2/CH_4 GC-TCD and GC-IRMS

NGS MiSeq (Eub/Arch) DNA/cDNA

qPCR 16S rRNA and mcrA (DNA/cDNA)

Compound Stable Isotopic Analysis (CSIA) of biogas: Unlabelled experiments

Apparent fractionation factor (α_c) (Conrad 2005, Conrad et al. 2009)

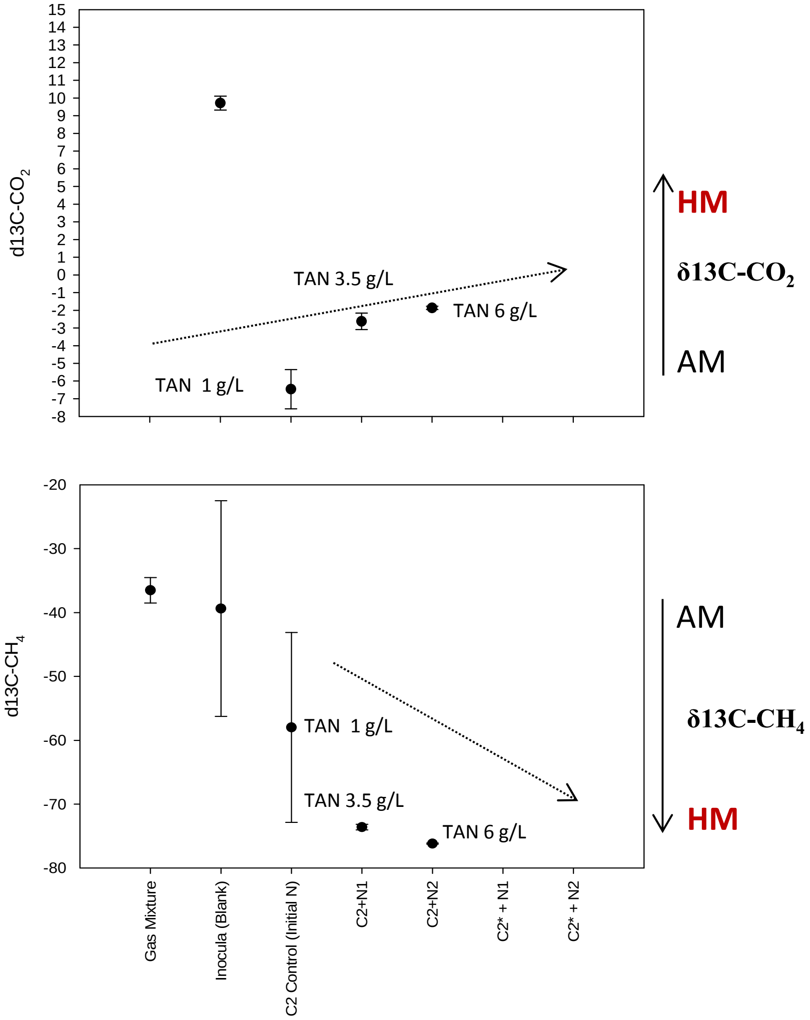
$$\boxed{\alpha_c = (\delta^{13}CO_2 + 1000)/ (\delta^{13}CH_4 + 1000)}$$

$\alpha_c < 1.055$ Dominance of acetotrophic methanogenesis
$\alpha_c > 1.065$ Dominance of hydrogenotrophic methanogenesis
$\alpha_c > 1.080$ Exclusively hydrogenotrophic methanogenesis

1gN-TAN $\cdot L^{-1}$ **α_c = 1.054** ± 0.017 **Acetotrophic**

3gN-TAN $\cdot L^{-1}$ **α_c = 1.077** ± 0.001 **Hydrogenotrophic**

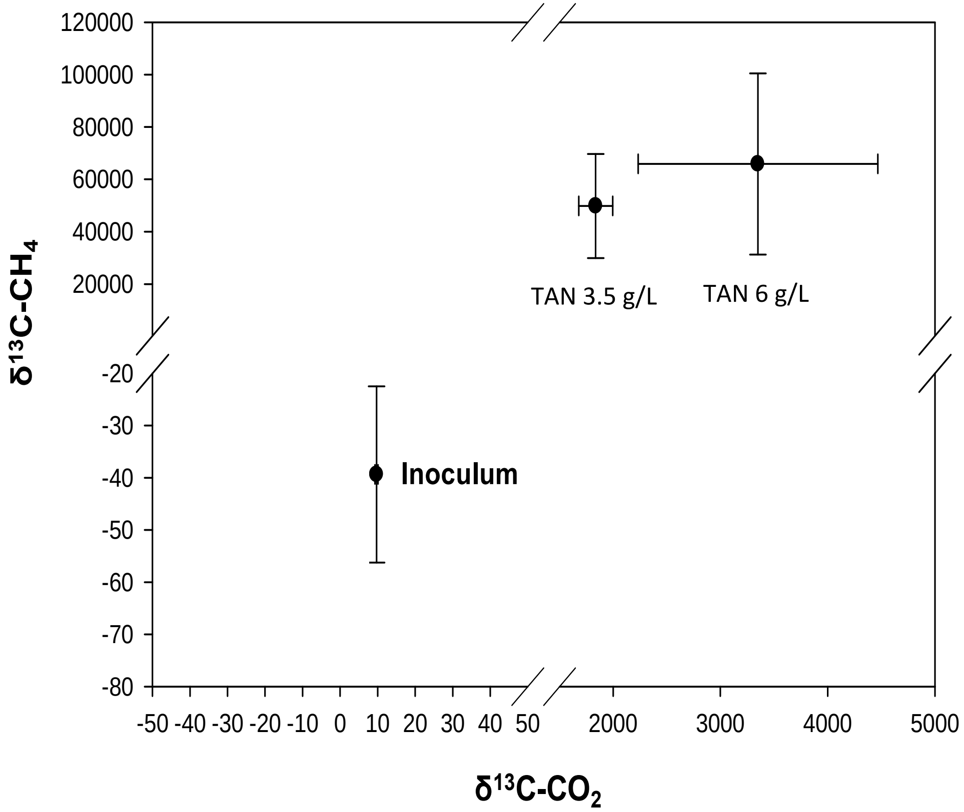
6gN-TAN $\cdot L^{-1}$ **α_c = 1.080** ± 0.001 **Hydrogenotrophic**



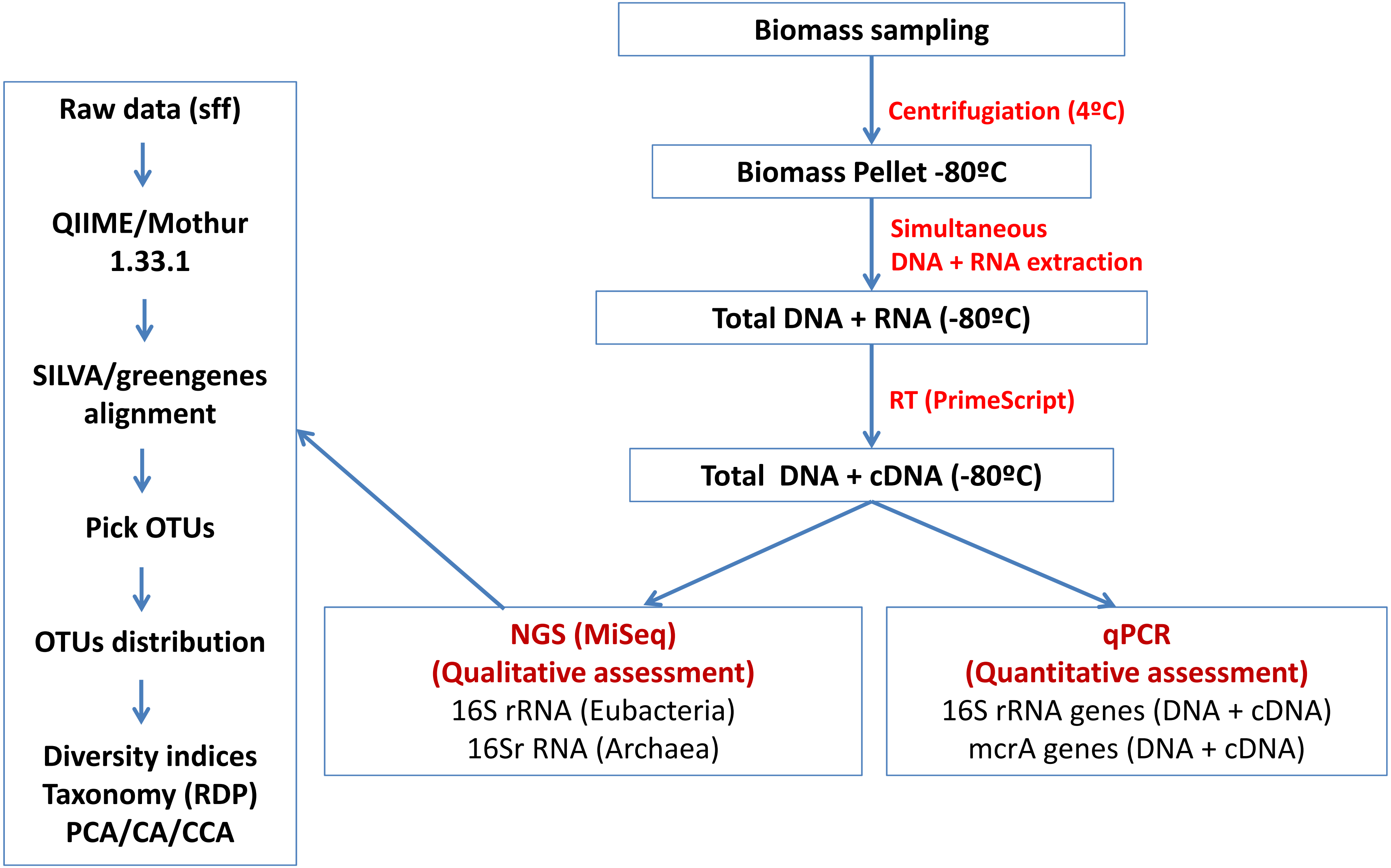
Compound Stable Isotopic Analysis (CSIA) of biogas: labelled experiments

AM $^{13}CH_3\text{-}COOH \longrightarrow {}^{13}CH_4 + CO_2$

SAO $^{13}CH_3\text{-}COOH + 2H_2O \xrightarrow[\text{SAOB}]{} {}^{13}CO_2 + CO_2 + 4H_2 \xrightarrow[\text{SAOA}]{} {}^{13}CH_4 / CH_4 + {}^{13}CO_2 / CO_2$



Microbial molecular characterization



Biomass sampling

Centrifugiation (4ºC)

Biomass Pellet -80ºC

Simultaneous
DNA + RNA extraction

Total DNA + RNA (-80ºC)

RT (PrimeScript)

Total DNA + cDNA (-80ºC)

NGS (MiSeq)
(Qualitative assessment)
16S rRNA (Eubacteria)
16Sr RNA (Archaea)

qPCR
(Quantitative assessment)
16S rRNA genes (DNA + cDNA)
mcrA genes (DNA + cDNA)

Raw data (sff)

QIIME/Mothur
1.33.1

SILVA/greengenes
alignment

Pick OTUs

OTUs distribution

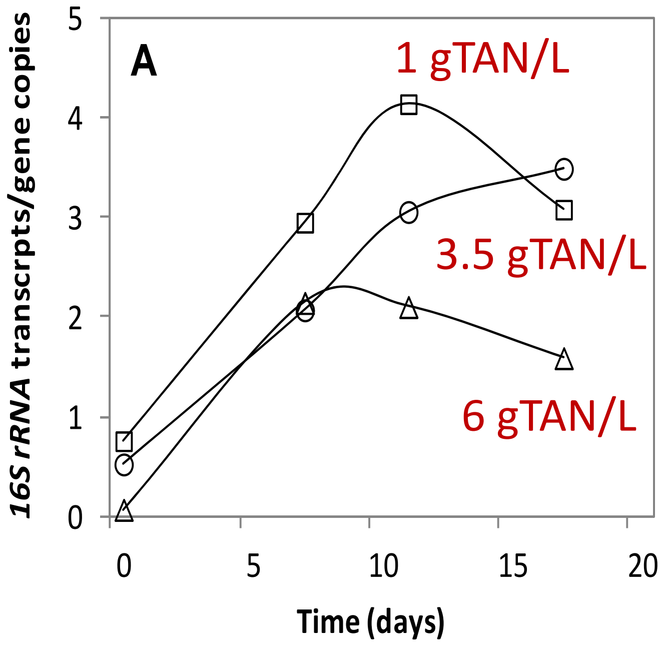
Diversity indices
Taxonomy (RDP)
PCA/CA/CCA

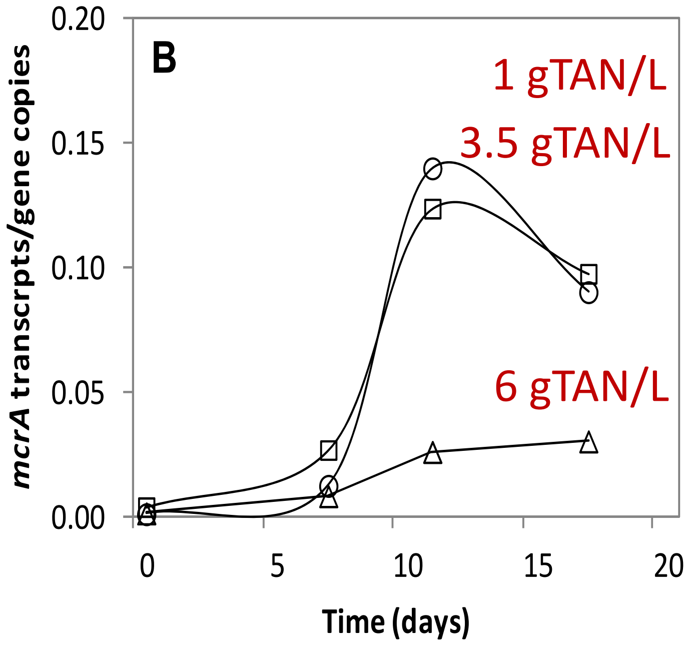
Ammonia versus transcription level (RT-qPCR)

Molecular targets:

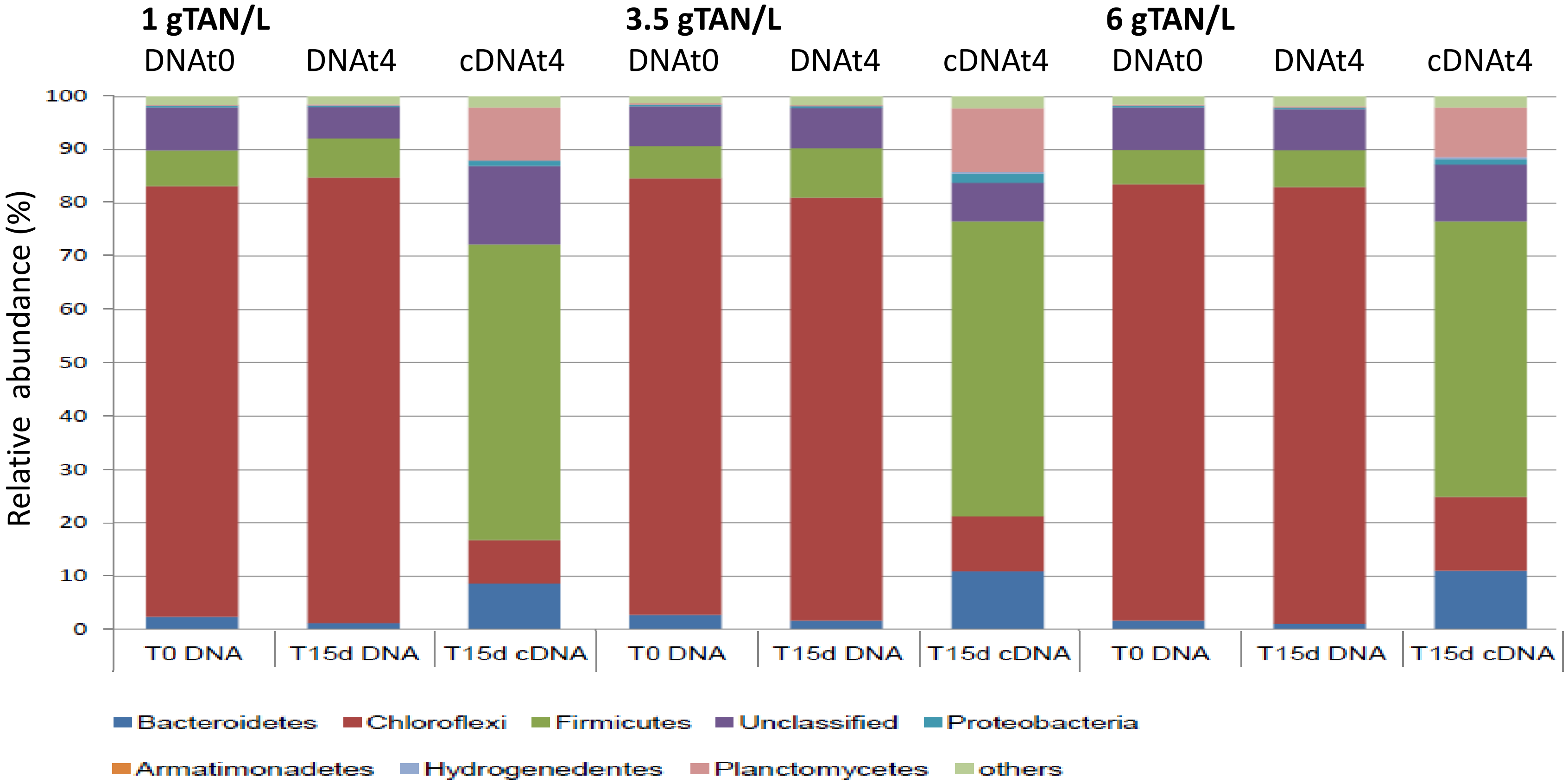
The hypervariable V3-V5 region from eubacterial *16S rRNA* genes

The functional *mcrA* gene (methyl coenzyme-M reductase) specific of methanogenic archaea


Total eubacteria (*16S rRNA*)

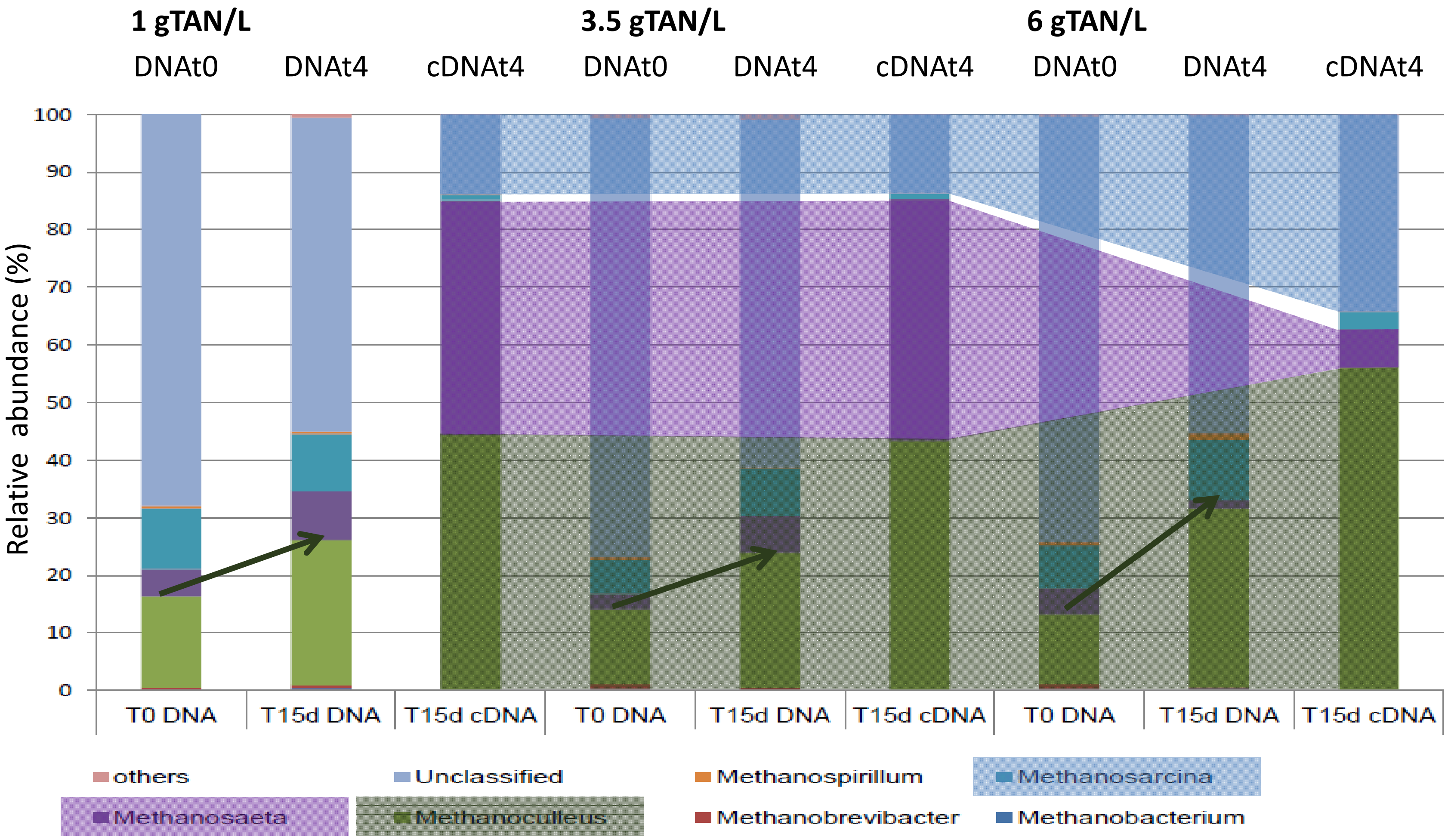

Methanogenic archaea (*mcrA*)

NGS of eubacterial *16S rRNA* libraries



Some of the identified ribotypes were somewhat homologous to the SAOB *Clostridium ultunense* and *Tepidanaerobacter acetatoxydans*

NGS of archaeal *16S rRNA* libraries



Implementation in high-rate anaerobic digesters

Enrichment of SAO biofilm on different support materials (nylon, zeolite, magnetite, steal, graphite, etc.)

Process optimization in a continuous lab-scale anaerobic filter

Conclusions

1. A diversified approach (batch methanogenic activity tests, biogas isotopic fractionation, and molecular characterization of microbial communities) demonstrated the occurrence of SAO activity in a full-scale anaerobic digester.

2. Methanogenic activity switched from predominantly acetotrophic to hydrogenotrophic (linked to SAO activity) at 3 gN-TAN L^{-1} and could be sustained up to 6 gN-TAN L^{-1}, but a slight inhibition could already be observed.

3. No significant microbial shift upon ammonia supplementation were apparent for the *Eubacteria*. Members of the phylum *Chloroflexi* were predominant, but the most active belonged to the *Firmicutes*.

4. About 5% - 10% of identified OTUs were related to homoacetogenic bacteria. Some of them were homologous to the SAOB *Clostridium ultunense* and *Tepidanaerobacter acetatoxydans*.

5. Concerning the *Archaea*, representatives of the genera *Methanoculleus* and *Methanosarcina* appear to be fundamental hydrogenotrophic syntrophic partners (SAOA).

6. Current efforts are aimed at lab-scale process implementation and optimization in high-rate anaerobic digesters based on SAO biofilms.

Acknowledgements

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