KITP Tutorial:
An Introduction to Environmental Microbiology

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Outline

- Introduction to cultivation-independent methods
- Metagenomics
- Activity assays
- Linking Function to Identity

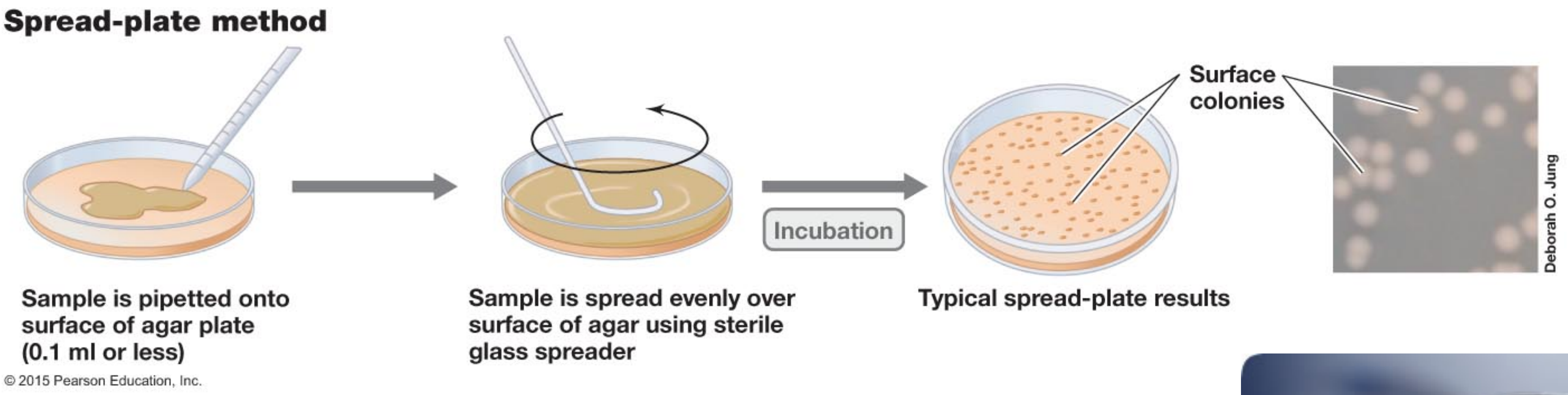
Microbial Ecology: Who, when, where and why?

Microbial Diversity

One gram of soil
>1,000,000,000 microbes
>10,000 species

People on our planet:
7,847,645,500 (22 Feb 2021)

Traditional Microbiological Analysis



Sample is pipetted onto surface of agar plate (0.1 ml or less)

Sample is spread evenly over surface of agar using sterile glass spreader

Typical spread-plate results

Majority of Prokaryotes is Unculturable

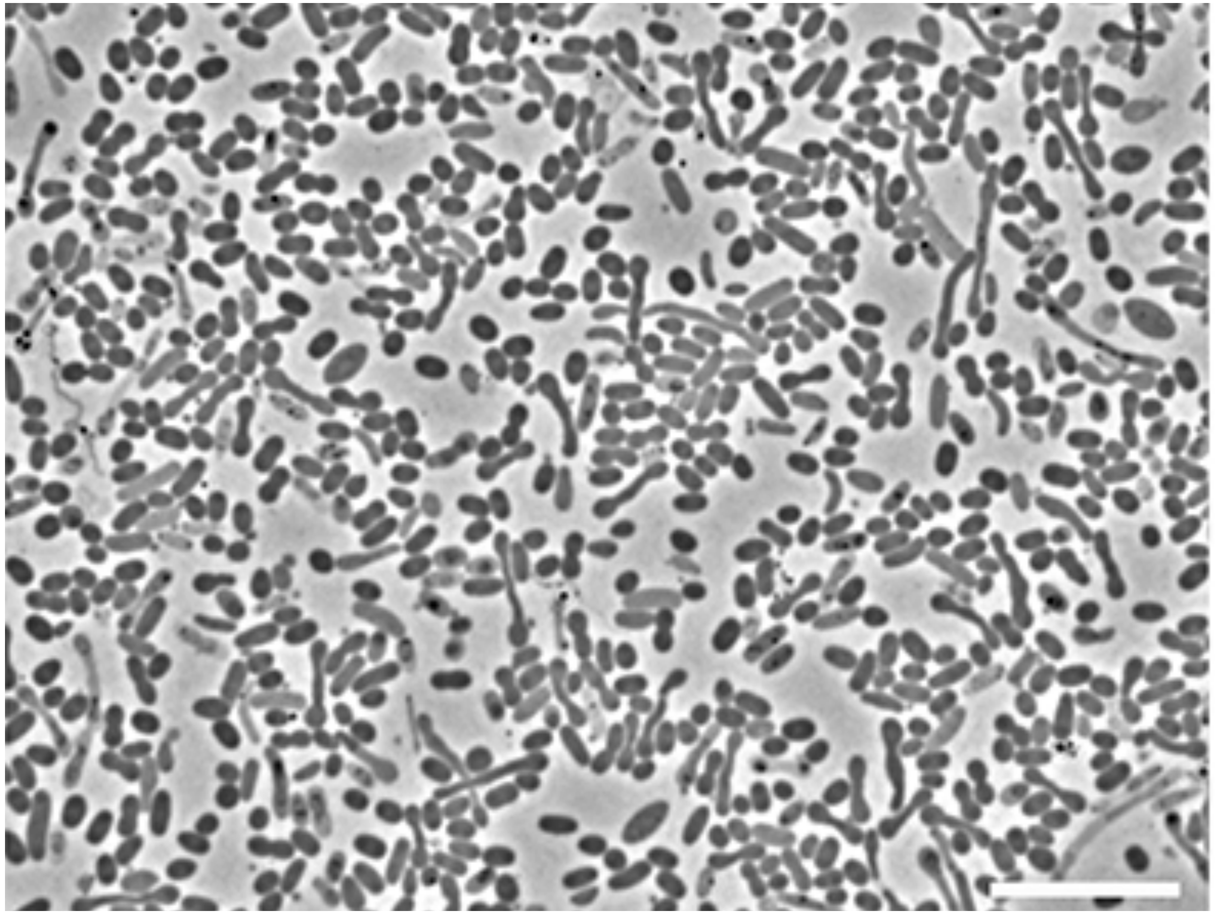
Habitat	Cultured (%)
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic lakes	0.1-1
Estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

Cultivation Introduces Bias

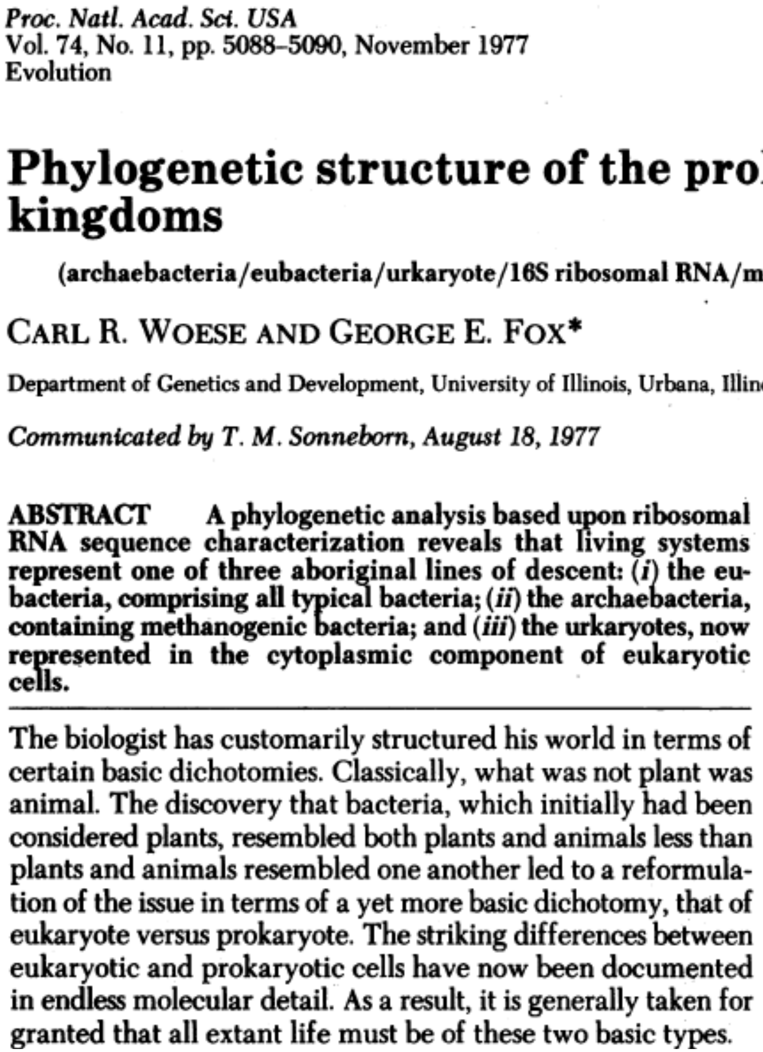
What you think you study

What you actually study

Uniform Bacterial Morphology



Molecular phylogeny



Proc. Natl. Acad. Sci. USA
Vol. 74, No. 11, pp. 5088–5090, November 1977
Evolution

Phylogenetic structure of the prokaryotic domain: The primary kingdoms

(archaebacteria/eubacteria/urkaryote/16S ribosomal RNA/molecular phylogeny)

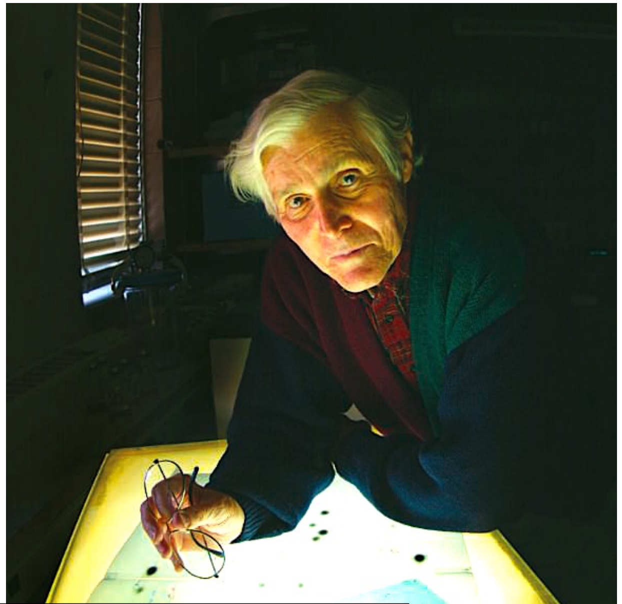
CARL R. WOESE AND GEORGE E. FOX*

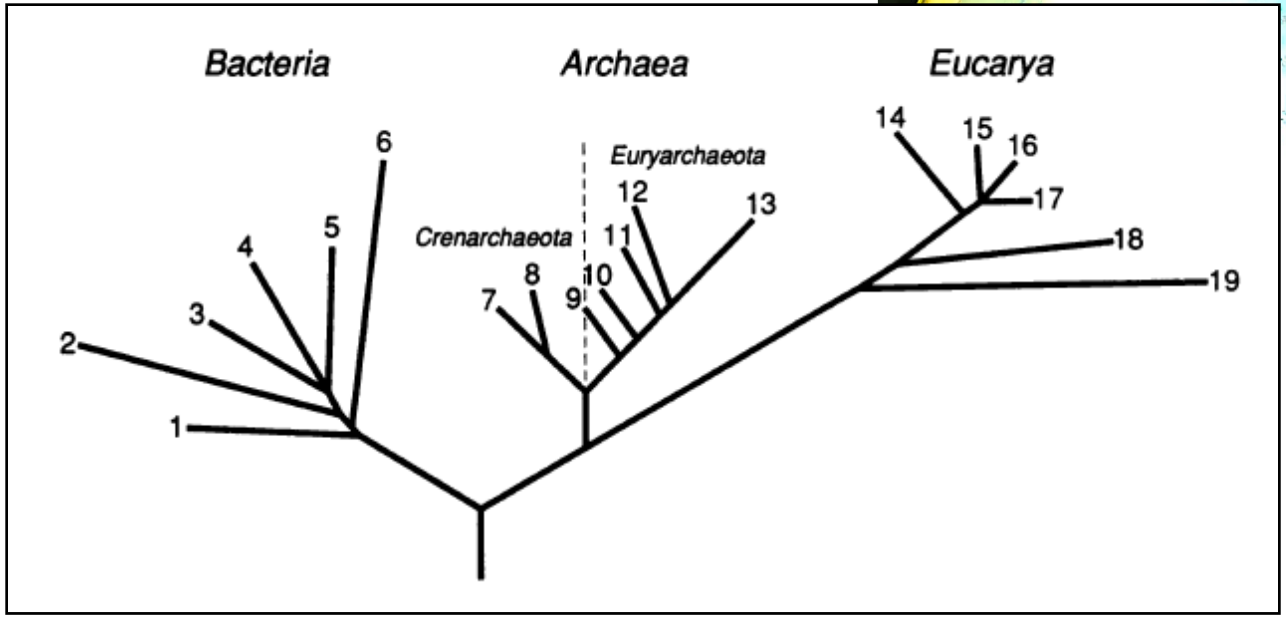
Department of Genetics and Development, University of Illinois, Urbana, Illinois 61801

Communicated by T. M. Sonneborn, August 18, 1977

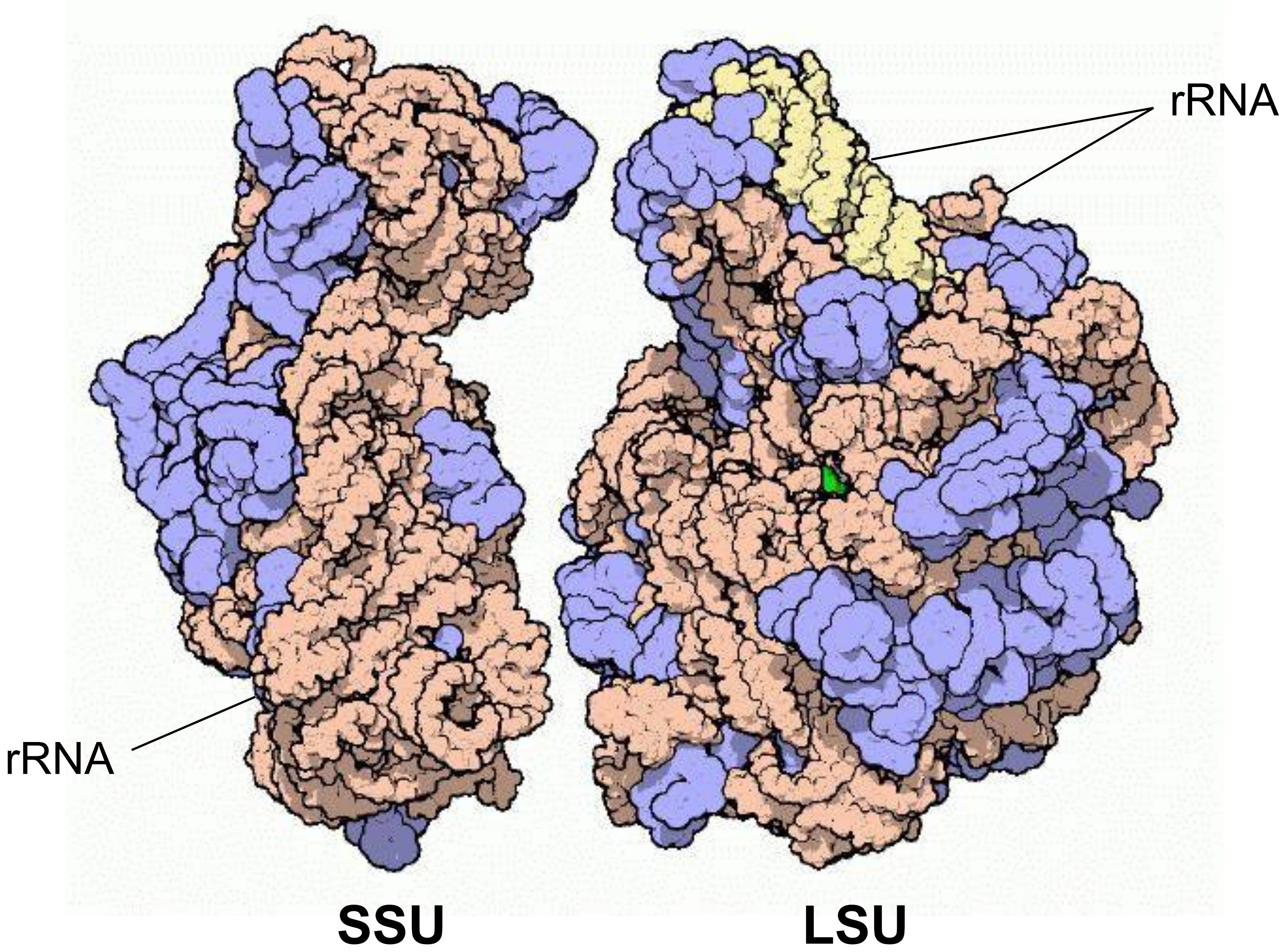
ABSTRACT A phylogenetic analysis based upon ribosomal RNA sequence characterization reveals that living systems represent one of three aboriginal lines of descent: (*i*) the eubacteria, comprising all typical bacteria; (*ii*) the archaebacteria, containing methanogenic bacteria; and (*iii*) the urkaryotes, now represented in the cytoplasmic component of eukaryotic cells.

The biologist has customarily structured his world in terms of certain basic dichotomies. Classically, what was not plant was animal. The discovery that bacteria, which initially had been considered plants, resembled both plants and animals less than plants and animals resembled one another led to a reformulation of the issue in terms of a yet more basic dichotomy, that of eukaryote versus prokaryote. The striking differences between eukaryotic and prokaryotic cells have now been documented in endless molecular detail. As a result, it is generally taken for granted that all extant life must be of these two basic types.

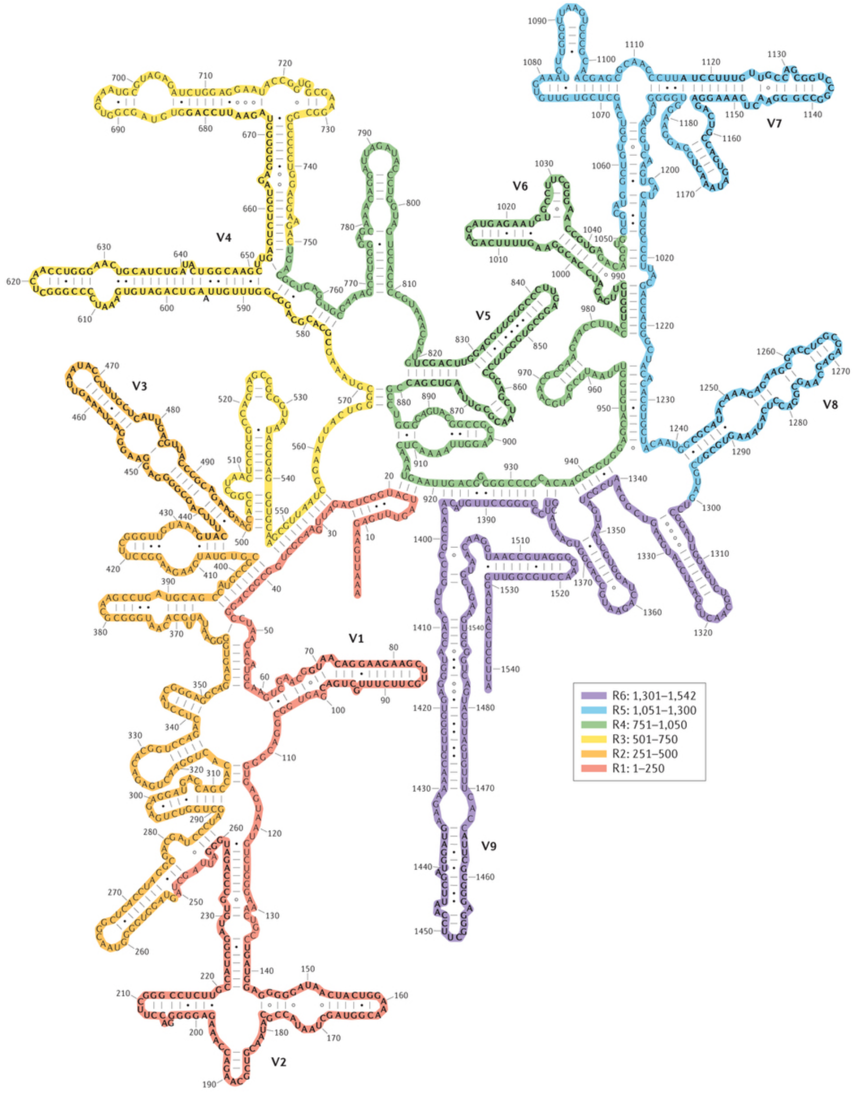




Ribosomal RNA



16S rRNA as Phylogenetic Marker

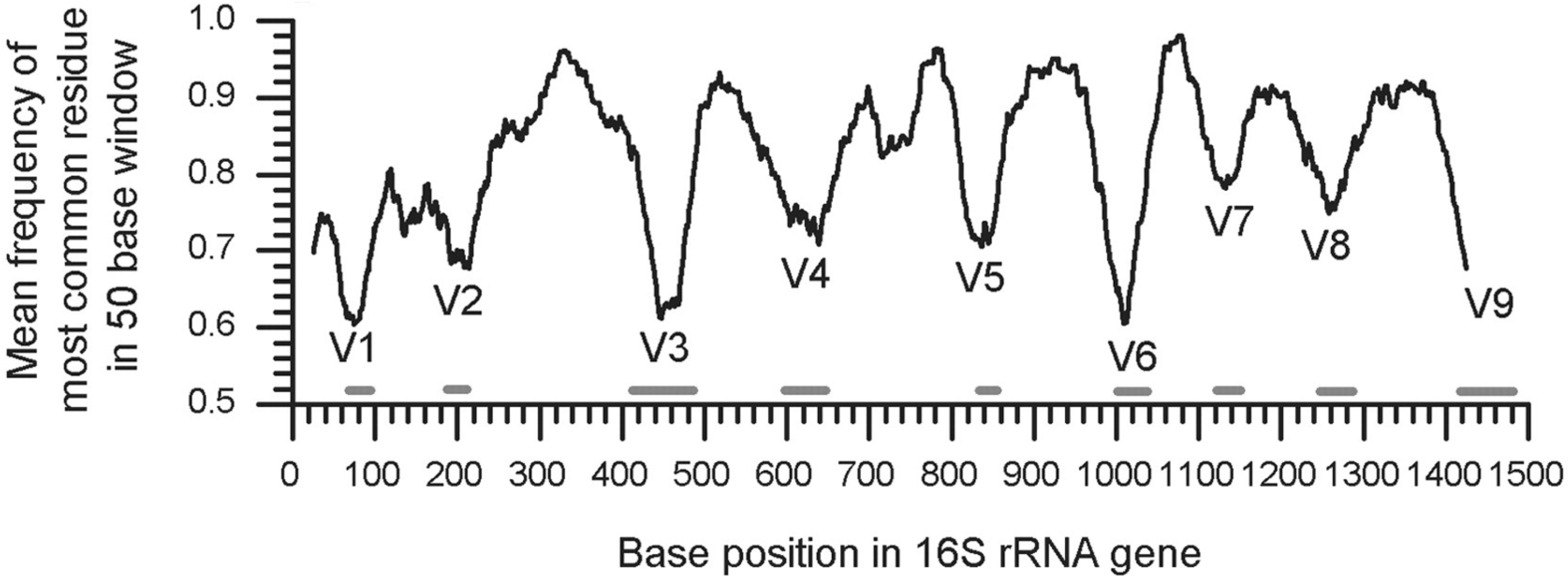


Nature Reviews | Microbiology

Advantages :

- Functionally constant
- Ubiquitous in all organisms
- High information content
- Varying sequence conservation
- Large dataset available

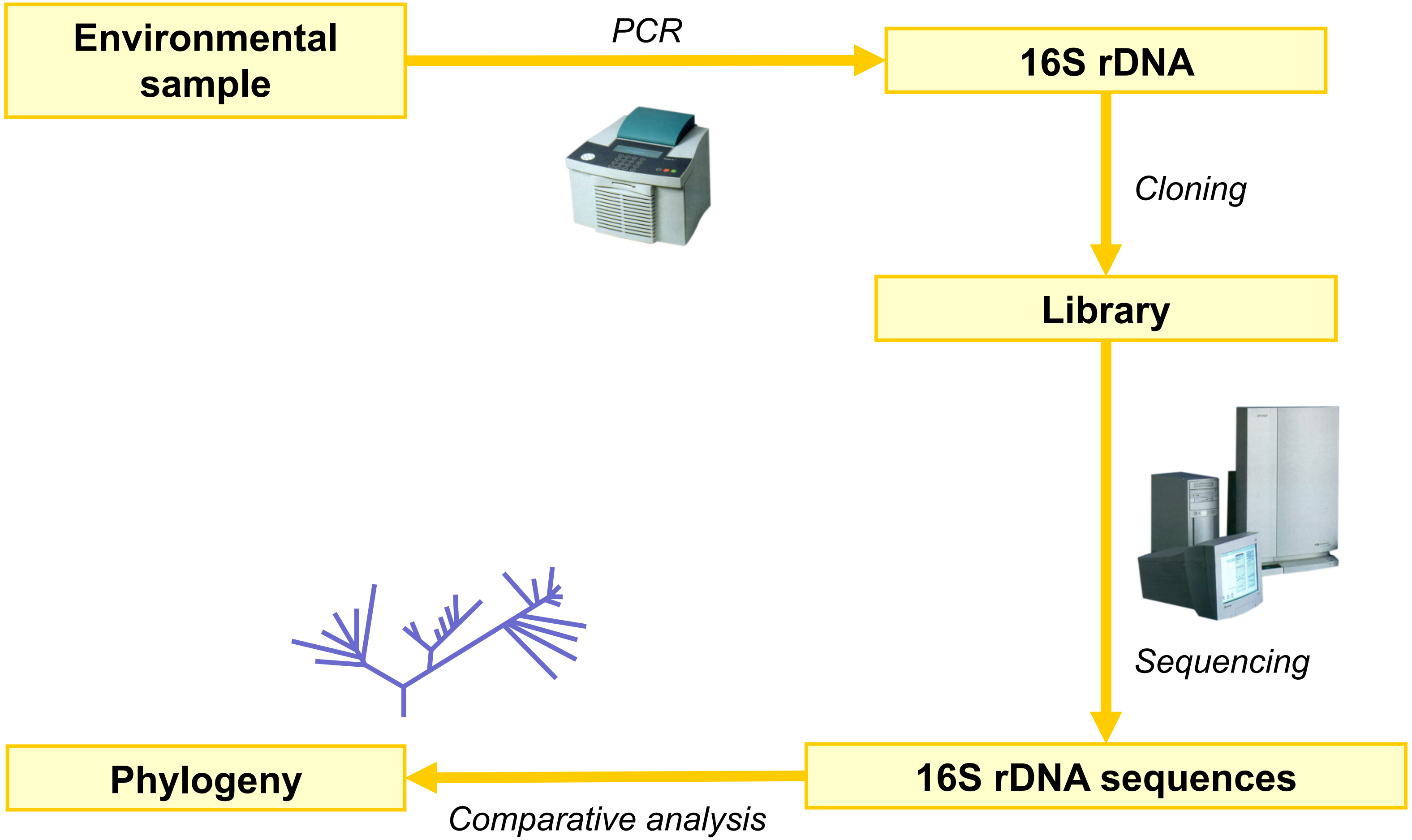
Base conservation level across the 16S rRNA



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

The rRNA Approach



Environmental sample

PCR

16S rDNA

Cloning

Library

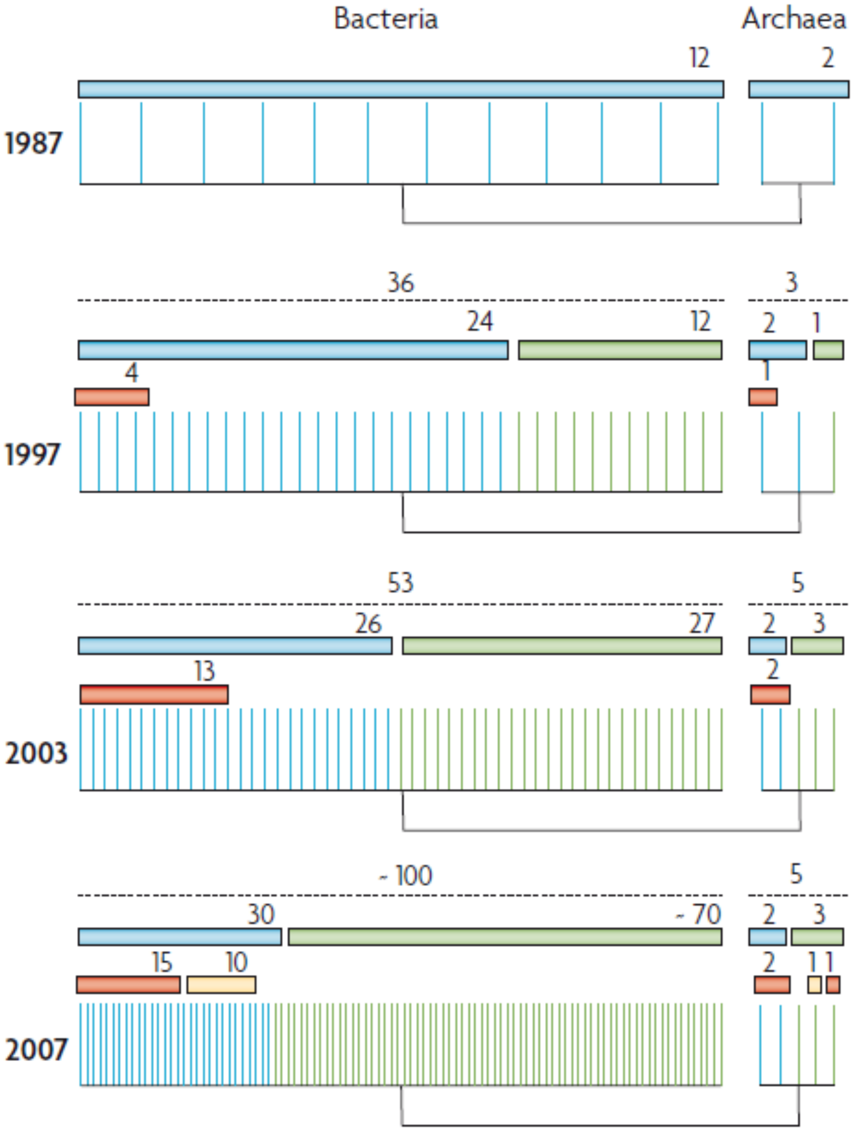
Sequencing

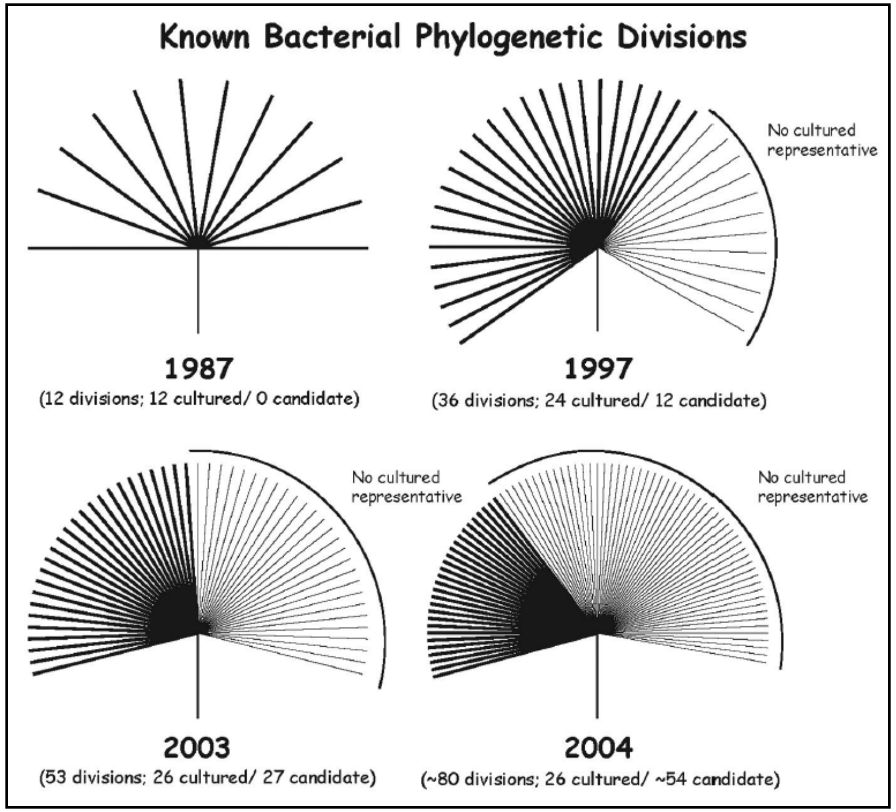
16S rDNA sequences

Comparative analysis

Phylogeny

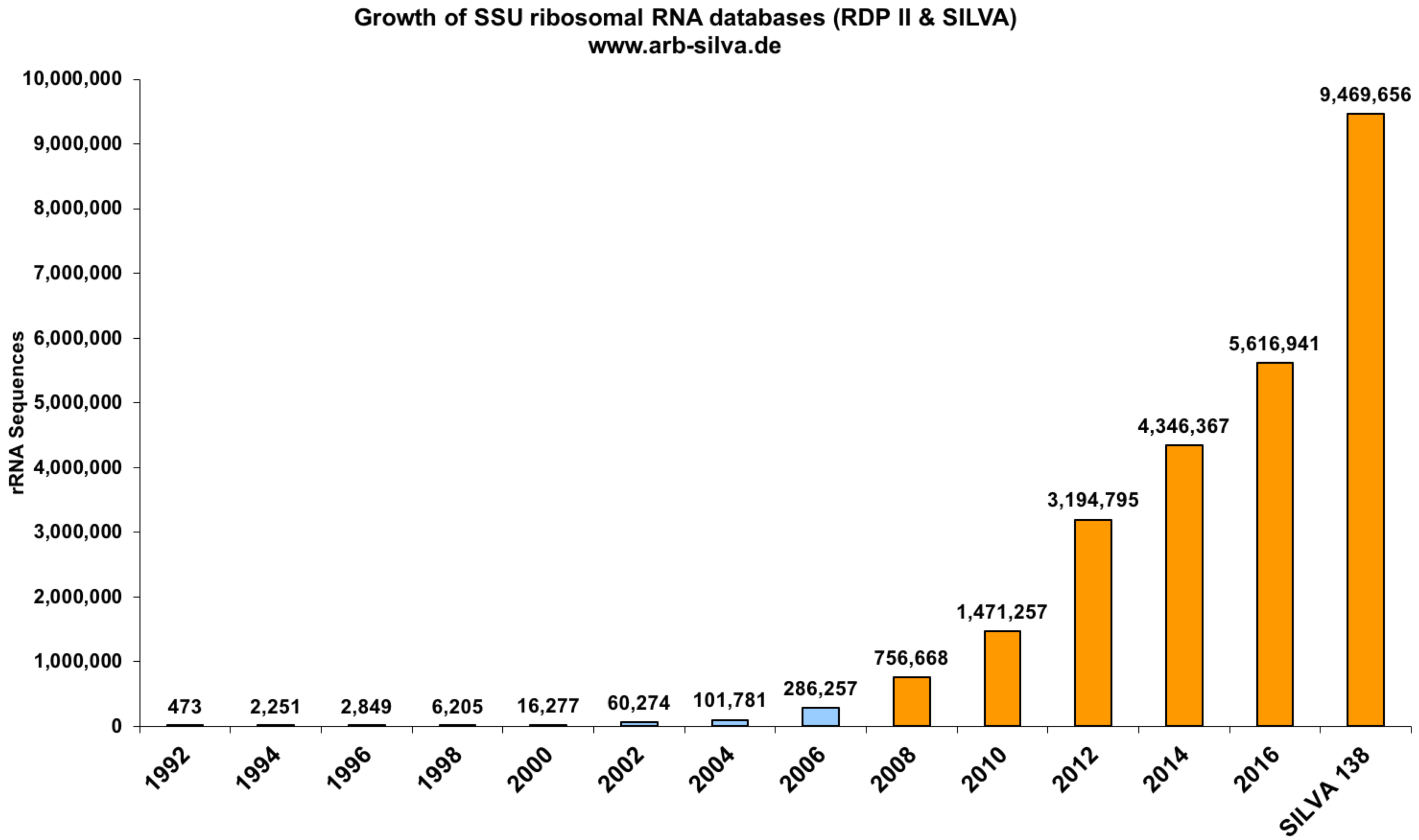
The Discovery Phase of Microbial Ecology



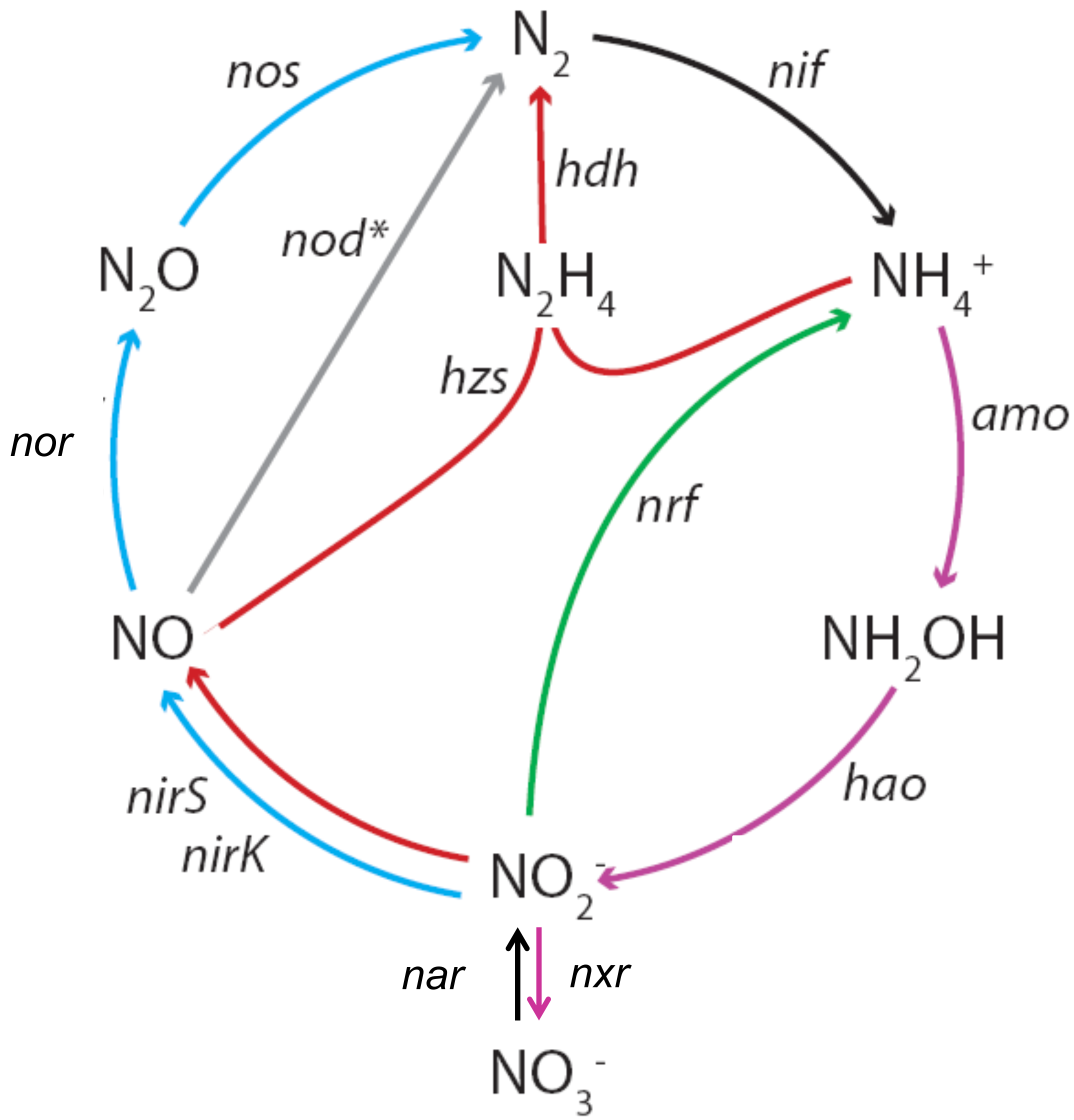


🟦 Cultivated
🟩 Uncultivated
🟥 Sequence complete
🟨 Sequence in progress

The rRNA Approach is a Success Story



Functional Marker Genes for the Nitrogen Cycle



Nitrogen fixation

nif - nitrogenase

Nitrification

amo - ammonia monooxygenase
hao - hydroxylamine oxidoreductase
nxr - nitrite reductase

Denitrification

nar - nitrate reductase
nir - nitrite reductase
nor - nitric oxide reductase
nos - nitrous oxide reductase

Anammox

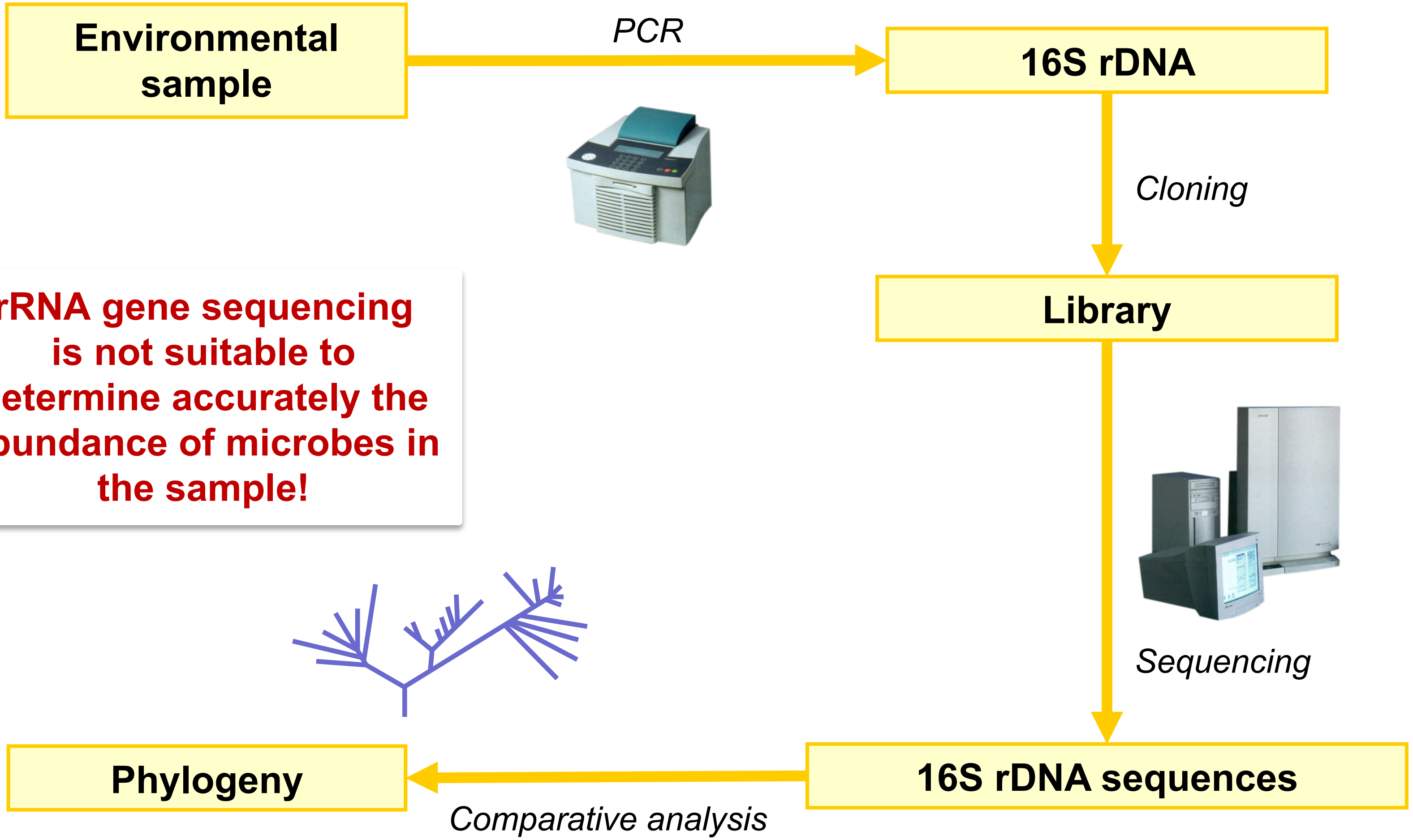
hzs - hydrazine synthase
hdh - hydrazine dehydrogenase

DNRA

nrf - pentaheme nitrite reductase

nod - NO dismutase

The rRNA Approach



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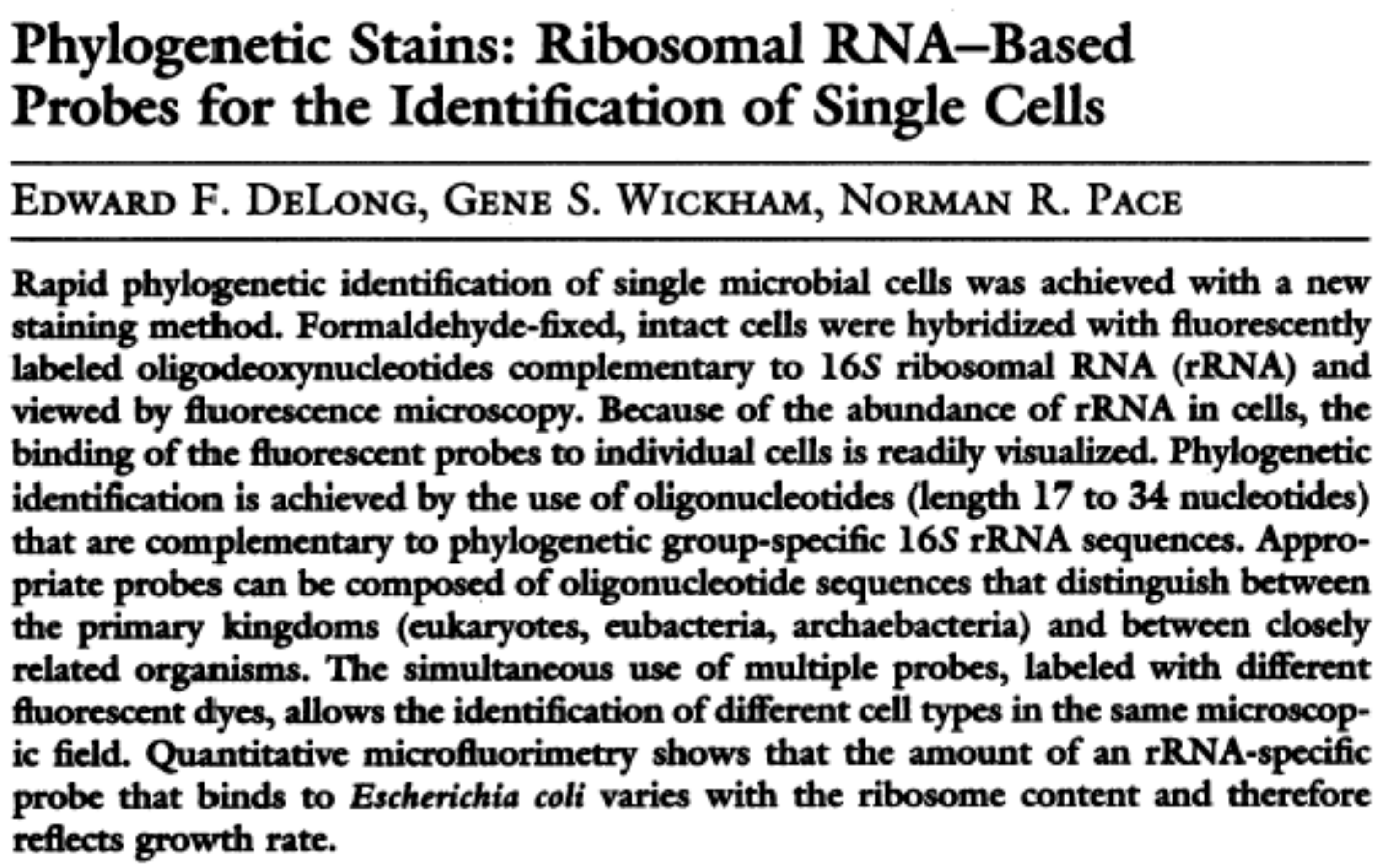
rRNA gene sequencing is not suitable to determine accurately the abundance of microbes in the sample!

Fluorescence *In Situ* Hybridization (FISH)



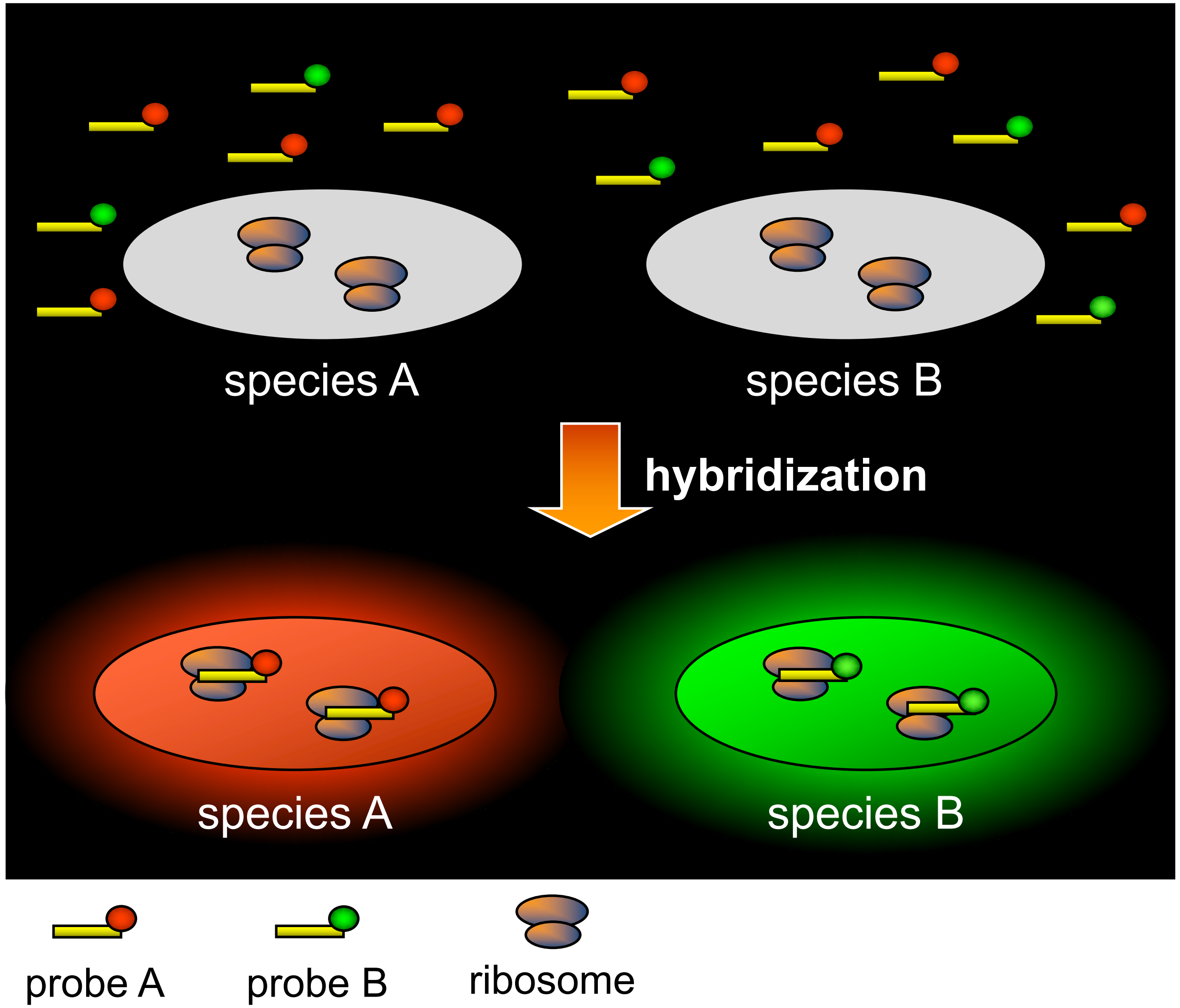
Phylogenetic Stains: Ribosomal RNA–Based Probes for the Identification of Single Cells

EDWARD F. DELONG, GENE S. WICKHAM, NORMAN R. PACE

Rapid phylogenetic identification of single microbial cells was achieved with a new staining method. Formaldehyde-fixed, intact cells were hybridized with fluorescently labeled oligodeoxynucleotides complementary to 16*S* ribosomal RNA (rRNA) and viewed by fluorescence microscopy. Because of the abundance of rRNA in cells, the binding of the fluorescent probes to individual cells is readily visualized. Phylogenetic identification is achieved by the use of oligonucleotides (length 17 to 34 nucleotides) that are complementary to phylogenetic group-specific 16*S* rRNA sequences. Appropriate probes can be composed of oligonucleotide sequences that distinguish between the primary kingdoms (eukaryotes, eubacteria, archaebacteria) and between closely related organisms. The simultaneous use of multiple probes, labeled with different fluorescent dyes, allows the identification of different cell types in the same microscopic field. Quantitative microfluorimetry shows that the amount of an rRNA-specific probe that binds to *Escherichia coli* varies with the ribosome content and therefore reflects growth rate.

Science 243:1360-3 (1989)

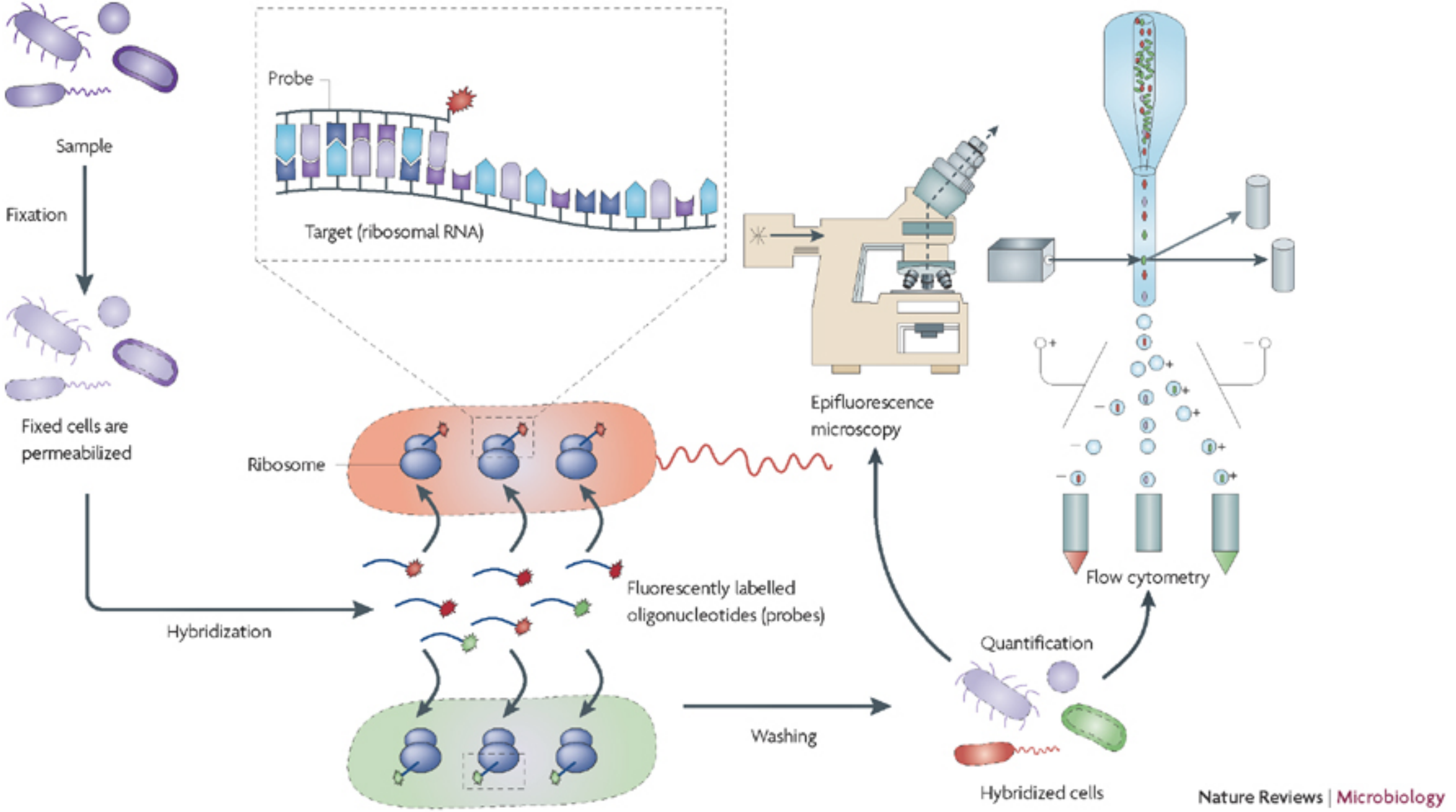
Fluorescence *In Situ* Hybridization (FISH)



All advantages of rRNA as phylogenetic marker apply.

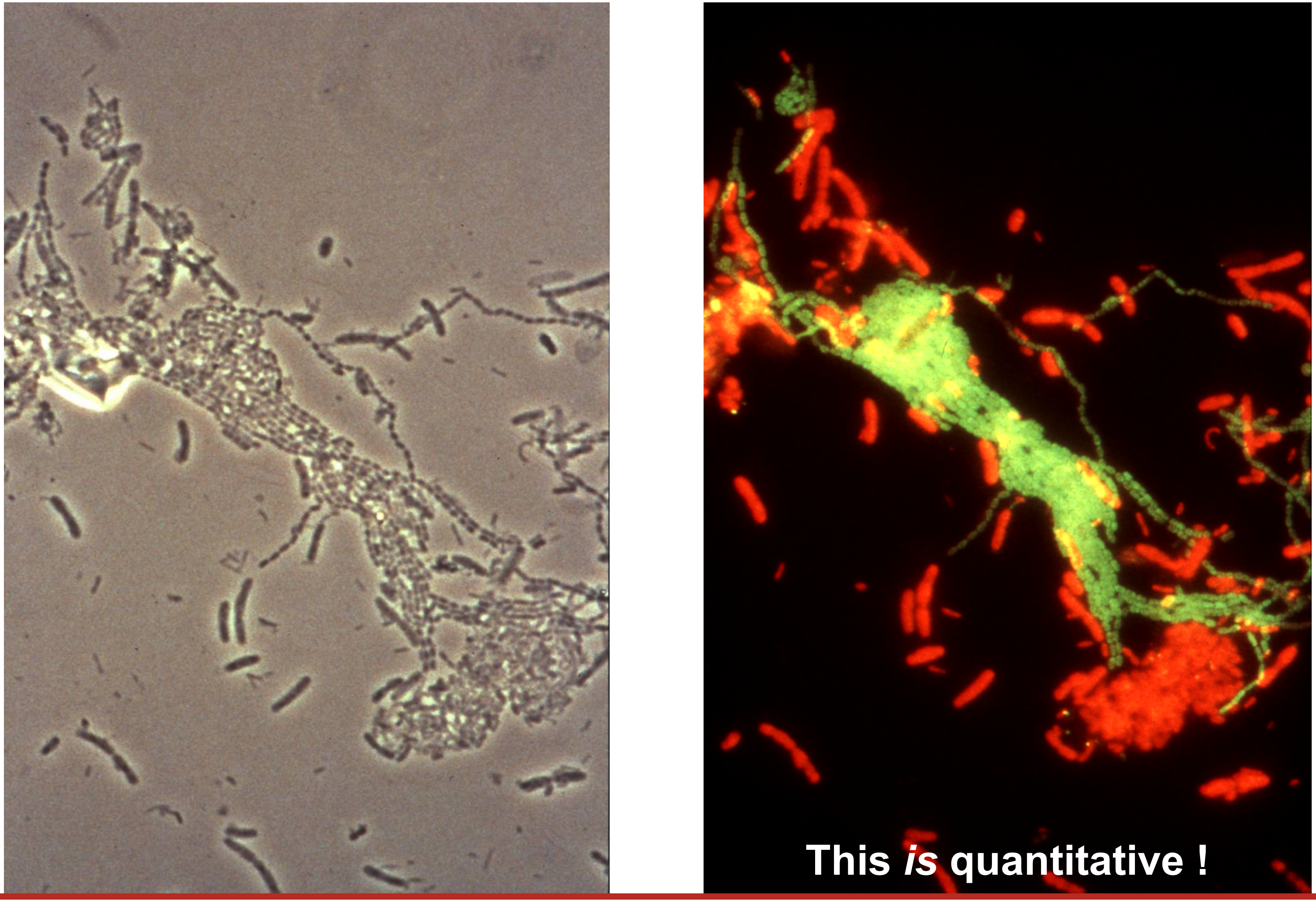
rRNA is a naturally amplified target molecule.

Fluorescence *In Situ* Hybridization (FISH)



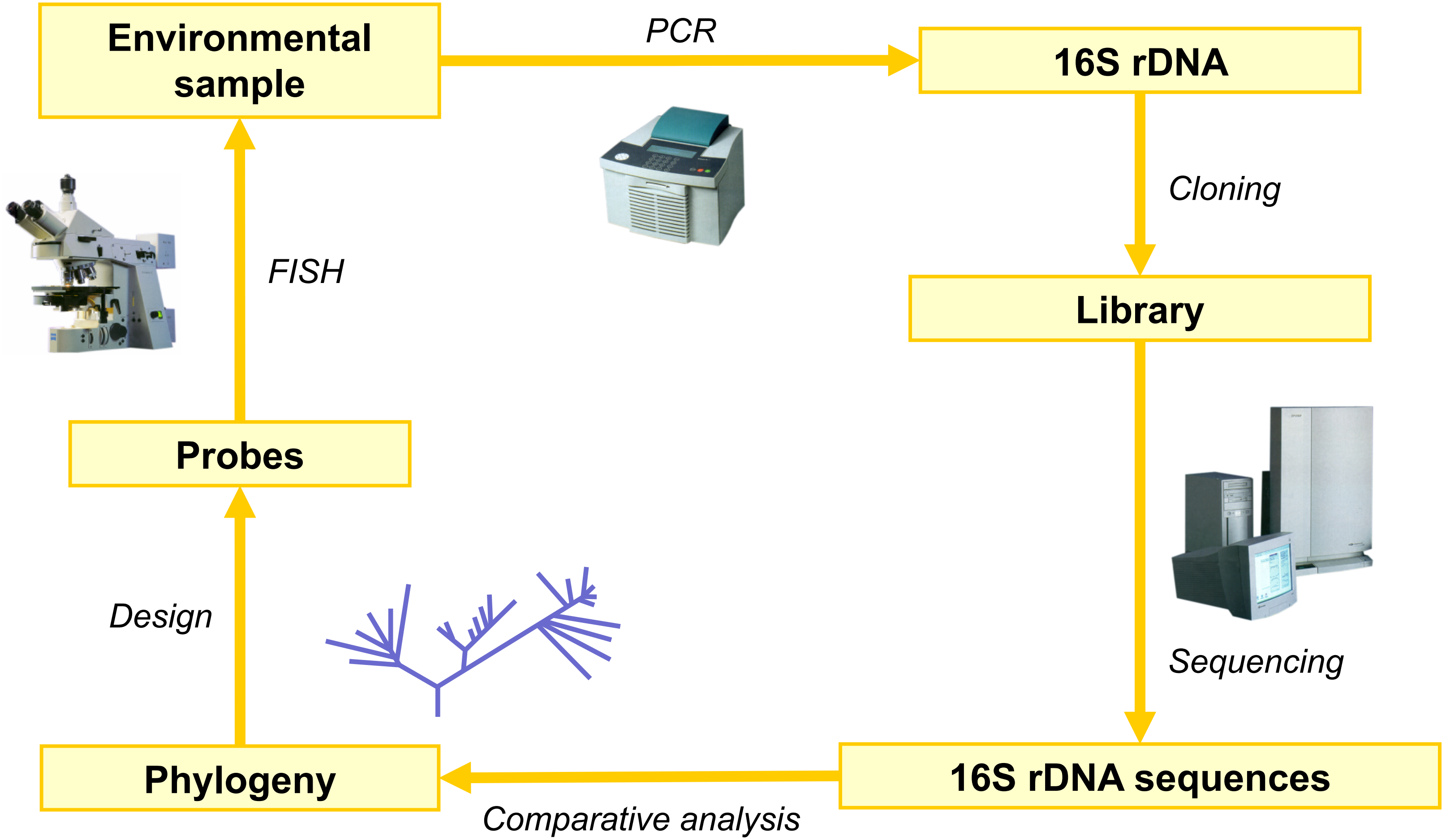
Amann & Fuchs, 2008

Fluorescence *In Situ* Hybridization (FISH)



This *is* quantitative !

The Full Cycle rRNA Approach



Environmental sample

PCR

16S rDNA

Cloning

Library

Sequencing

16S rDNA sequences

Comparative analysis

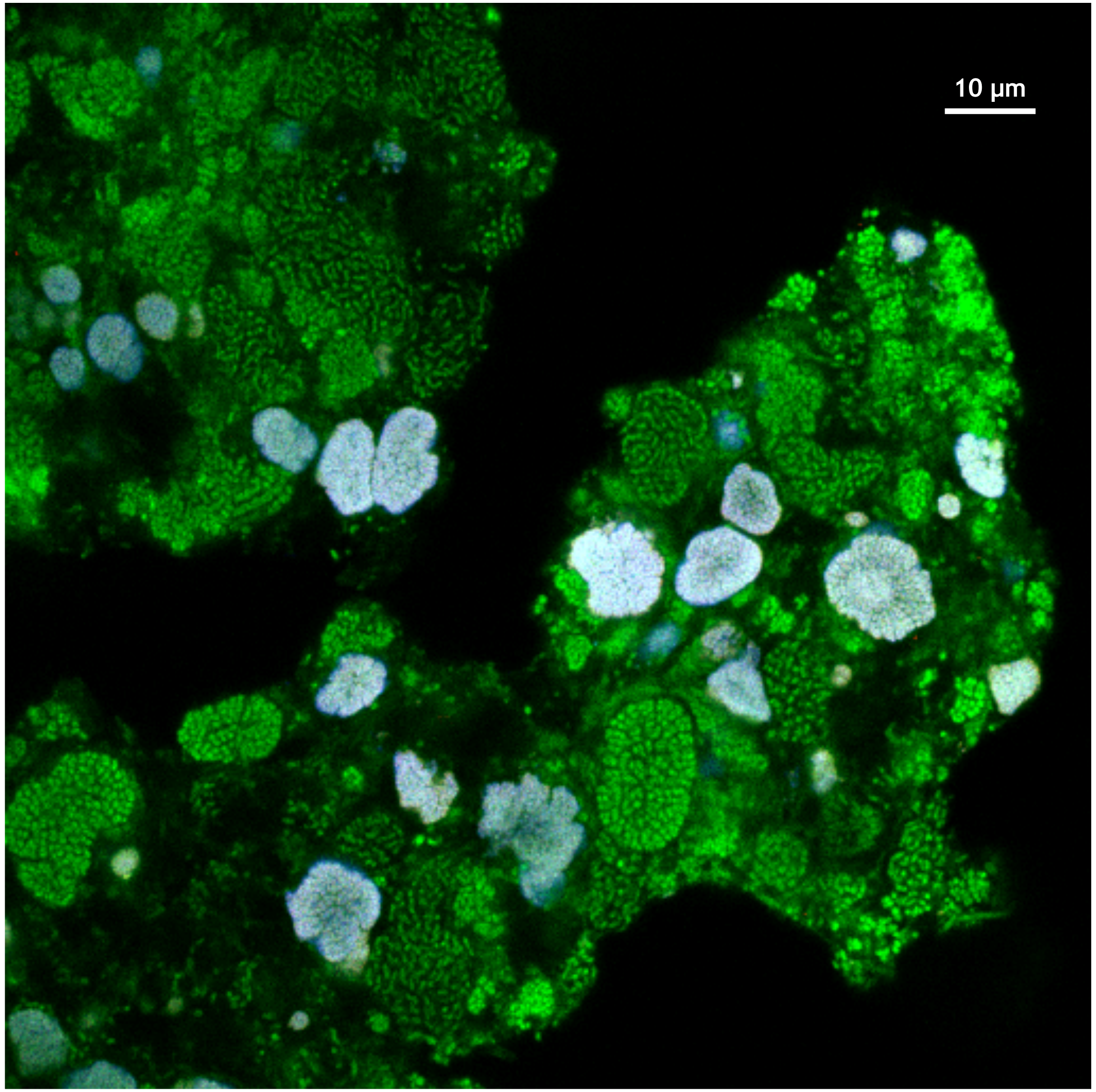
Phylogeny

Design

Probes

FISH

FISH to Study Microorganisms in Environmental Samples



***Nitrospira*-like bacteria in nitrifying biofilm**

Oligonucleotide probes:

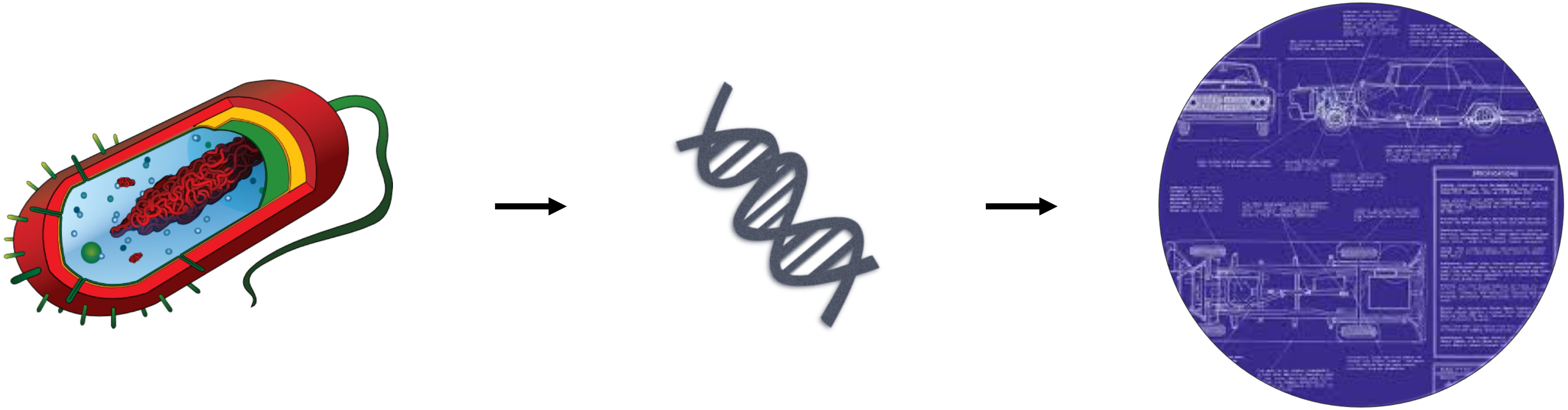
- **EUB338 probe mix (Domain *Bacteria*)**
- **Ntspa712 (Phylum *Nitrospirae*)**
- **Ntspa662 (Genus *Nitrospira*)**

© Holger Daims

Outline

- Introduction to cultivation-independent methods
- **Metagenomics**
- Activity assays
- Linking Function to Identity

What is a genome?



Genome = Parts list of a single species

How do we get microbial genomes?

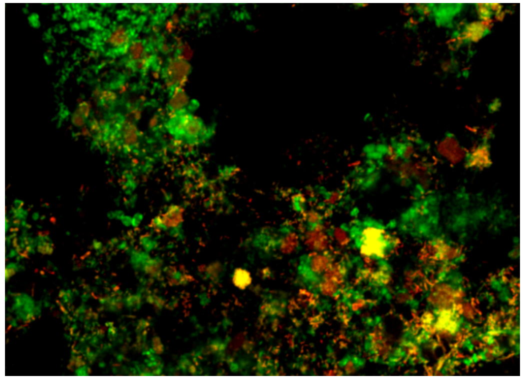
Culturing

Few microorganisms can be easily cultured (<<5%)

How do we get microbial genomes?

Culturing

Few microorganisms can be easily cultured (<<5%)



Metagenomics

Analyses of microbial genomes directly from the environment

What is metagenomics?

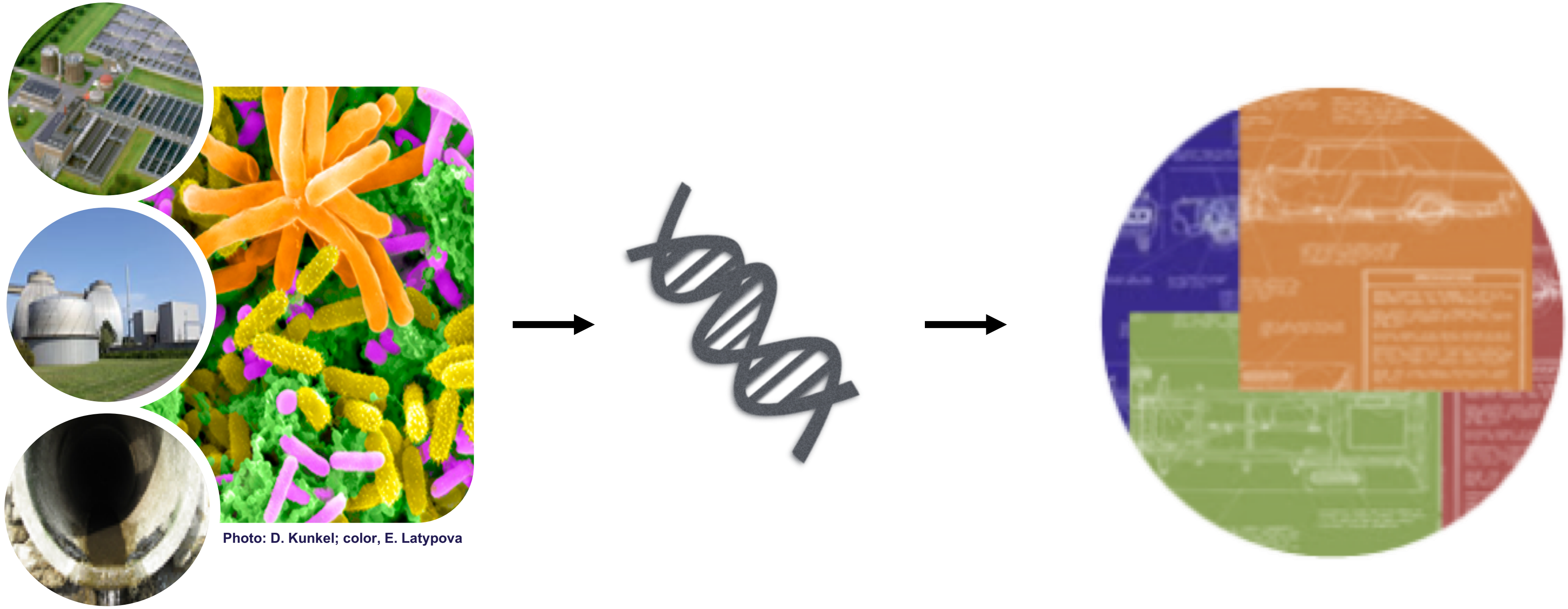
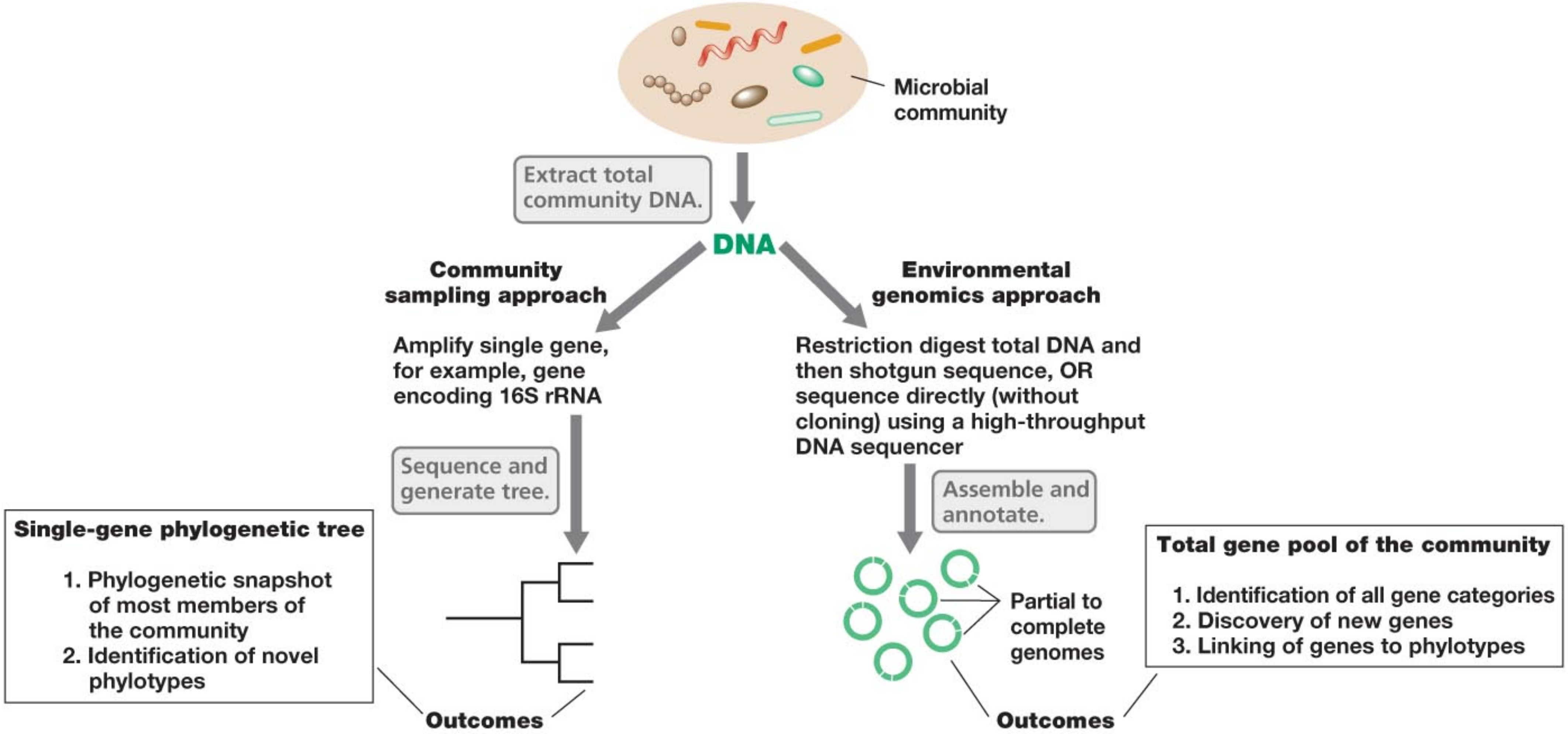


Photo: D. Kunkel; color, E. Latypova

Metagenome = Parts list of the community

The Environmental Genomics Approach



Microbial community

Extract total community DNA.

DNA

Community sampling approach

Amplify single gene, for example, gene encoding 16S rRNA

Sequence and generate tree.

Single-gene phylogenetic tree

1. Phylogenetic snapshot of most members of the community
2. Identification of novel phylotypes

Outcomes

Environmental genomics approach

Restriction digest total DNA and then shotgun sequence, OR sequence directly (without cloning) using a high-throughput DNA sequencer

Assemble and annotate.

Partial to complete genomes

Total gene pool of the community

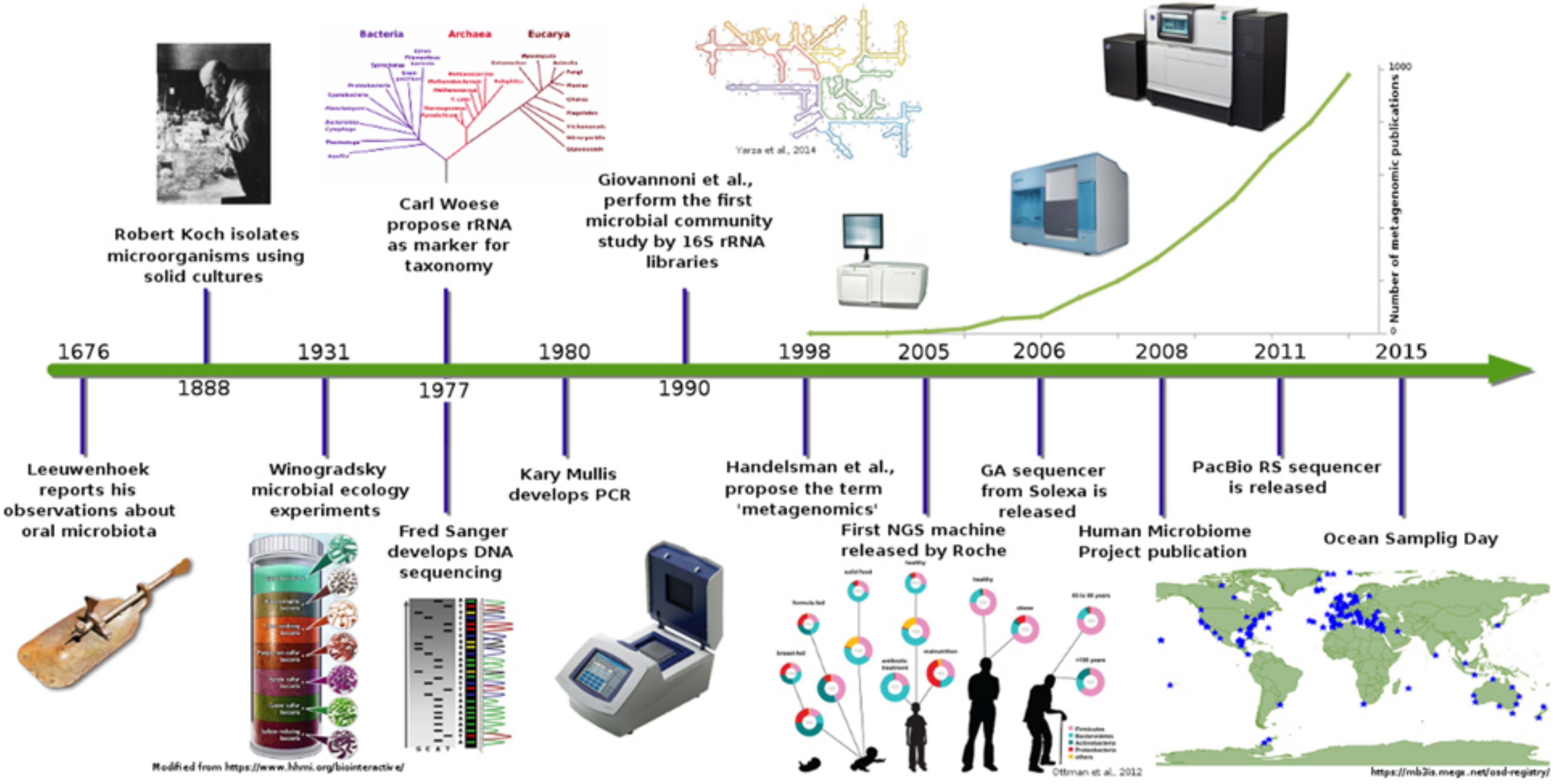
1. Identification of all gene categories
2. Discovery of new genes
3. Linking of genes to phylotypes

Outcomes

Amplicon sequencing

Metagenomics

(Meta)genomic Sequencing Timeline and Milestones



1676
Leeuwenhoek reports his observations about oral microbiota

1888
Robert Koch isolates microorganisms using solid cultures

1931
Winogradsky microbial ecology experiments

Modified from https://www.hhmi.org/biointeractive/

1977
Carl Woese propose rRNA as marker for taxonomy

Fred Sanger develops DNA sequencing

1980
Kary Mullis develops PCR

1990
Giovannoni et al., perform the first microbial community study by 16S rRNA libraries

Yarza et al., 2014

1998
Handelsman et al., propose the term 'metagenomics'

2005
First NGS machine released by Roche

2006
GA sequencer from Solexa is released

Ottman et al., 2012

2008
Human Microbiome Project publication

2011
PacBio RS sequencer is released

2015
Ocean Samplig Day

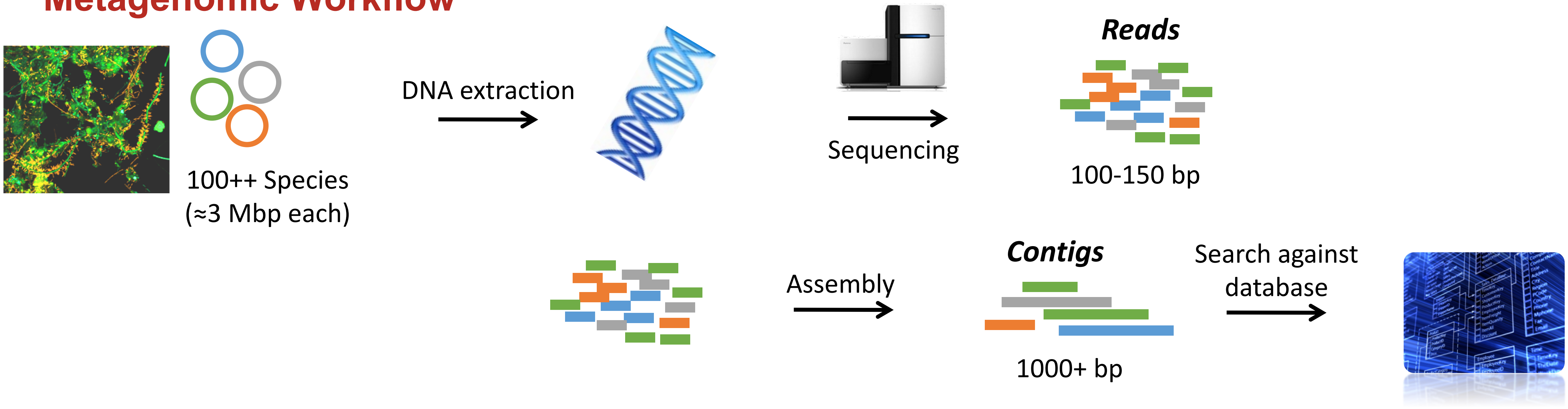
https://mb3is.megx.net/osd-registry/

https://doi.org/10.3389/fgene.2015.00348

Sequencing Technologies | Short and long read sequencing

	 Illumina MiSeq	 Nanopore MinION	 PacBio Sequel
DNA requirements	Low 1 ng – 50 ng	Moderate/high 10 ng – 1,500 ng	High 100 ng – 5,000 ng
Amplification	Yes PCR, Bridge amplification	No Single molecule sequencing	No Single molecule sequencing
Genome coverage	Biased	Some bias	Unbiased
Read length	Short 2 x 300 bp	Long 1 Kb to >100 Kb	Long Mean 30 Kb, up to 100 Kb
Throughput	High 15 Gb	High 10 – 30 Gb	High Up to 20 Gb
Accuracy	High *systematic* error rate: ~0.1%	Low *Random* and *systematic* error rate 5% – 10%	Low/high *Random* error rate: ~13% Consensus error rate: 0.001%
Other features	Paired-end sequencing	Portable, inexpensive, fast, real-time results	Detect DNA modifications

Metagenomic Workflow



100++ Species
(≈3 Mbp each)

DNA extraction

Sequencing

Reads

100-150 bp

Assembly

Contigs

1000+ bp

Search against
database

Pure Culture Genomics

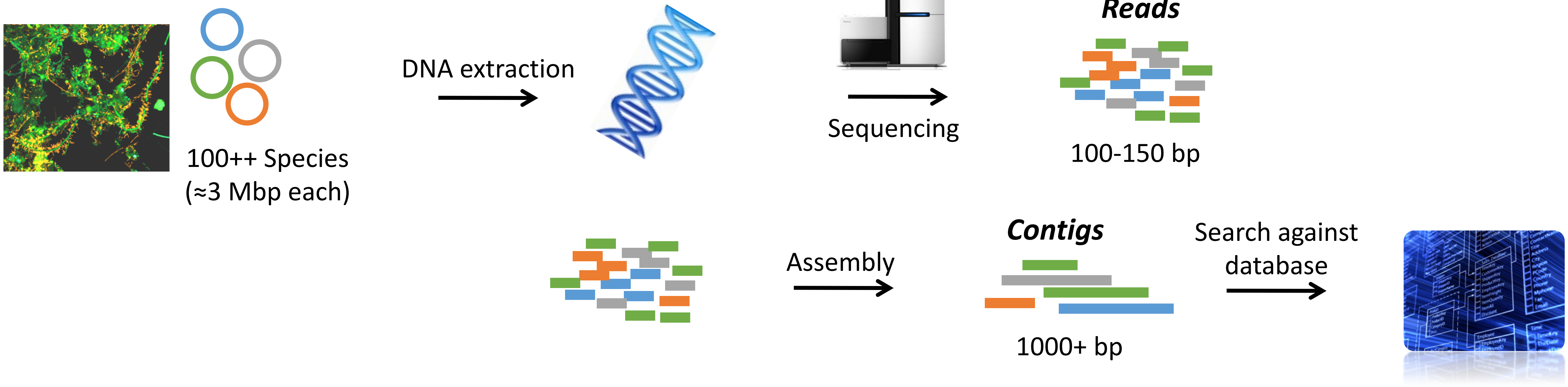
Sequencing →

Assembly →

Recovering Genomes from Metagenomes

Sequencing
→
Assembly

Metagenomic workflow



100++ Species
(≈3 Mbp each)

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Sequencing

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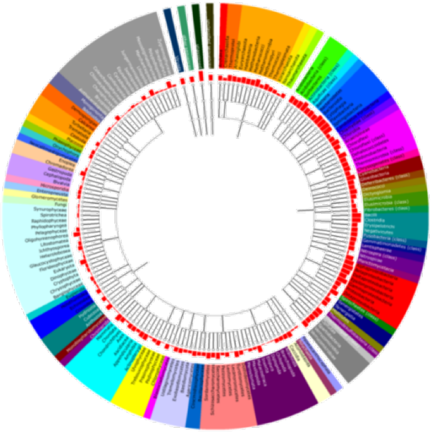
Phylogenetic classification

Who is there?

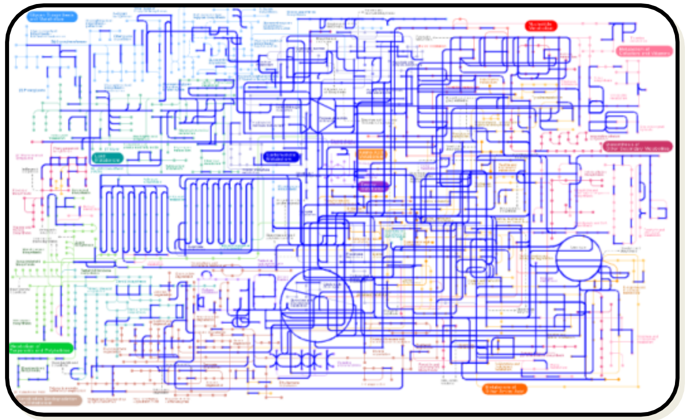
Bacterium A
Bacterium B
...
Bacterium X



✚

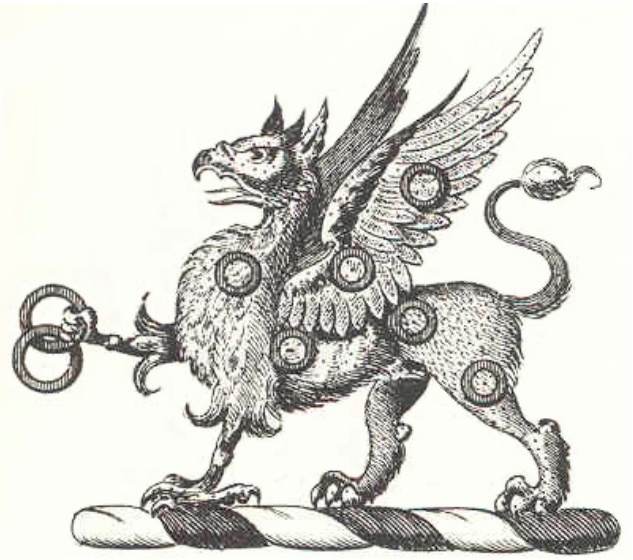
Functional classification

What can they do?



Gene A
Gene B
...
Gene X

Metagenomics



Lion + Eagle ≠ Flying Lion

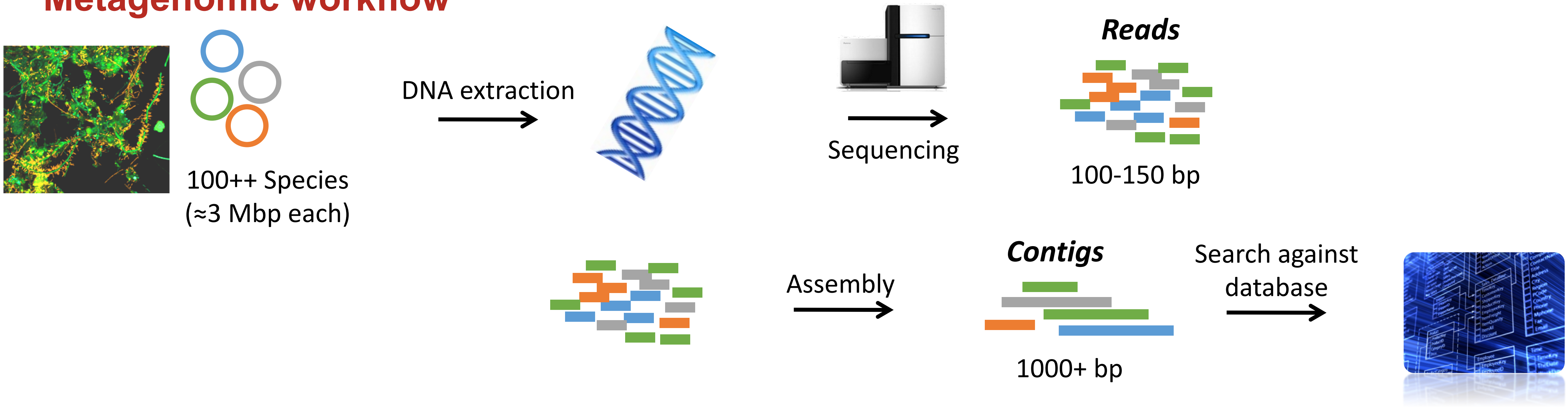
If you want to understand the ecosystem

you need to

understand the individual species

in the ecosystem

Metagenomic workflow



100++ Species
(≈3 Mbp each)

DNA extraction →

Sequencing →

Reads

100-150 bp

Assembly →

Contigs

1000+ bp

Search against
database →

Binning →

Binning =
Separation of genomes

Who is there and what can every <u>individual</u> do?

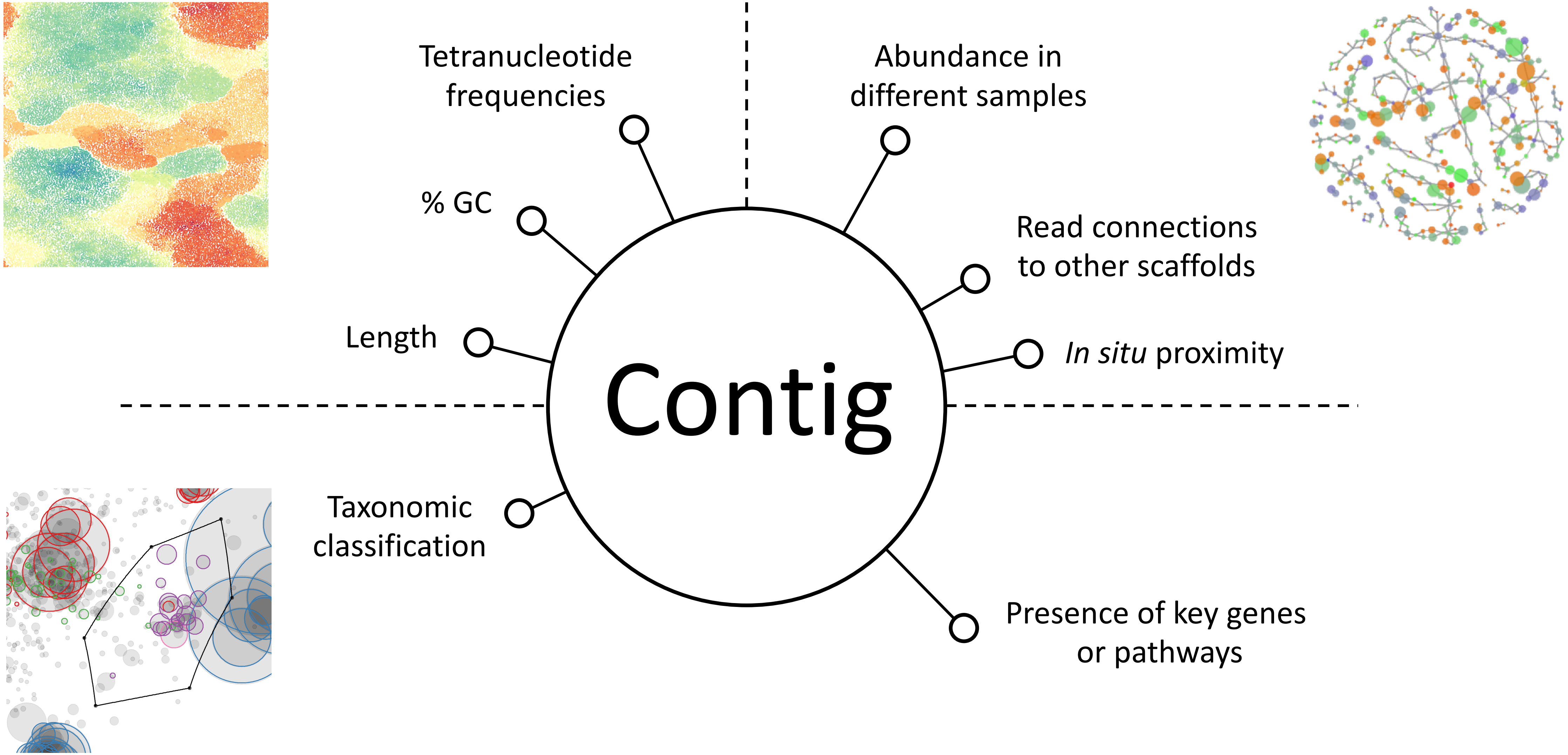
Binning

PhD student

Complex sample

Binning

Binning



Contig

Tetranucleotide frequencies

% GC

Length

Abundance in different samples

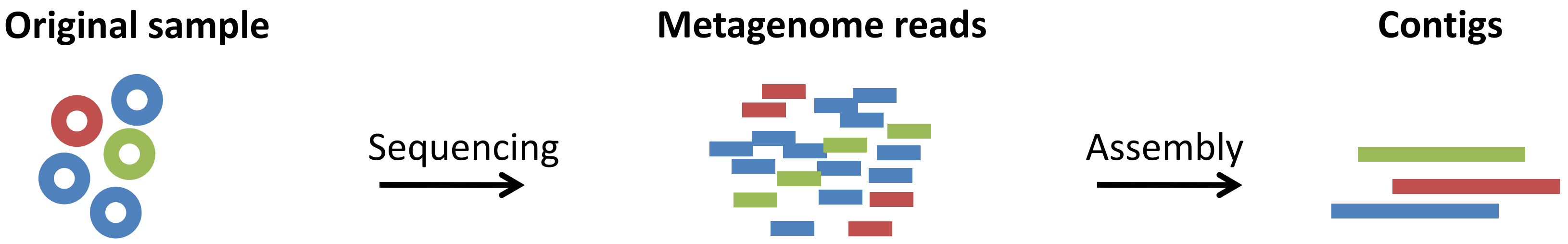
Read connections to other scaffolds

In situ proximity

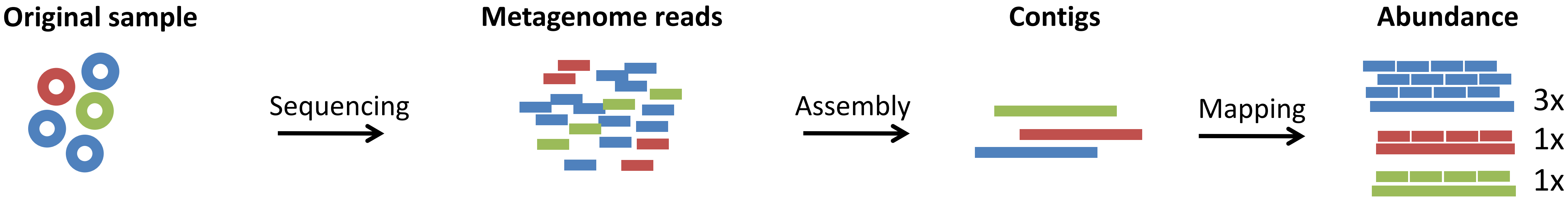
Taxonomic classification

Presence of key genes or pathways

Using Abundance Data for Binning



Using Abundance Data for Binning



Original sample

Sequencing

Metagenome reads

Assembly

Contigs

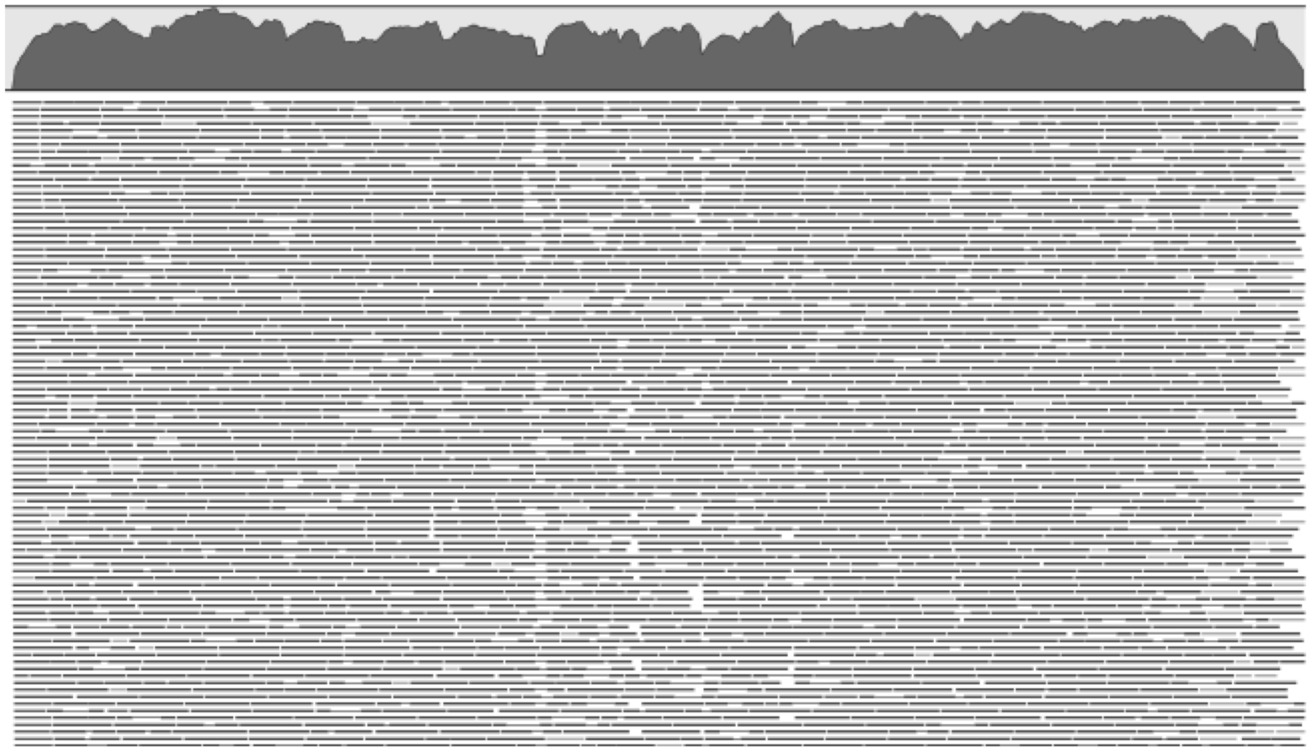
Mapping

Abundance

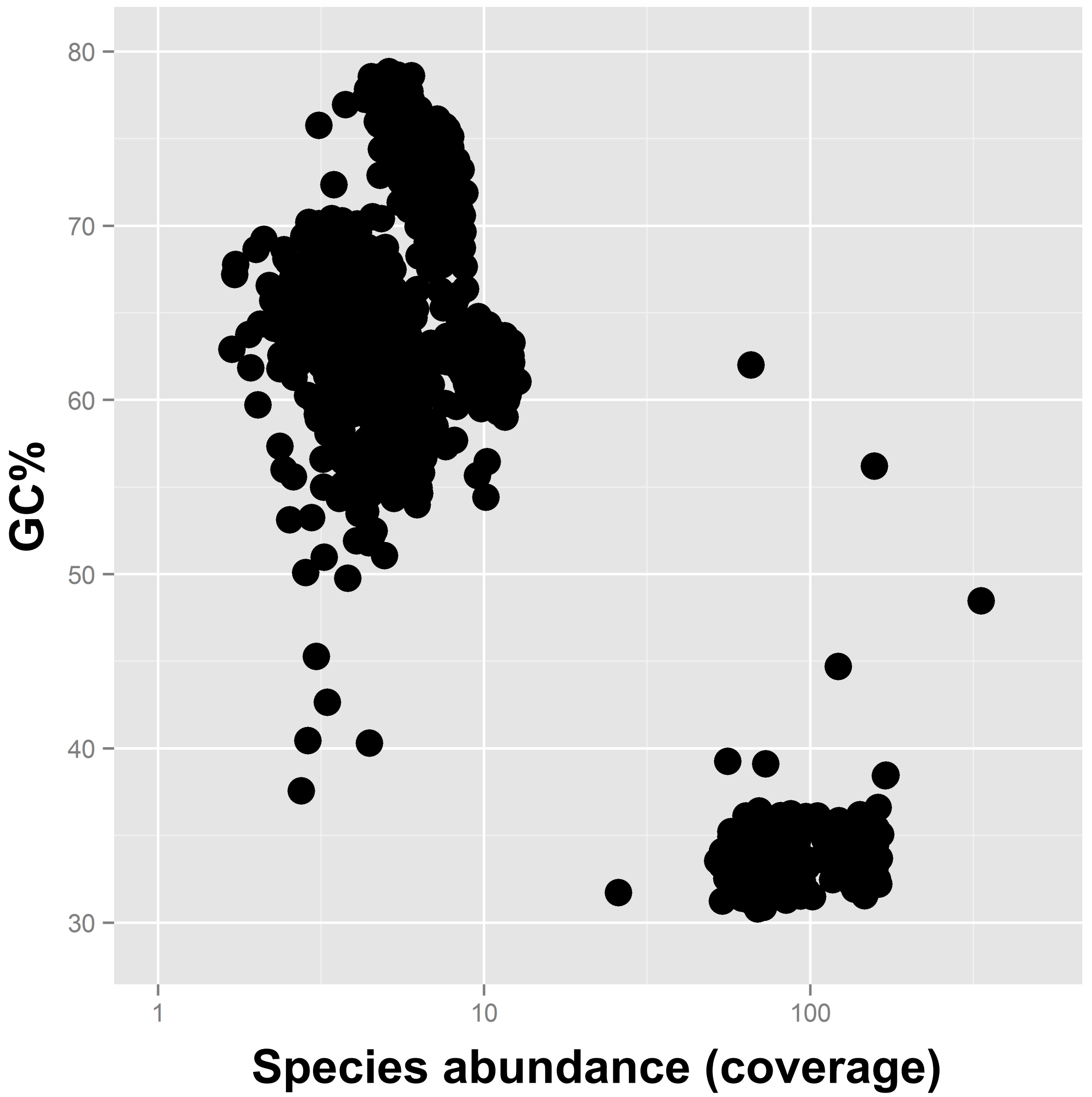
3x

1x

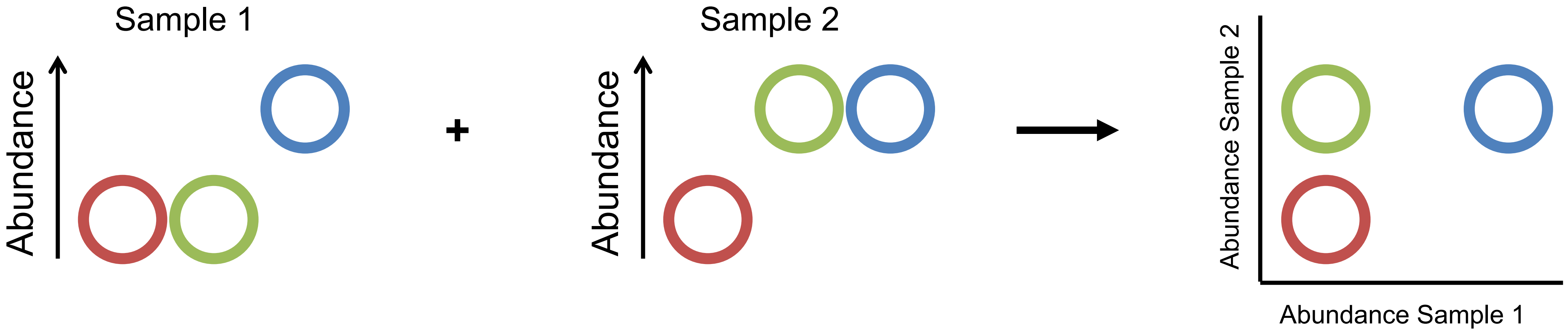
1x



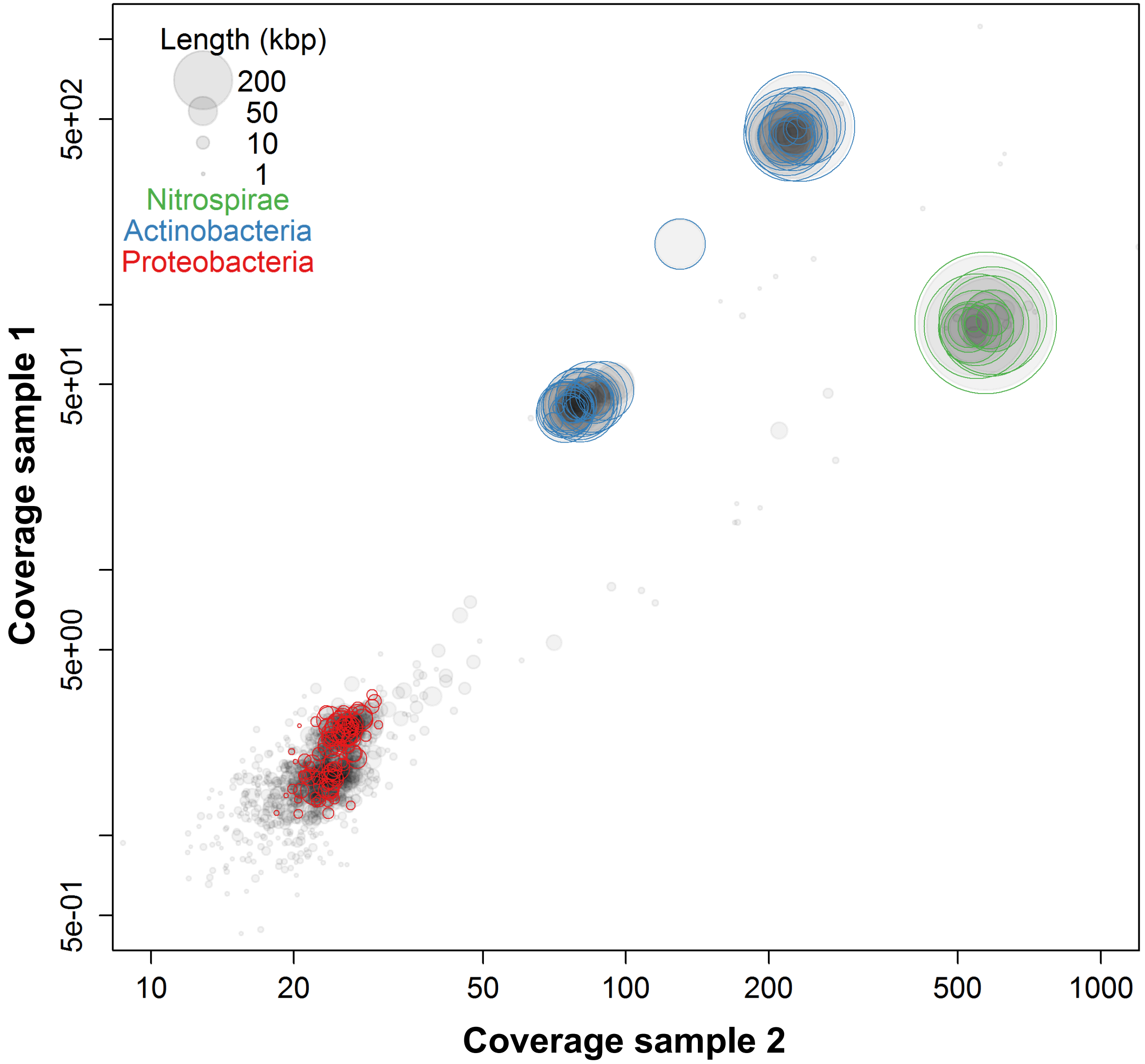
Sequence Composition-Based Binning



Sequence Composition-Independent Binning



Sequence Composition-Independent Binning



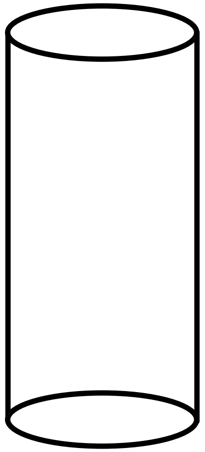
Reduction of Diversity

Environmental sample

Short term enrichment

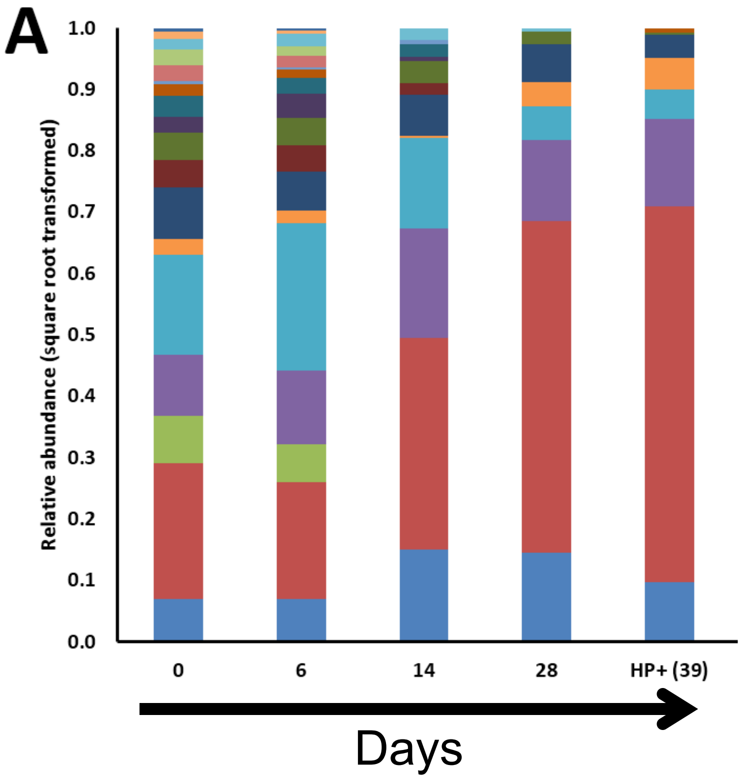
→

SBR reactor

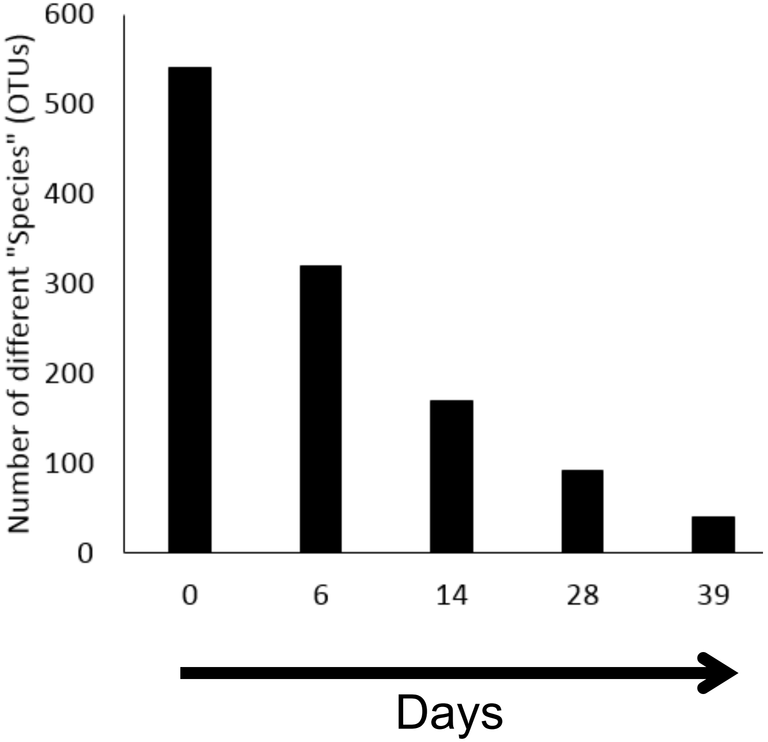


Reduction of Diversity

Relative abundance



Species diversity

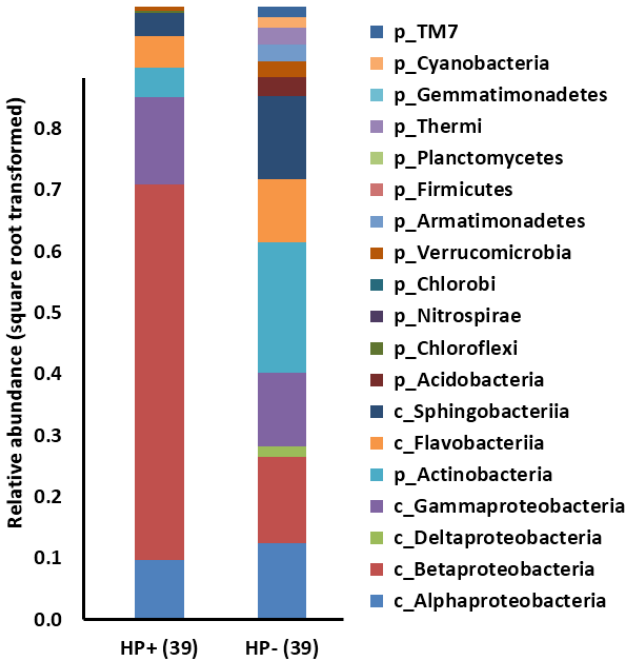


Albertsen *et al.*, 2013 **Nat. Biotech.**

Sequence Composition-Independent Binning

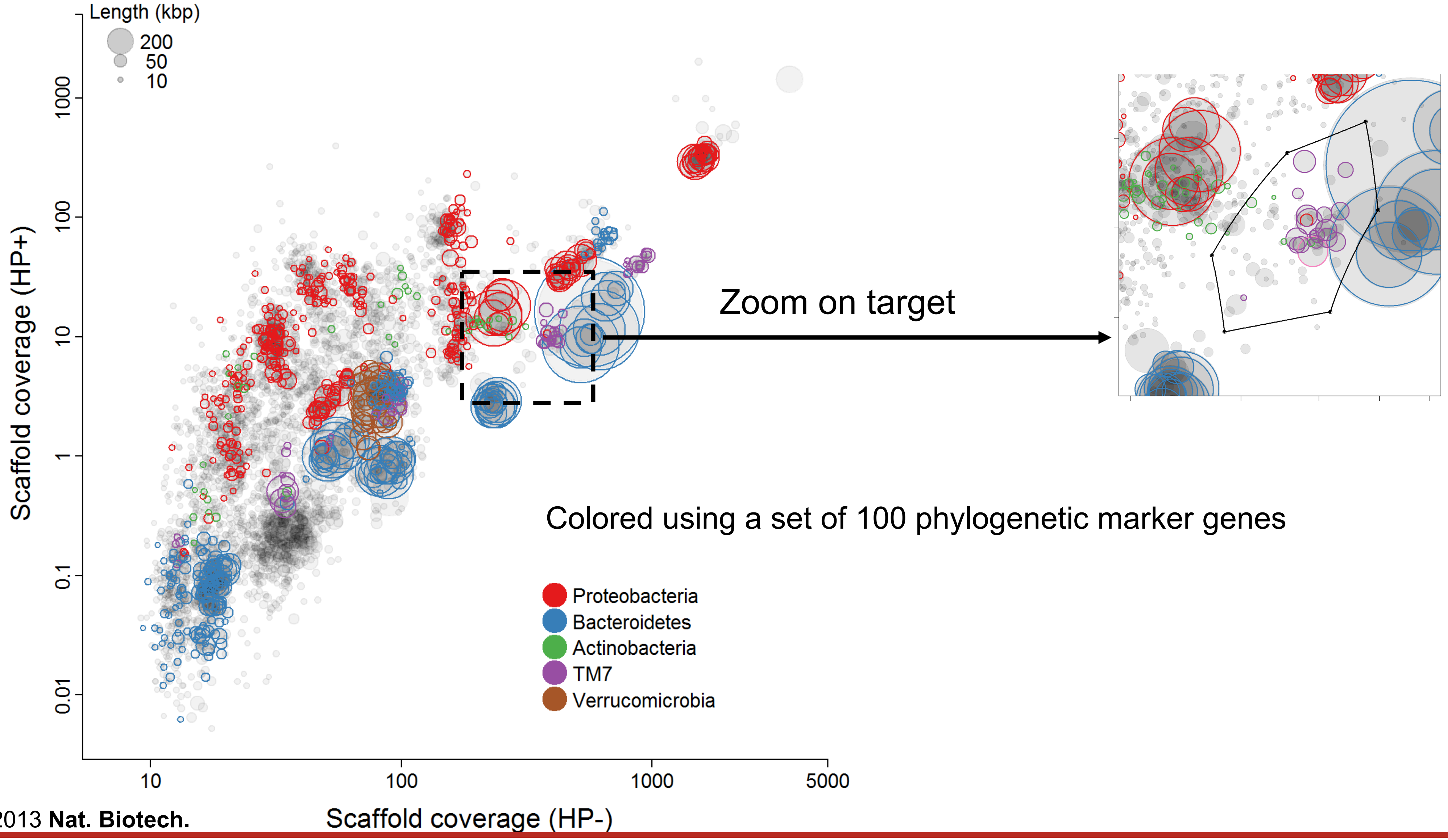
Environmental sample

Two different DNA extraction methods



Albertsen *et al.*, 2013 **Nat. Biotech.**

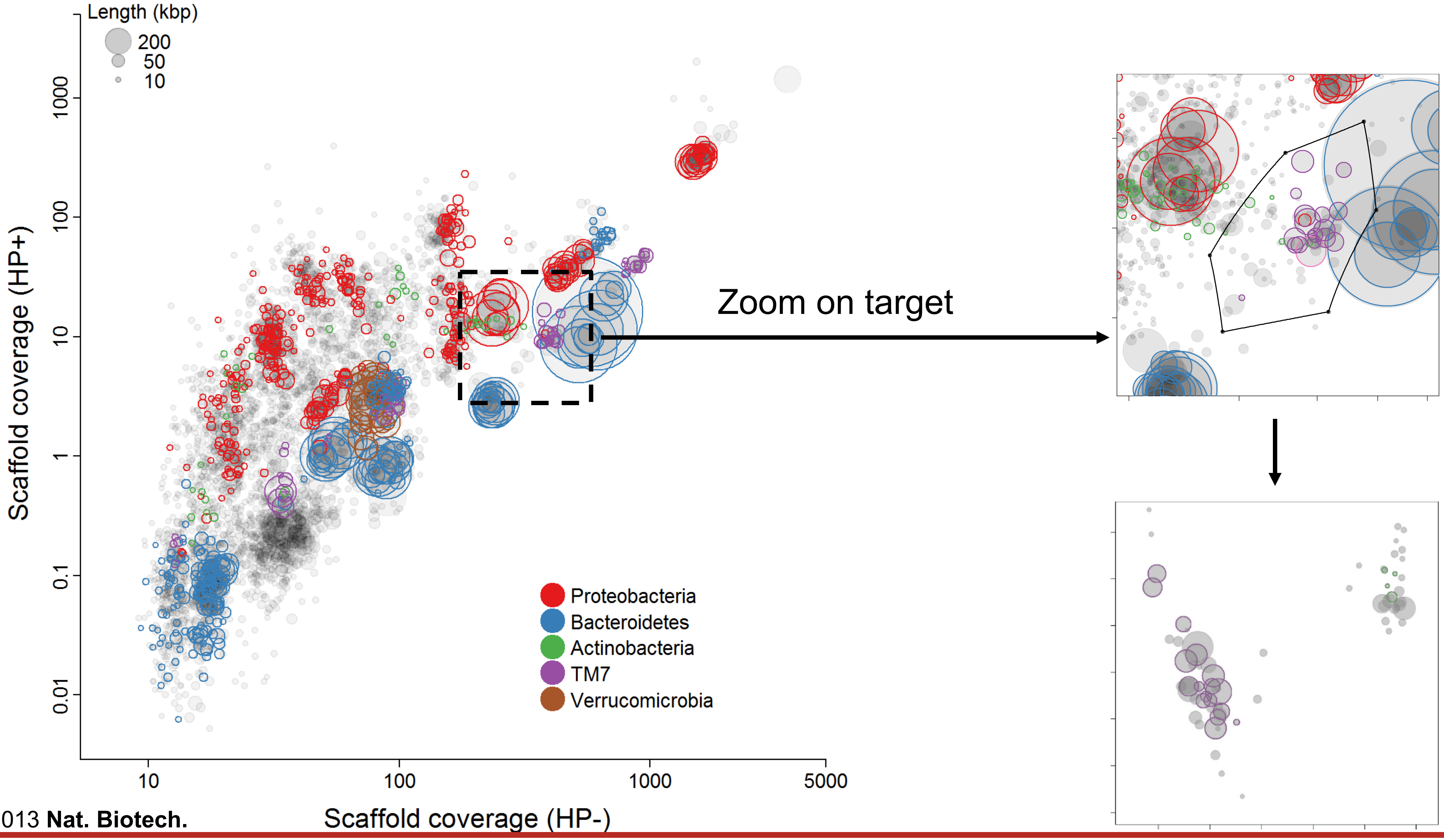
Sequence Composition-Independent Binning



Colored using a set of 100 phylogenetic marker genes

Albertsen *et al.*, 2013 **Nat. Biotech.**

Sequence Composition-Independent Binning



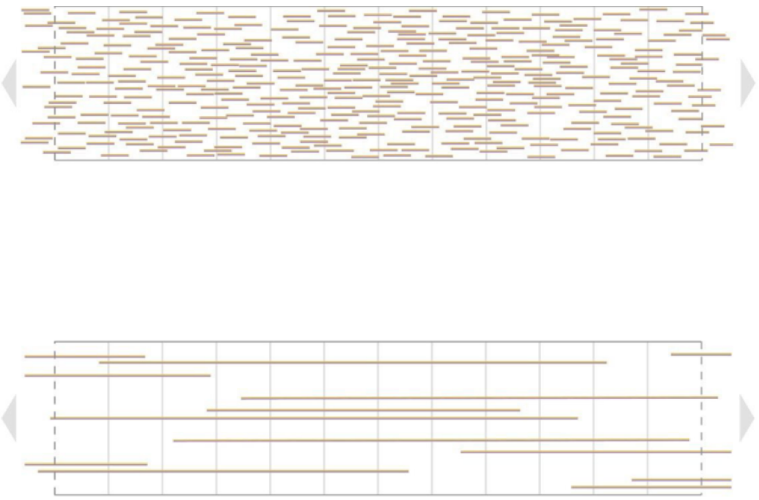
Albertsen *et al.*, 2013 **Nat. Biotech.**

Advantages of Long-Read Sequencing

Concept | Long reads are more *specific* and significantly reduce complexity of *de novo* assembly

You get a bigger piece of the puzzle..

..making the puzzle easier to solve.



Long reads can:

- Span large repetitive regions
- Resolve low-complexity and homopolymer regions, big structural variants & polymorphisms
- Identify long palindromes, determine microsatellite lengths, tandem repeats

Outline

- Introduction to cultivation-independent methods
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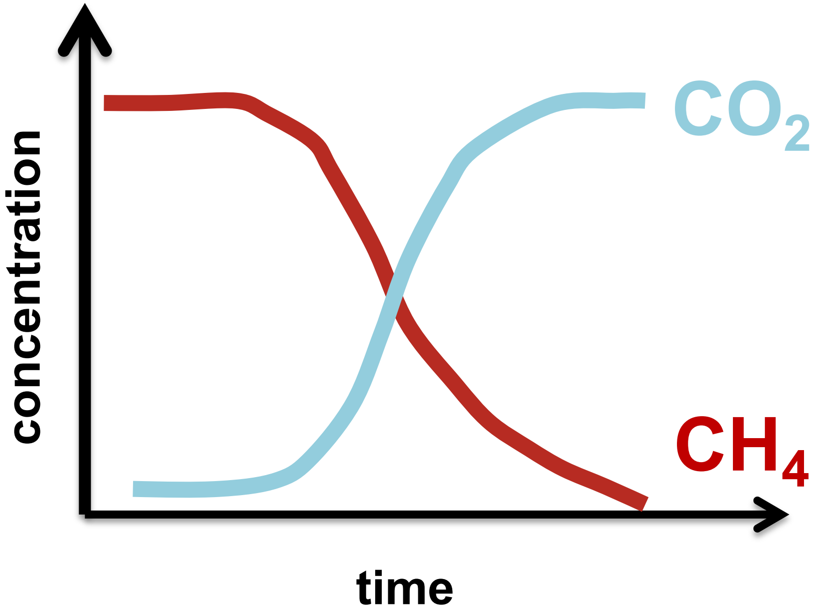
- **Activity assays**
- Linking Function to Identity

Measuring Direct Substrate Turnover

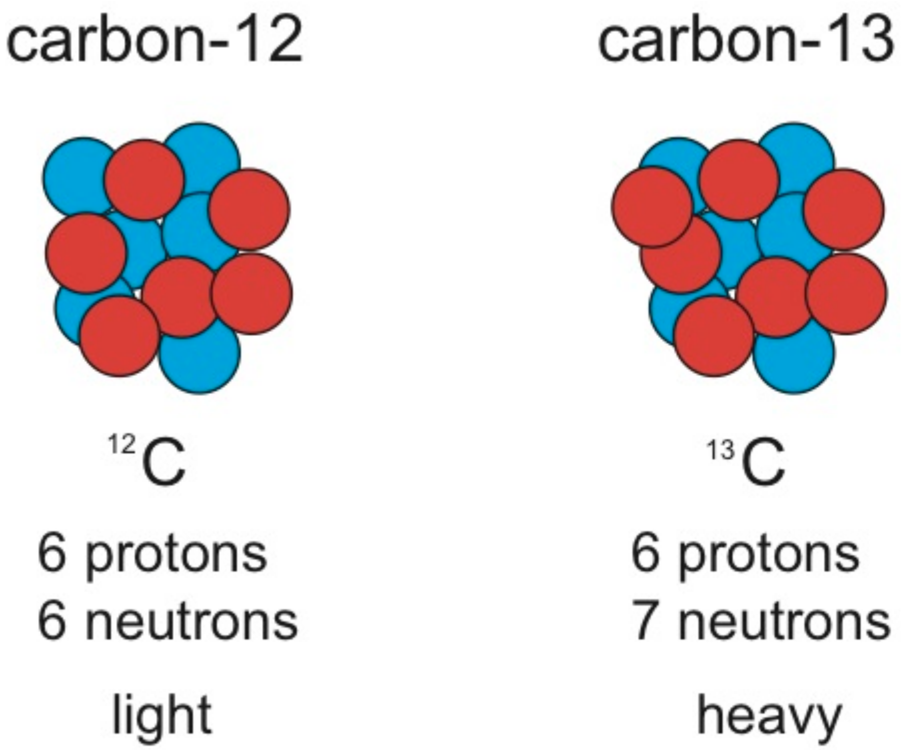
$$CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O$$



Not Always in Bottles

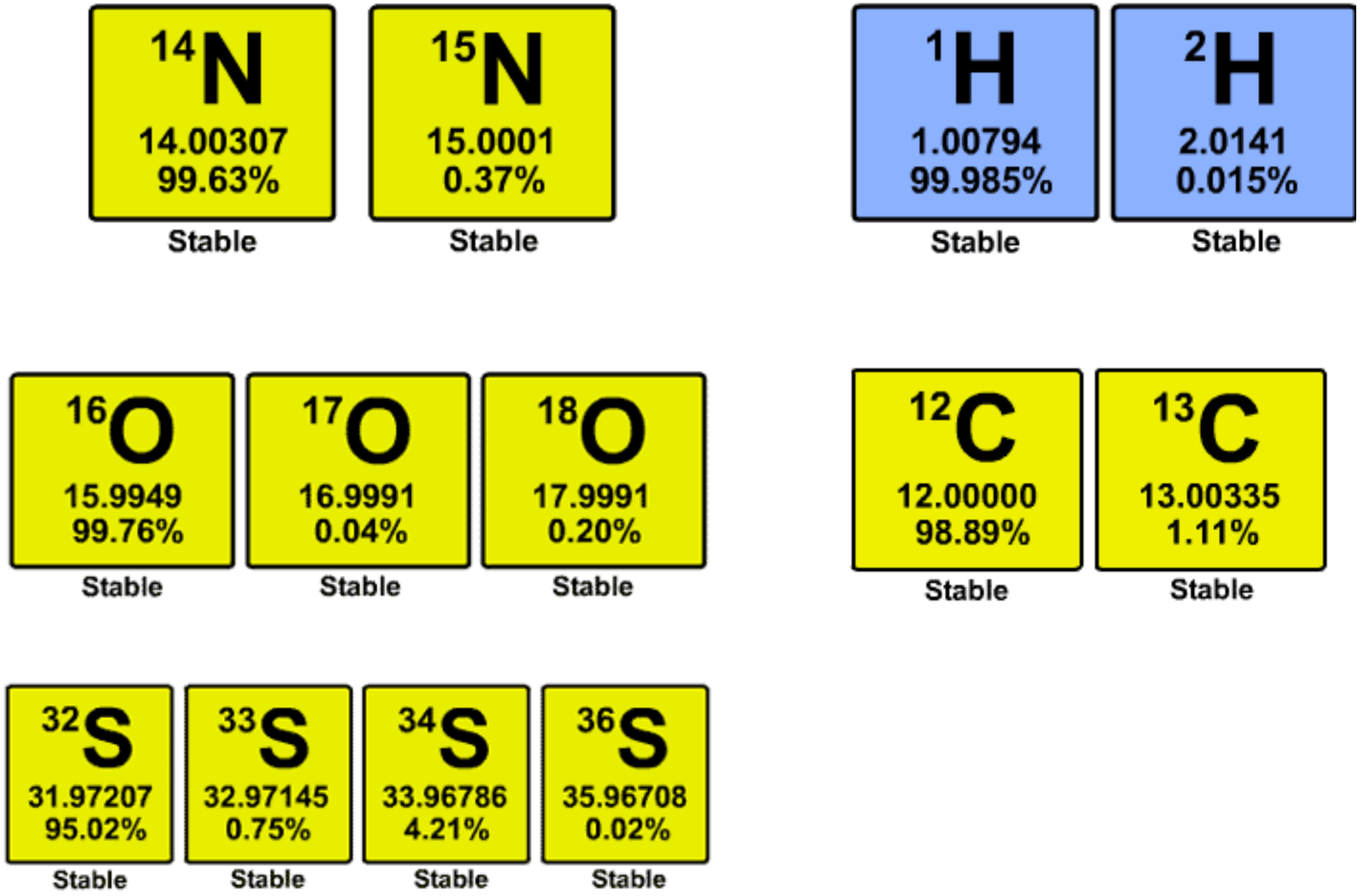
Stable Isotopes

- The number of protons in the nucleus defines an element
- The nucleus contains protons and neutrons
- Light isotopes *vs*. heavy isotopes



S. Montanari (2012)

Stable Isotopes Commonly Used in Environmental Microbiology



^{14}N	^{15}N
14.00307	15.0001
99.63%	0.37%
Stable	Stable

^{1}H	^{2}H
1.00794	2.0141
99.985%	0.015%
Stable	Stable

^{16}O	^{17}O	^{18}O
15.9949	16.9991	17.9991
99.76%	0.04%	0.20%
Stable	Stable	Stable

^{12}C	^{13}C
12.00000	13.00335
98.89%	1.11%
Stable	Stable

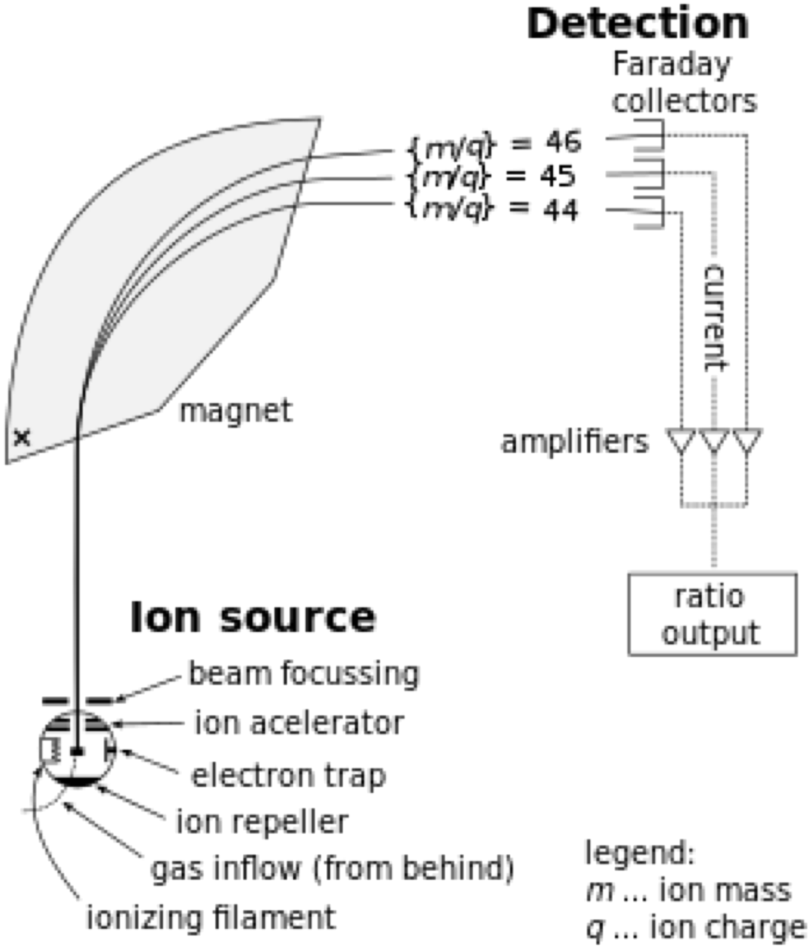
^{32}S	^{33}S	^{34}S	^{36}S
31.97207	32.97145	33.96786	35.96708
95.02%	0.75%	4.21%	0.02%
Stable	Stable	Stable	Stable

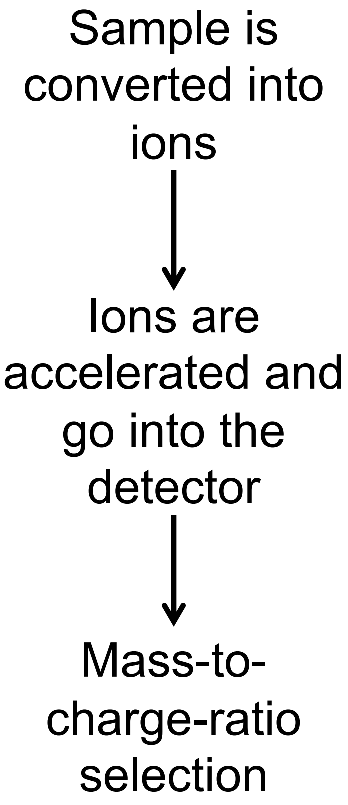
Activity Assays Using Stable Isotopes

- Feed labelled substrates
- Trace back the label
    - MS (mass spectrometry)
    - NMR (nuclear magnetic resonance)

→ Which processes take place?

Mass Spectrometry





Sample is converted into ions

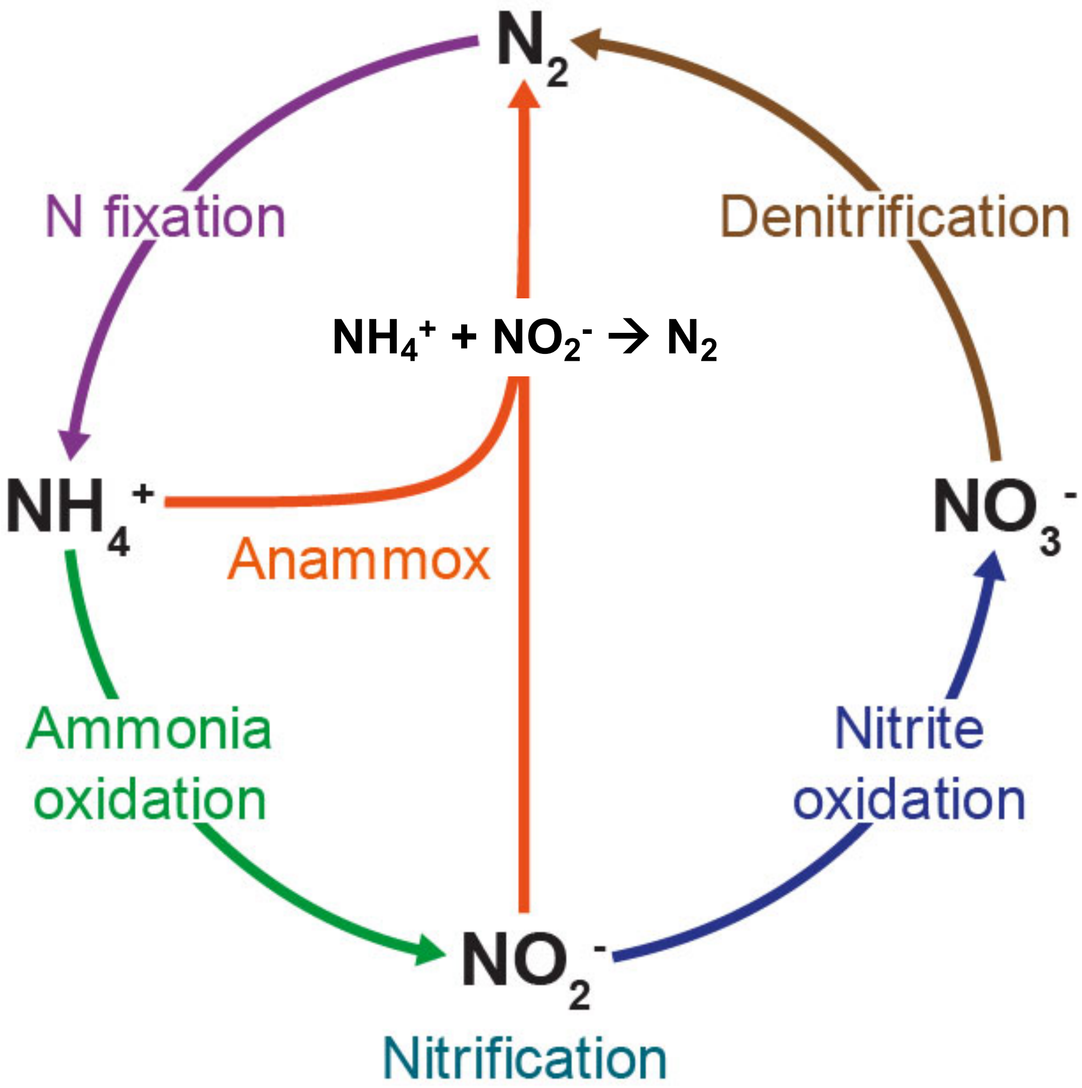
↓

Ions are accelerated and go into the detector

↓

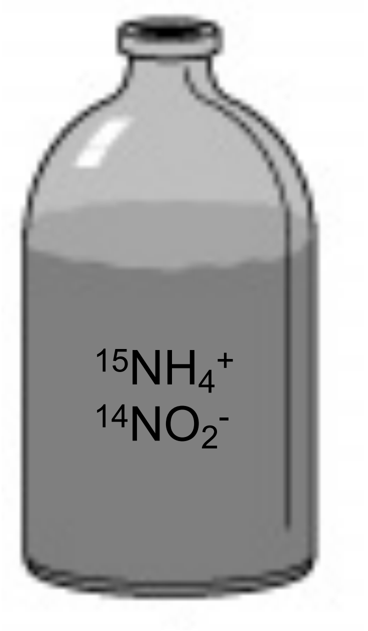
Mass-to-charge-ratio selection

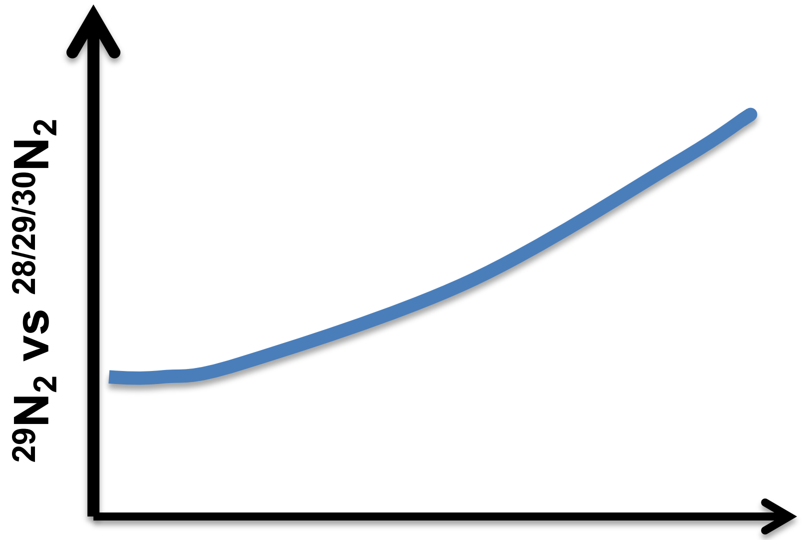
Detecting Anaerobic Ammonium Oxidation (Anammox)



Detecting Anammox Activity

- Add ^{15}N-labelled ammonium (or ^{15}N-nitrite)
- Anoxic conditions
- Measure $^{29}N_2$ in the headspace



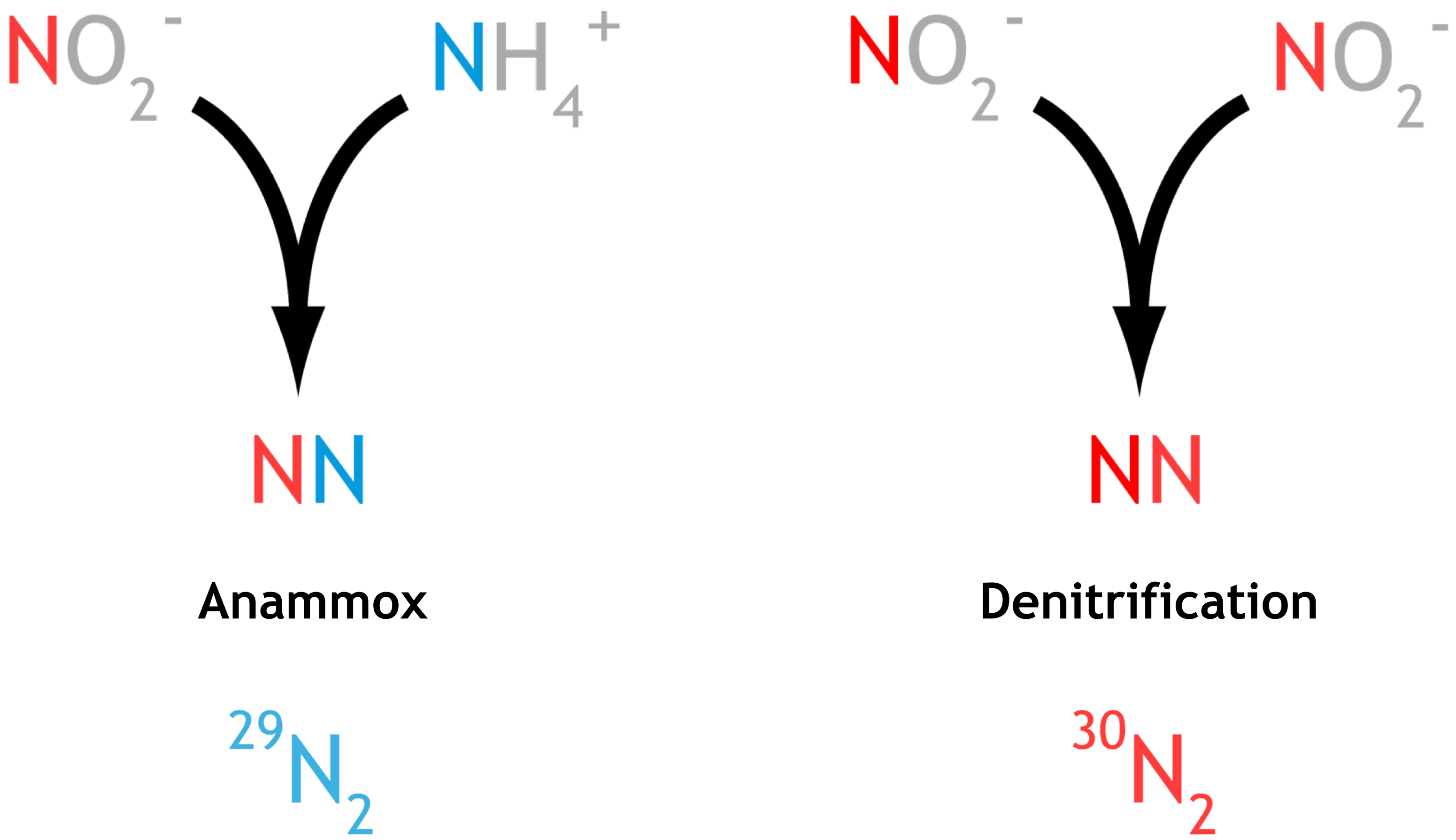


Change in ratio
=
conversion of substrate

Distinguish Processes that Have the Same End Product

$N = {}^{15}N$

$N = {}^{14}N$



NO_2^- NH_4^+

NN

Anammox

$^{29}N_2$

NO_2^- NO_2^-

NN

Denitrification

$^{30}N_2$

Outline

- Introduction to cultivation-independent methods
- Metagenomics
- Activity assays
- **Linking Function to Identity**

Functional Analysis - Substrate Uptake/Utilization

- Uptake of radioactive substrate
  - **FISH-MAR**
- Uptake of substrate labeled with stable isotopes
  - SIP
  - FISH-Raman
  - FISH-SIMS / HISH-SIMS

Radioactive Isotopes

- **Isotope:** Atoms of the same element that have same numbers of protons, but different numbers of neutrons

$^{12}_{6}C$

stable (98.89%)

$^{13}_{6}C$

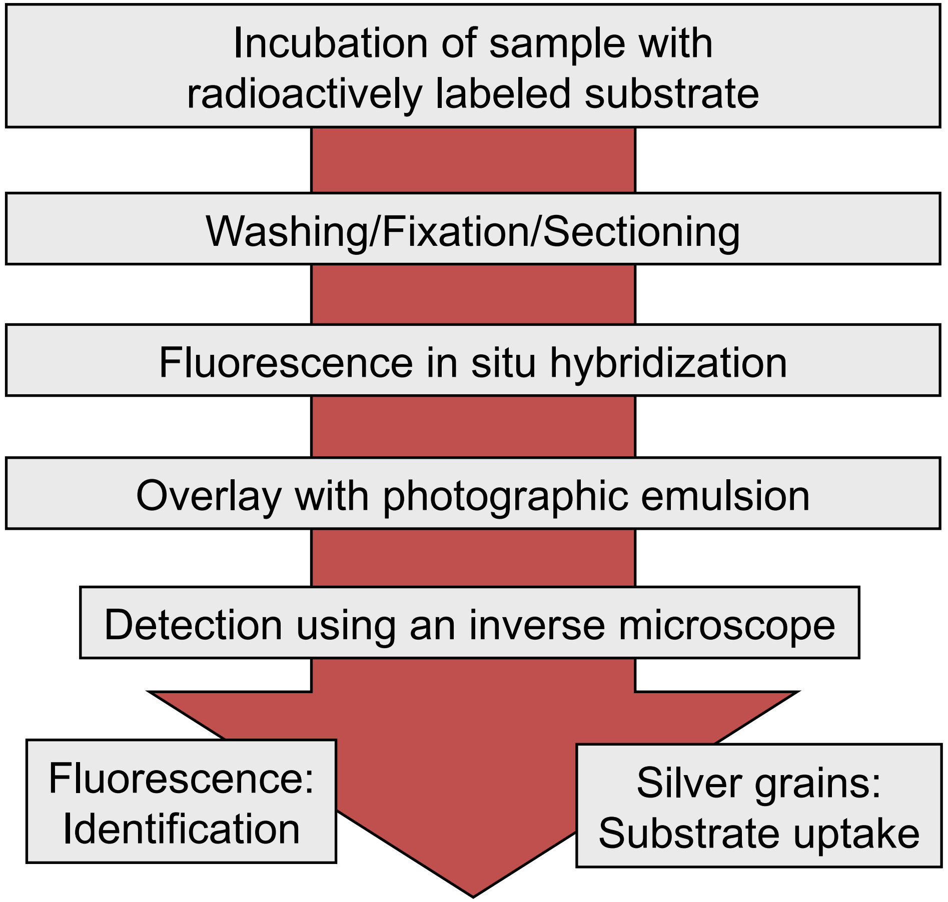
stable (1.11%)

$^{14}_{6}C$

instable (0.001%)
half life: 5730 years
β-decay into ^{14}N

FISH-MAR

- **combination of sample incubation with radioactively labeled substrate, FISH and microautoradiography**



Incubation of sample with radioactively labeled substrate

Washing/Fixation/Sectioning

Fluorescence in situ hybridization

Overlay with photographic emulsion

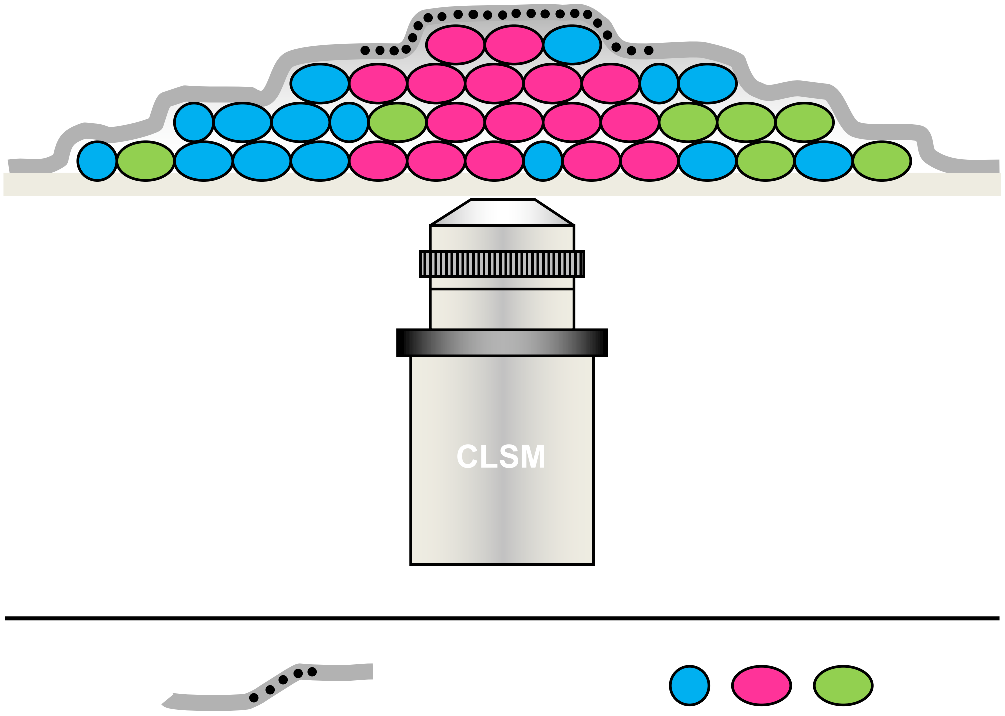
Detection using an inverse microscope

Fluorescence: Identification

Silver grains: Substrate uptake

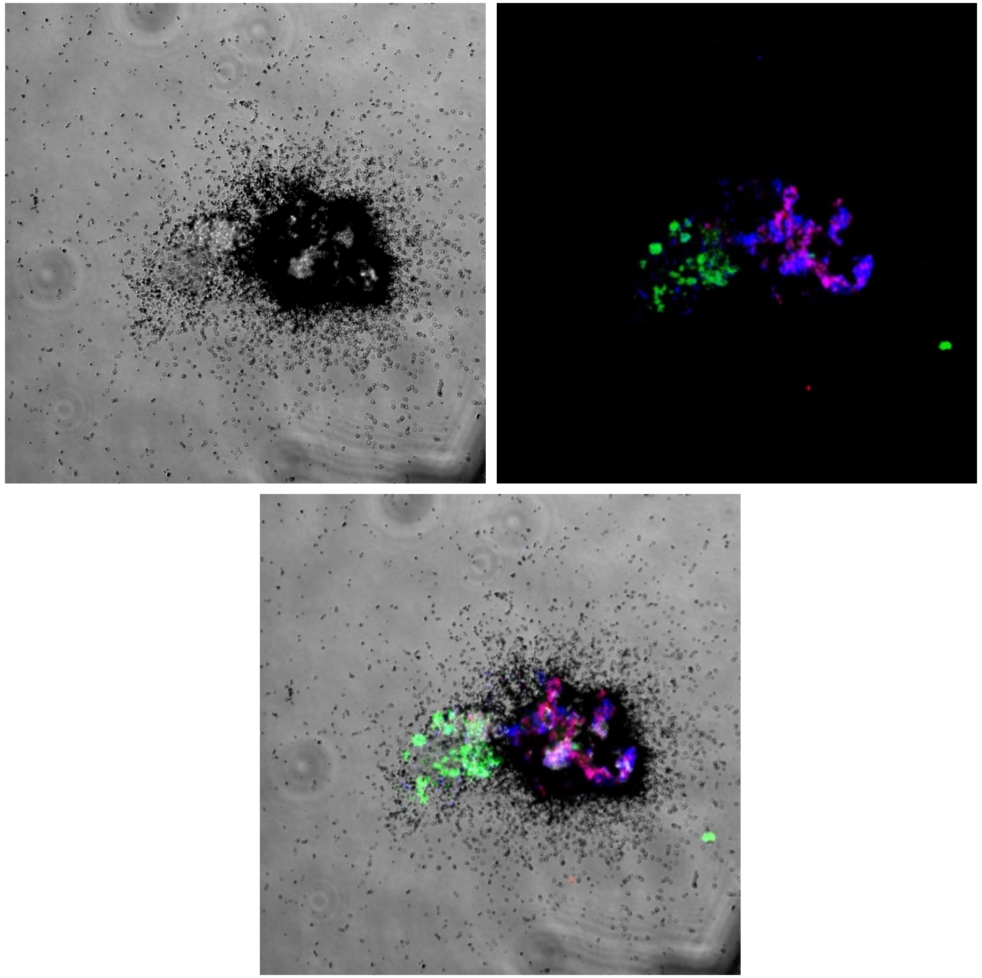
Appl Environ Microbiol 65: 1289-1297 (1999)

Radioisotope incorporation (FISH-MAR)



MAR: Cells which took up radioactively labeled substrate induce silver grain formation.

FISH: Bacteria stained with fluorescently labeled probes



KITP Lecture:
Complete Nitrification by a Single Microorganism

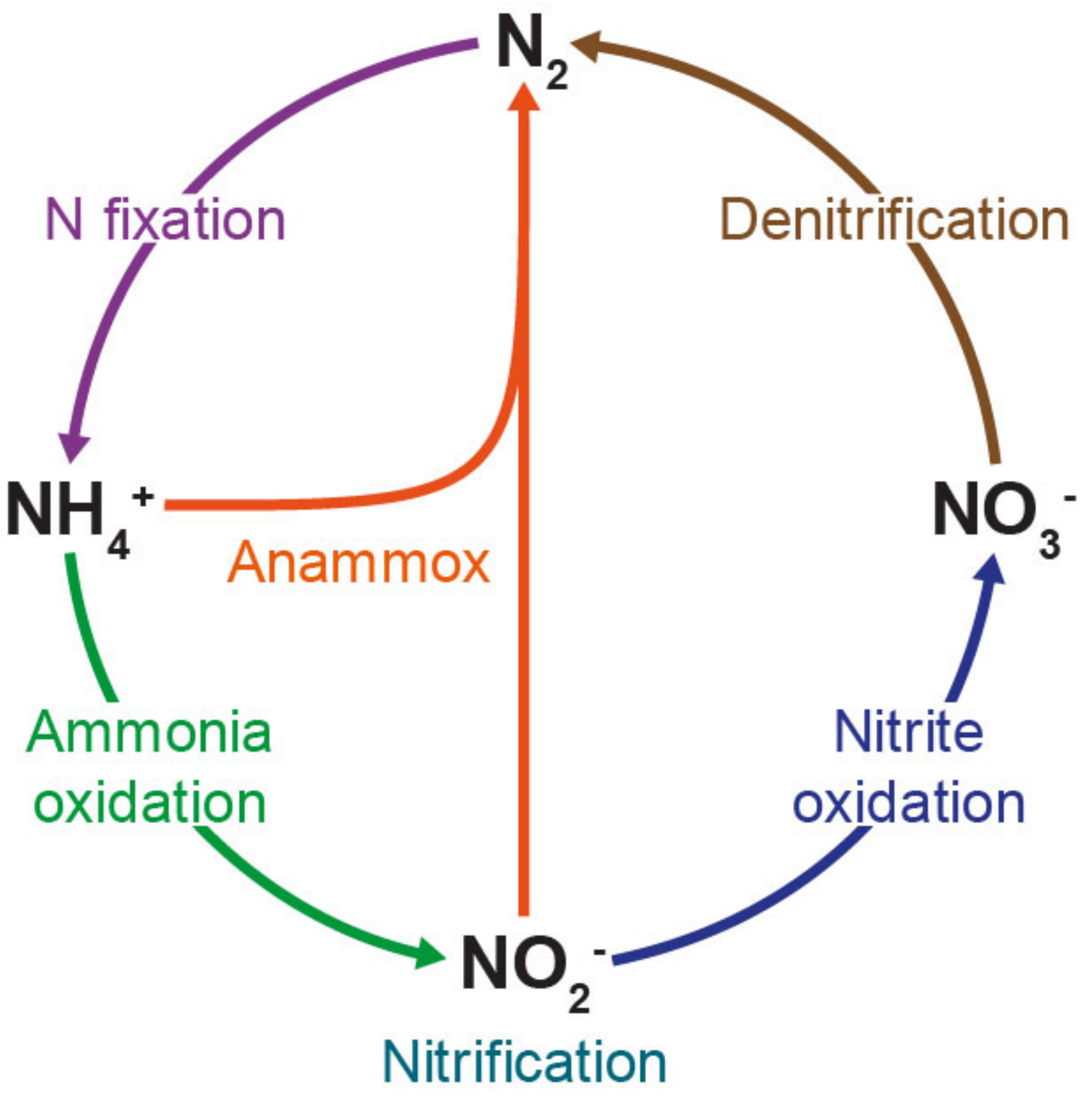
Sebastian Lücker

Department of Microbiology
Institute for Water and Wetland Research
Radboud University, Nijmegen

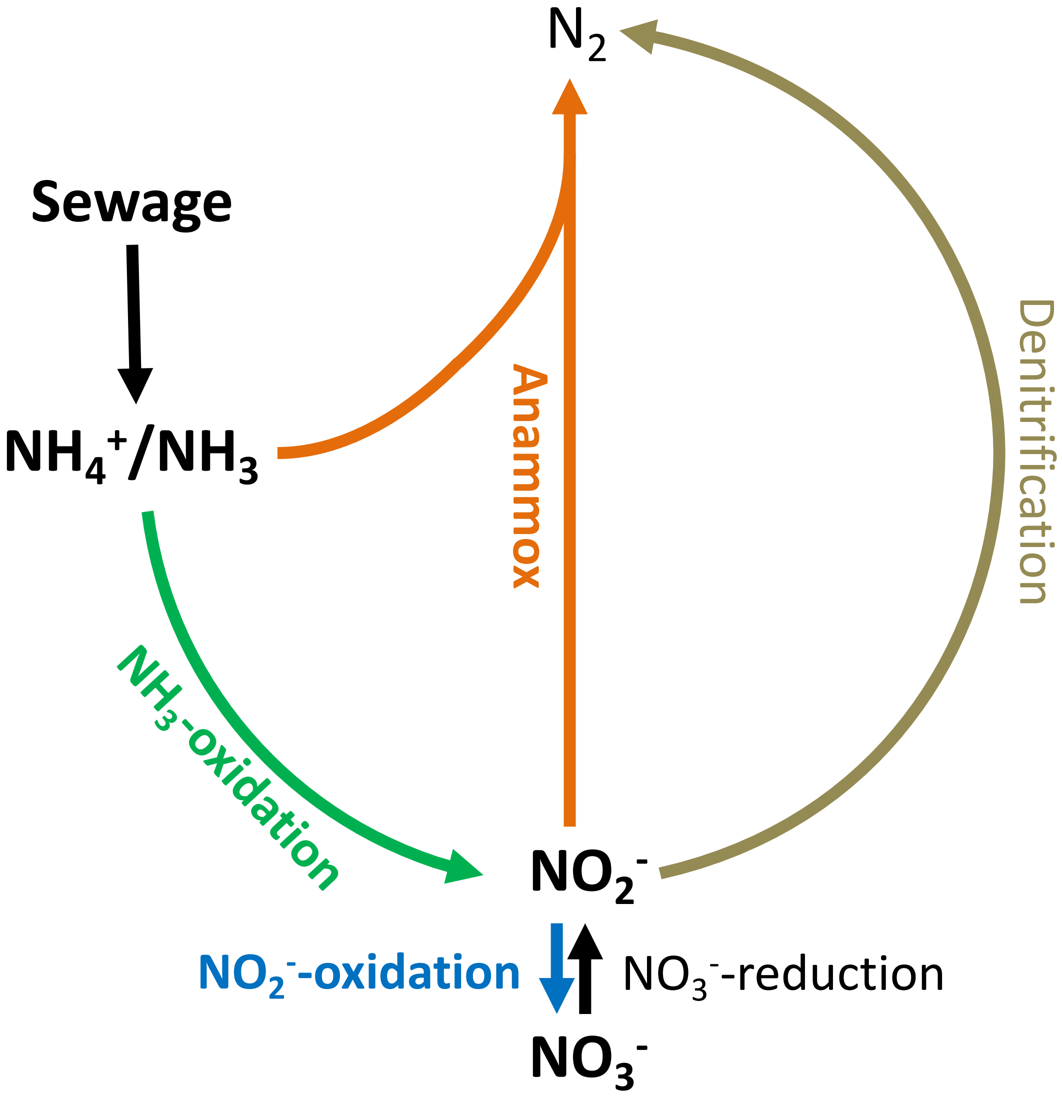
Outline

- Introduction
- Complete nitrification by *Nitrospira*
- Novel physiologies of comammox *Nitrospira*
- *In situ* detection of ammonia-oxidizing bacteria
- Ammonia oxidation kinetics of comammox *Nitrospira*

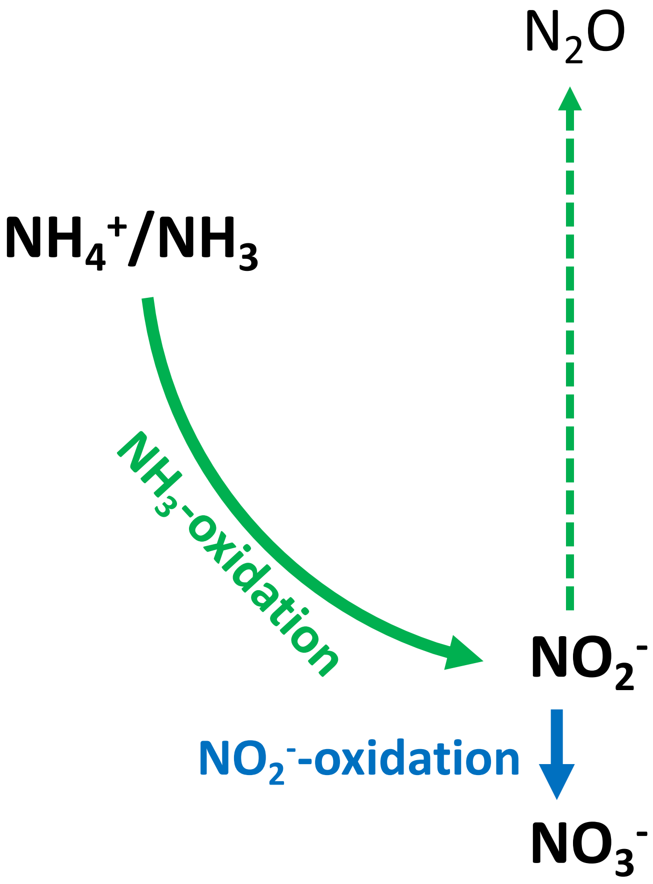
The biogeochemical nitrogen cycle



Nitrification essential for nitrogen removal from wastewater



Nitrification increases fertilizer runoff



N_2O

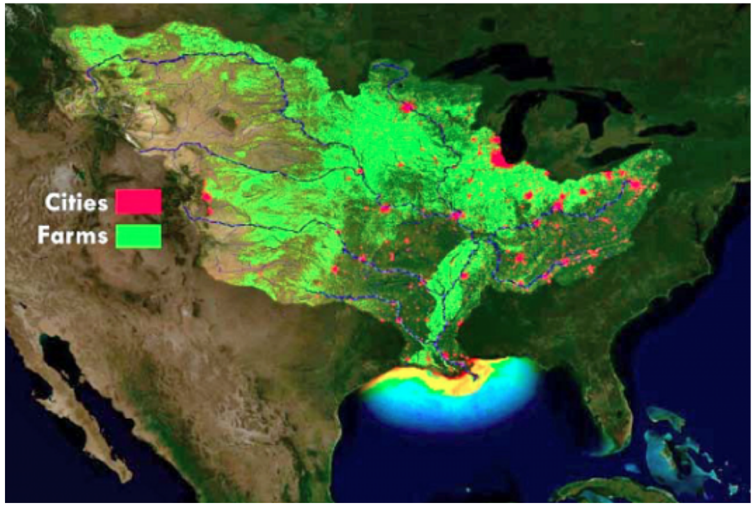
NH_4^+/NH_3

NH_3-oxidation

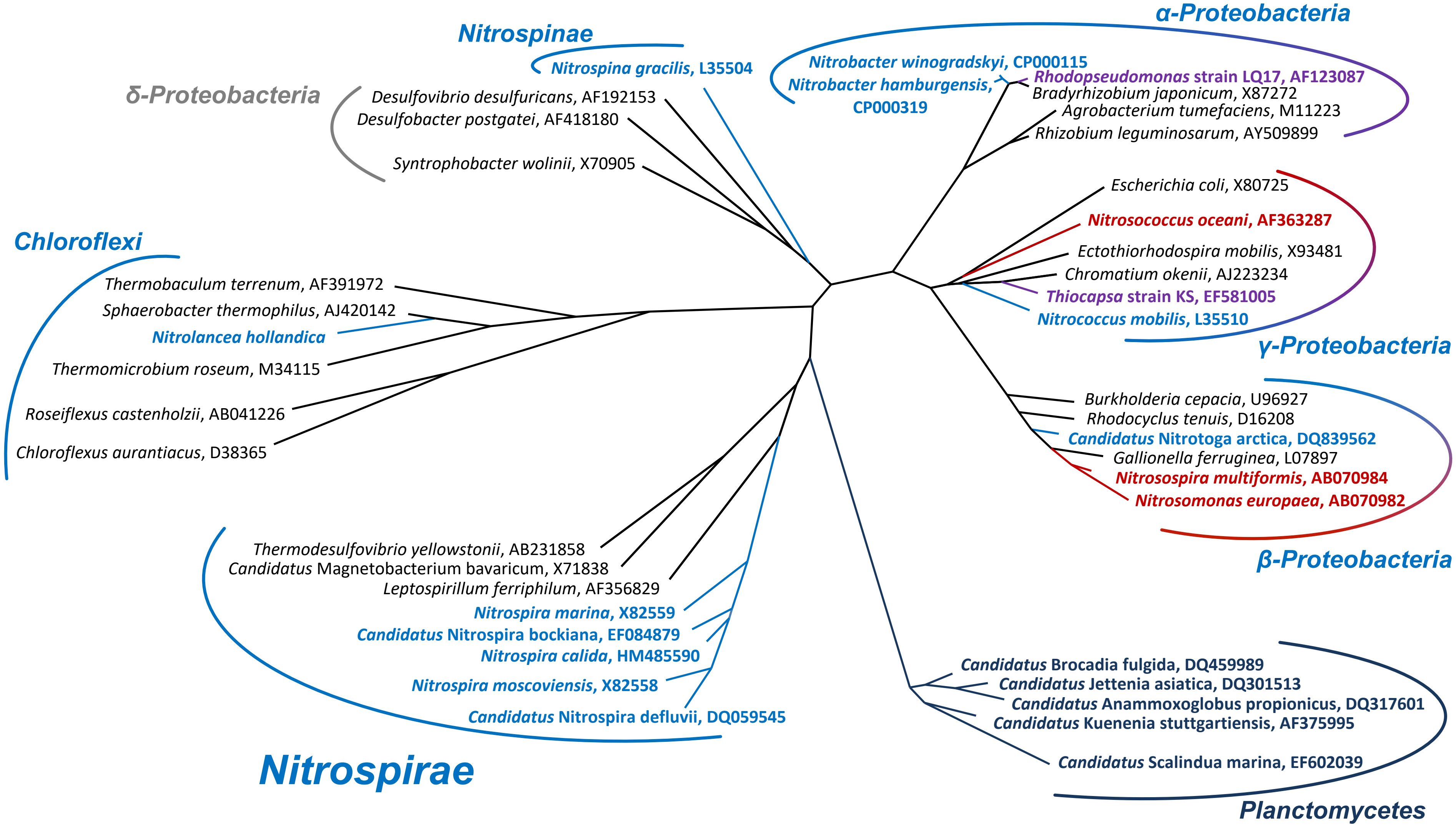
NO_2^-

NO_2^--oxidation

NO_3^-



Phylogeny of nitrifying bacteria



Nitrospinae

Nitrospina gracilis, L35504

δ-Proteobacteria

Desulfovibrio desulfuricans, AF192153
Desulfobacter postgatei, AF418180
Syntrophobacter wolinii, X70905

α-Proteobacteria

Nitrobacter winogradskyi, CP000115
Nitrobacter hamburgensis, CP000319
***Rhodopseudomonas* strain LQ17, AF123087**
Bradyrhizobium japonicum, X87272
Agrobacterium tumefaciens, M11223
Rhizobium leguminosarum, AY509899

Chloroflexi

Thermobaculum terrenum, AF391972
Sphaerobacter thermophilus, AJ420142
Nitrolancea hollandica
Thermomicrobium roseum, M34115
Roseiflexus castenholzii, AB041226
Chloroflexus aurantiacus, D38365

γ-Proteobacteria

Escherichia coli, X80725
Nitrosococcus oceani, AF363287
Ectothiorhodospira mobilis, X93481
Chromatium okenii, AJ223234
***Thiocapsa* strain KS, EF581005**
Nitrococcus mobilis, L35510

β-Proteobacteria

Burkholderia cepacia, U96927
Rhodocyclus tenuis, D16208
***Candidatus* Nitrotoga arctica, DQ839562**
Gallionella ferruginea, L07897
Nitrosospira multiformis, AB070984
Nitrosomonas europaea, AB070982

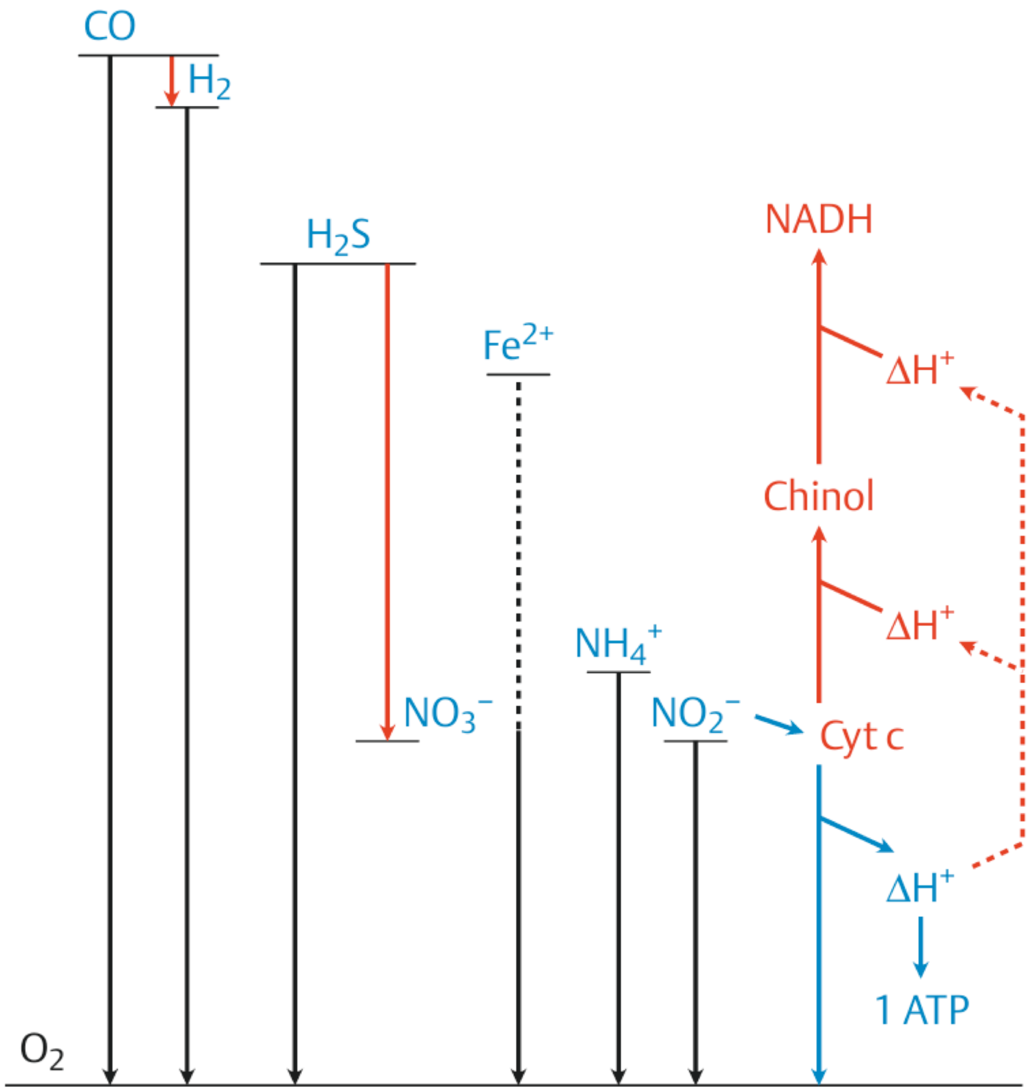
Nitrospirae

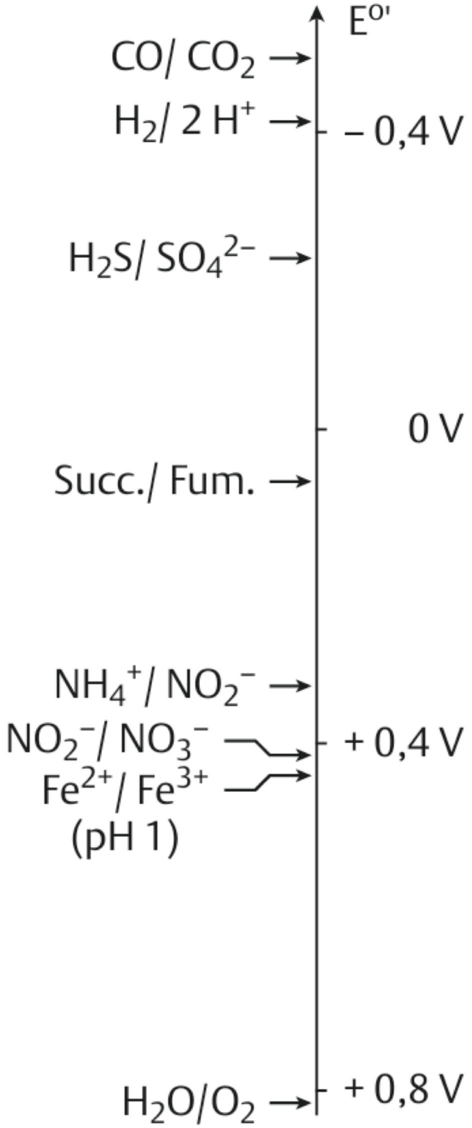
Thermodesulfovibrio yellowstonii, AB231858
Candidatus Magnetobacterium bavaricum, X71838
Leptospirillum ferriphilum, AF356829
Nitrospira marina, X82559
***Candidatus* Nitrospira bockiana, EF084879**
Nitrospira calida, HM485590
Nitrospira moscoviensis, X82558
***Candidatus* Nitrospira defluvii, DQ059545**

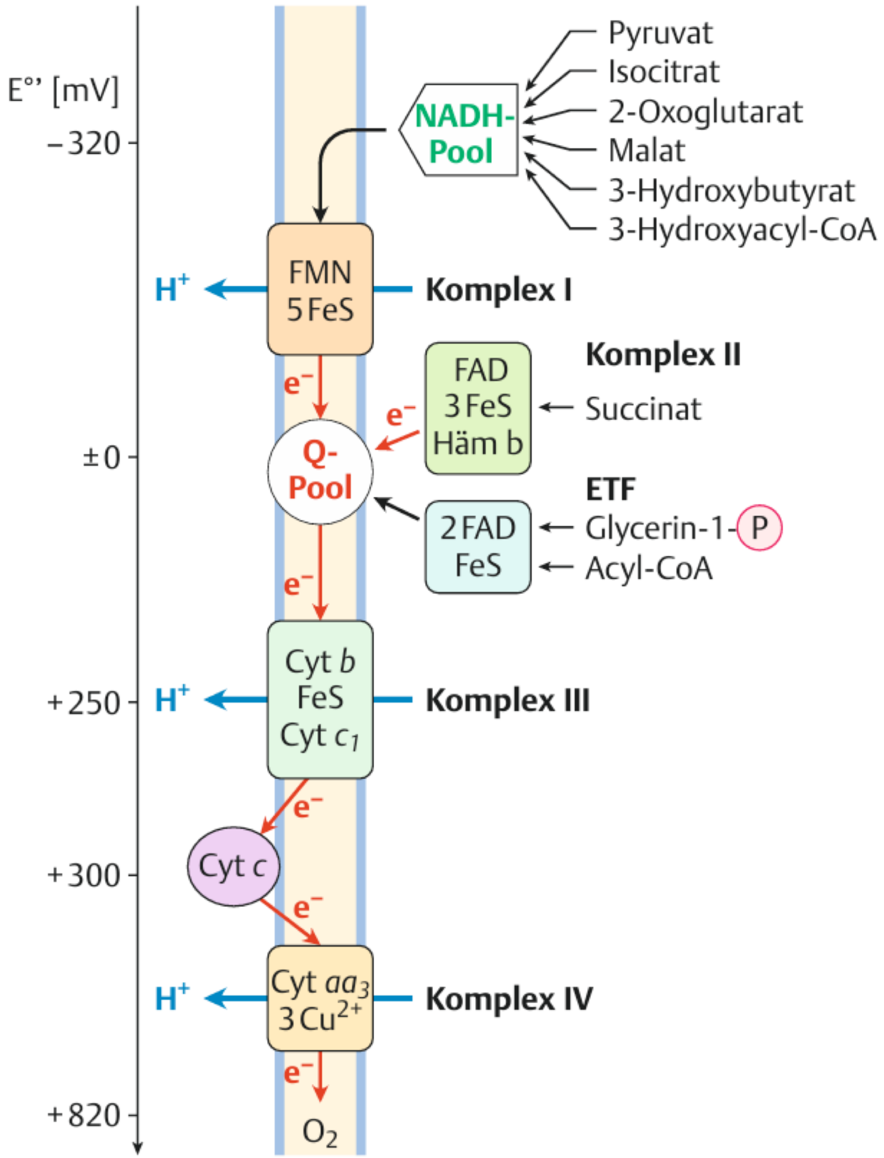
Planctomycetes

***Candidatus* Brocadia fulgida, DQ459989**
***Candidatus* Jettenia asiatica, DQ301513**
***Candidatus* Anammoxoglobus propionicus, DQ317601**
***Candidatus* Kuenenia stuttgartiensis, AF375995**
***Candidatus* Scalindua marina, EF602039**

Redox schemes of inorganic electron donors



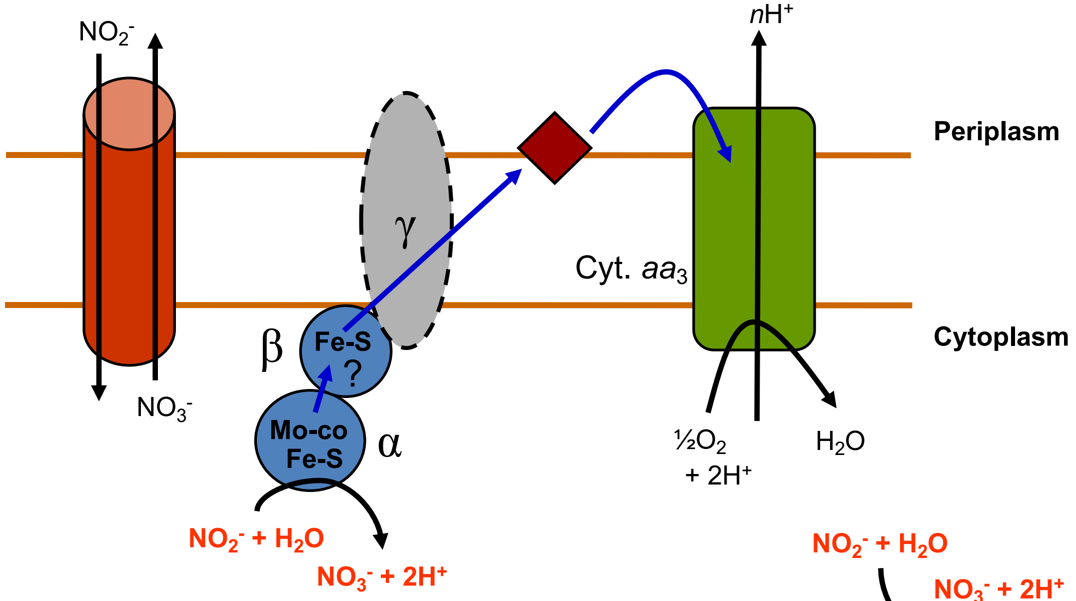


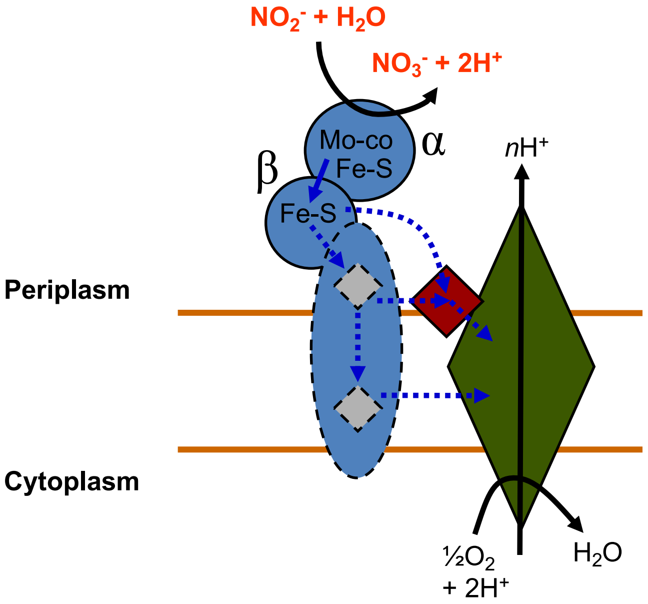


Different mechanisms of nitrite oxidation

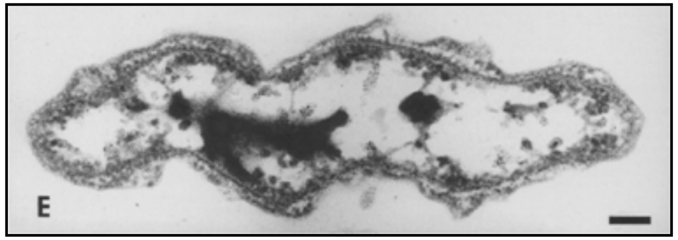
Bock *et al.*, 1991

Nitrobacter





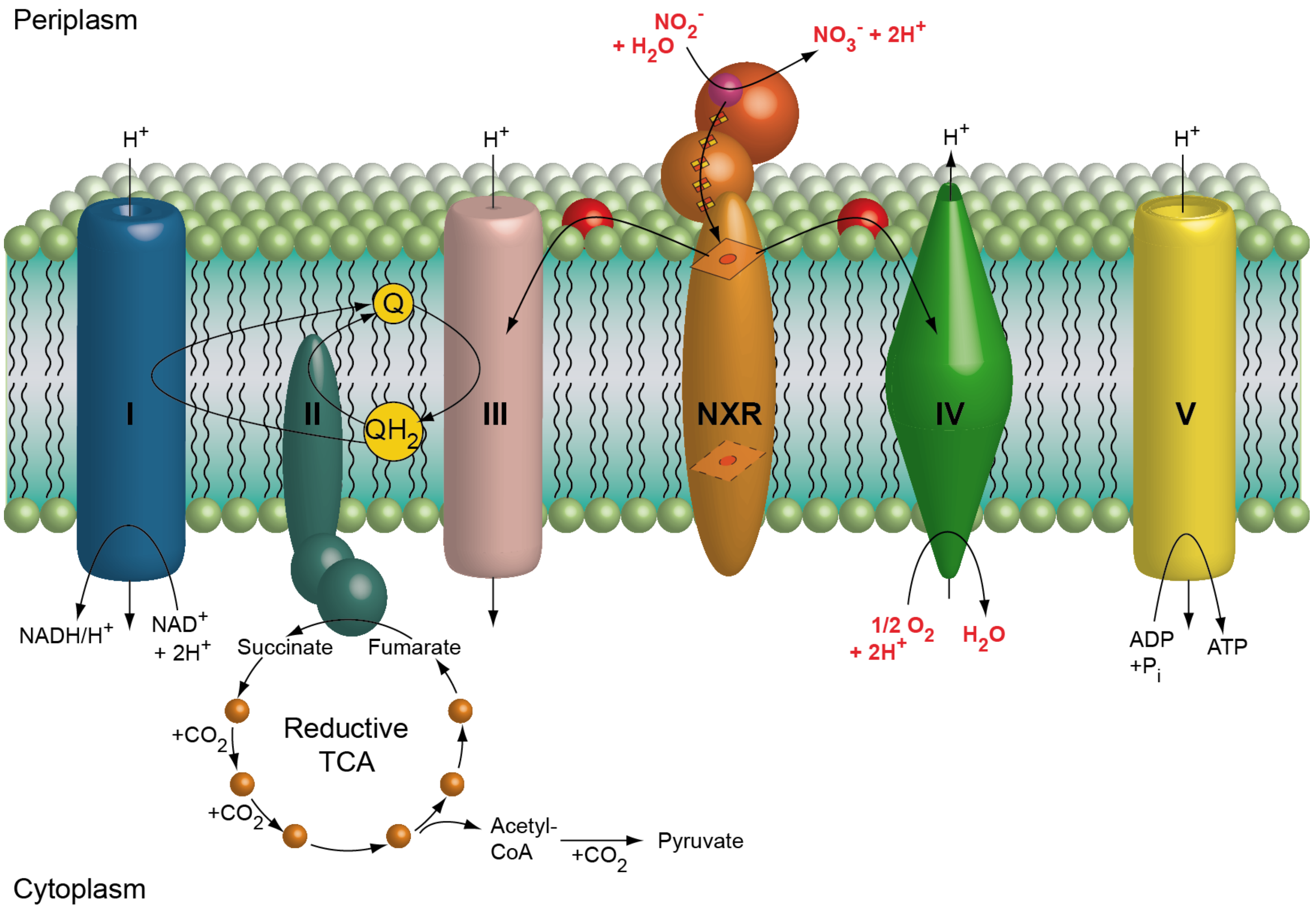
Ehrich *et al.*, 1995



Nitrospira

Lücker *et al.*, 2010

Respiratory chain of *Nitrospira*



Lücker *et al.*, 2010

Outline

- Introduction
- **Complete nitrification by *Nitrospira***
- Novel physiologies of comammox *Nitrospira*
- *In situ* detection of ammonia-oxidizing bacteria
- Ammonia oxidation kinetics of comammox *Nitrospira*

Opinion

TRENDS in Microbiology Vol.14 No.5 May 2006

Full text provided by www.sciencedirect.com
SCIENCE DIRECT

Why is metabolic labour divided in nitrification?

Engràcia Costa[1], Julio Pérez[1] and Jan-Ulrich Kreft[2]

[1]Department of Chemical Engineering, Autonomous University of Barcelona, ETSE-Campus de la UAB, 08193 Bellaterra (Cerdanyola del Vallès), Barcelona, Spain
[2]Theoretical Biology, IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Free energy in ammonia and nitrite oxidation

Ammonia oxidation:

$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$ ($\Delta G^{0'}$= -274.7 kJ·mol^{-1})

Nitrite oxidation

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ ($\Delta G^{0'}$= -74.1 kJ·mol^{-1})

Complete nitrification

$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2H^+$ ($\Delta G^{0'}$= -348.9 kJ·mol^{-1})

Opinion TRENDS in Microbiology Vol.14 No.5 May 2006

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[2]Theoretical Biology, IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

AOB: High growth rates, low yield

r strategist

Comammox: Low growth rates, high yield

K strategist

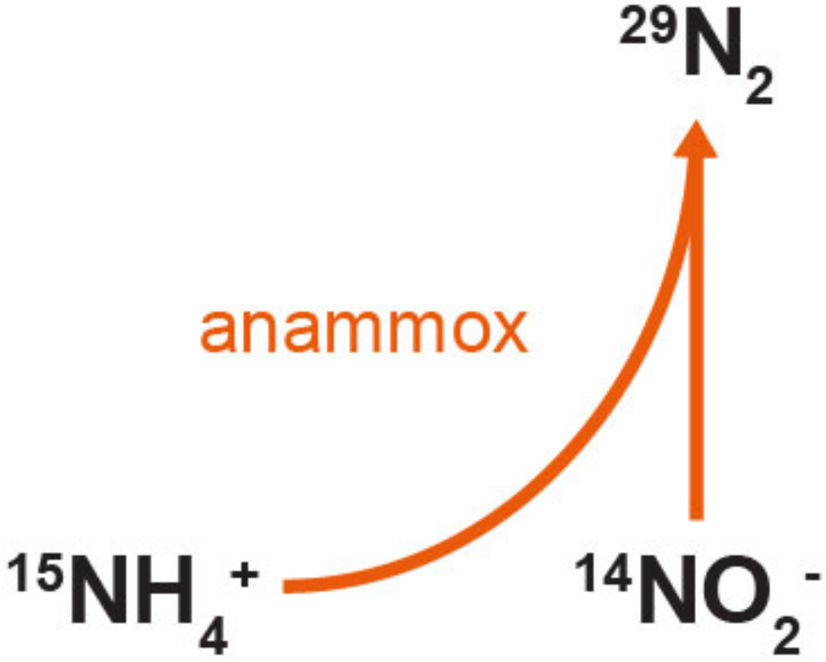
Bioreactor enrichment culture


Maartje van Kessel

- Inoculum:
  - Biofilm from aquaculture biofilter
- Medium:
  - Aquaculture water, supplemented with NH_4^+, NO_2^-, NO_3^-
  - No extra carbon source
- Hypoxic conditions (≤3.1 μM O_2)

van Kessel *et al.* (2015) Nature 528: 555-9

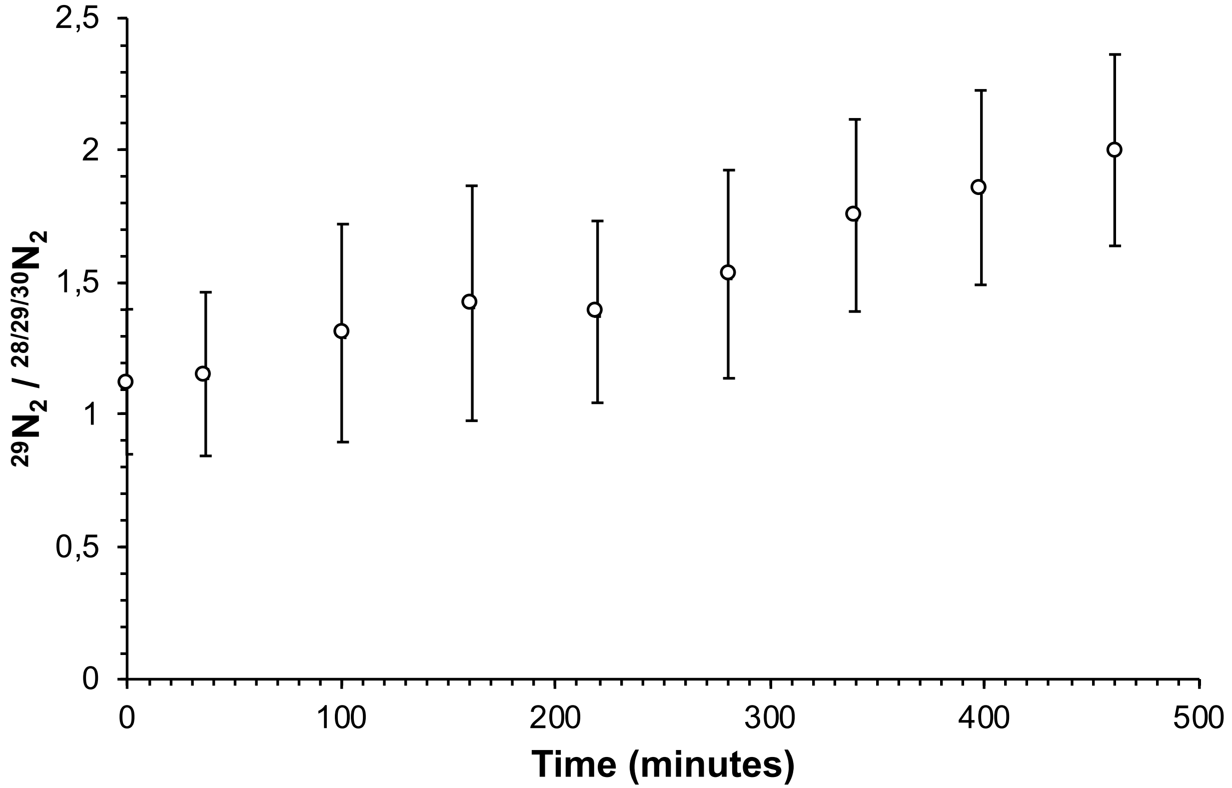
Measuring anammox avtivity



van Kessel *et al.* (2015) Nature 528: 555-9

Anammox activity assays

- Formation of $^{29}N_2$ in incubations with ^{15}N-labelled NH_4^+ confirms anammox activity

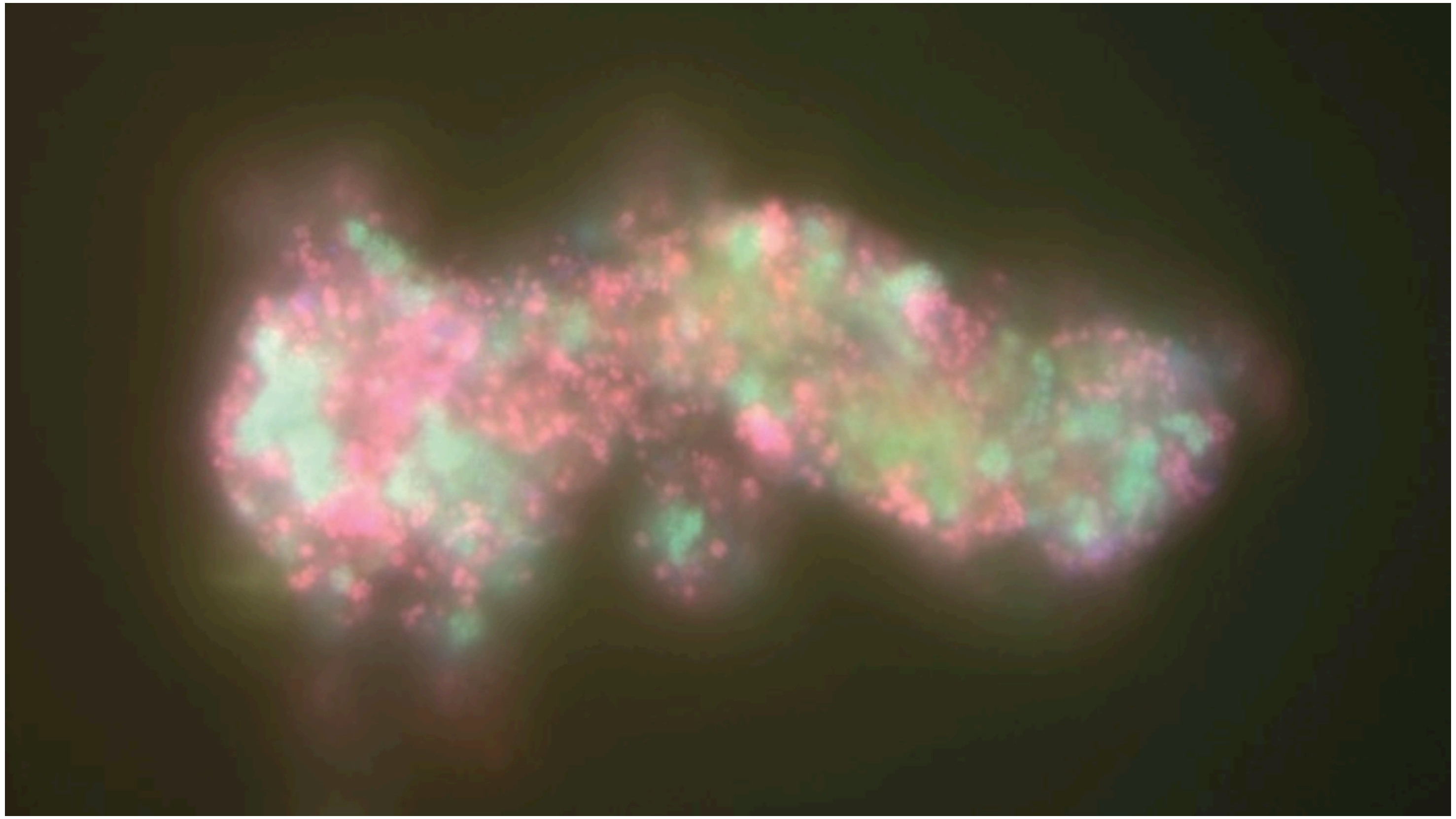


$^{29}N_2$ formation from $^{15}NH_4^+$

van Kessel *et al.* (2015) Nature 528: 555-9

FISH on bioreactor enrichment

- *Nitrospira* are always present in flocs with anammox (*Brocadia*)
- Stable coculture

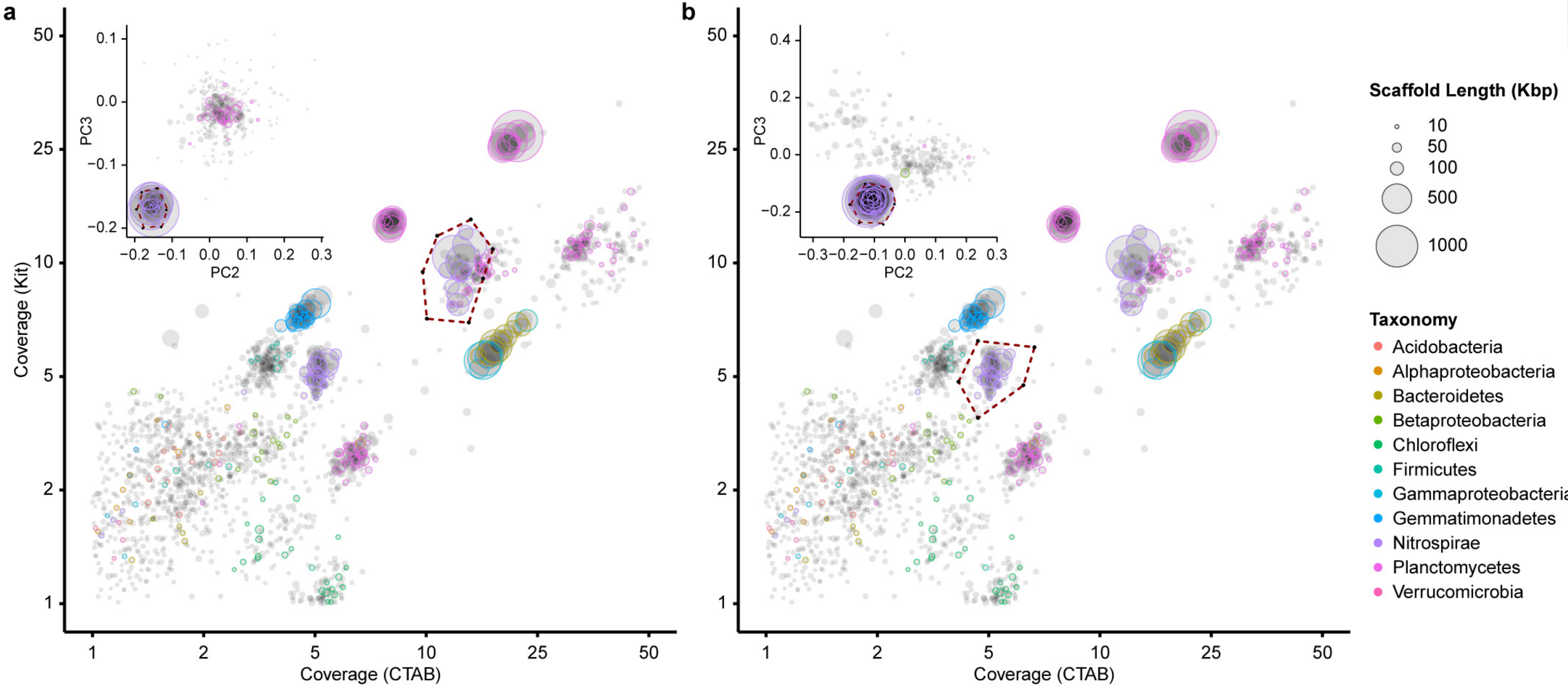


red = anammox; green = *Nitrospira*; blue= all bacteria

van Kessel *et al.* (2015) Nature 528: 555-9

Bioreactor metagenome sequencing

- Recovery of two high quality *Nitrospira* genomes



Mads Albertsen

van Kessel *et al.* (2015) Nature 528: 555-9

16S rRNA phylogeny

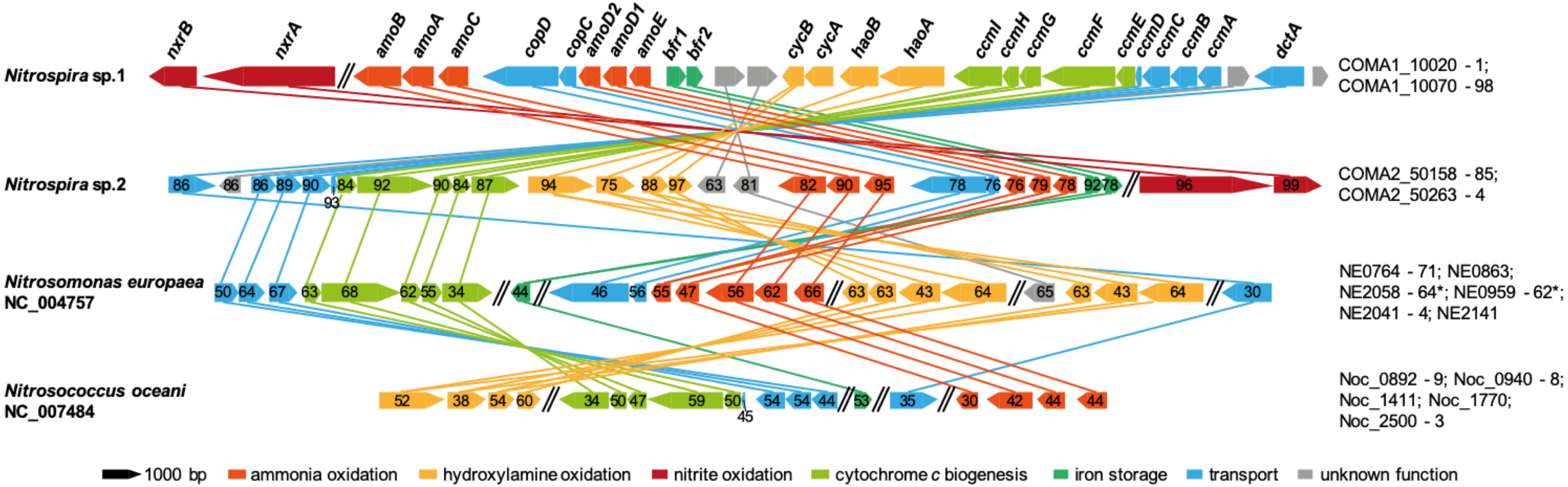
van Kessel *et al.* (2015) Nature 528: 555-9

Metagenomic analyses

- Both *Nitrospira* spp. genomes contain genes for
  - Ammonia monooxygenase
  - Hydroxylamine dehydrogenase
  - Nitrite oxidoreductase

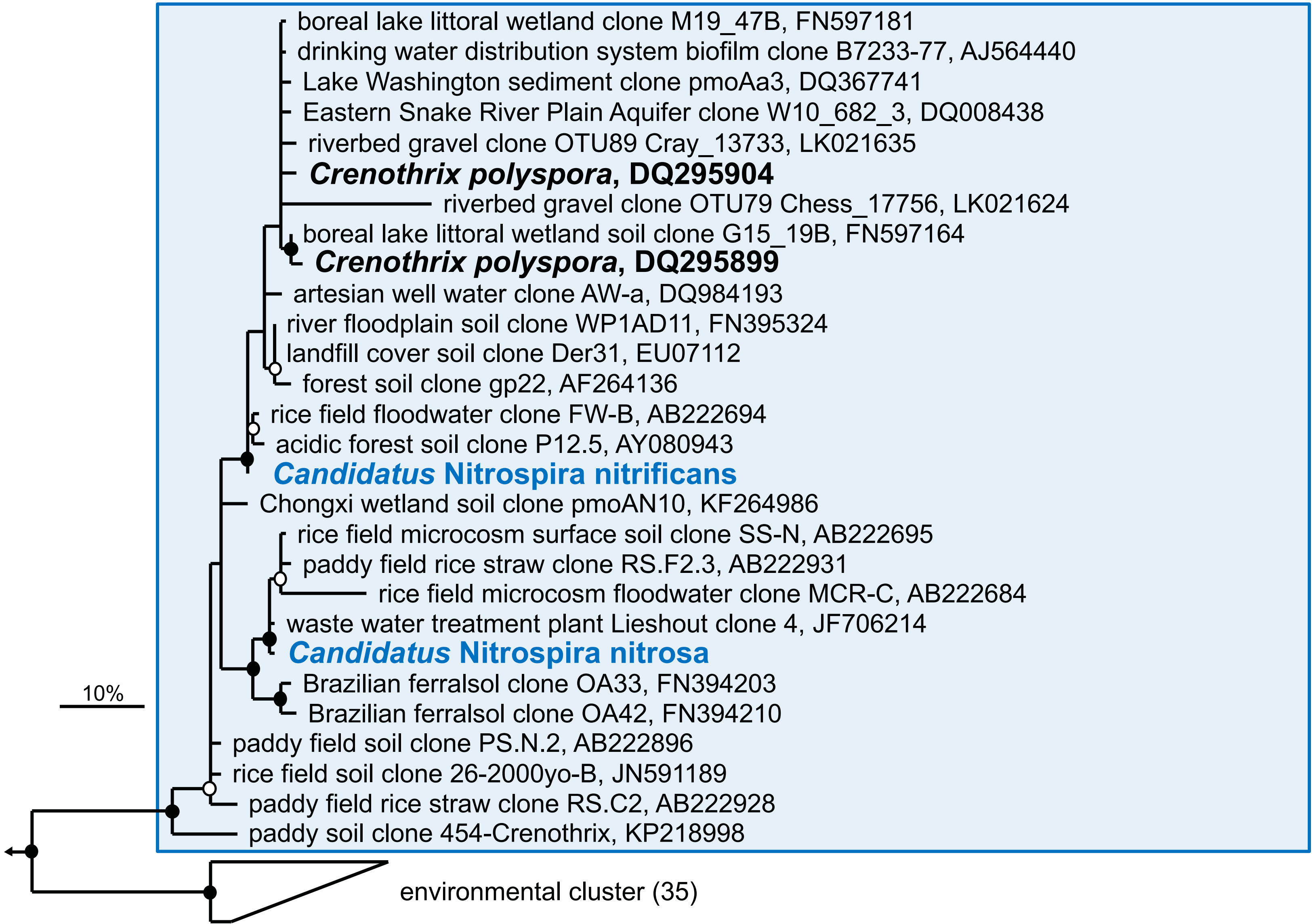
Only in ammonia oxidizers

Only in nitrite oxidizers



van Kessel *et al.* (2015) Nature 528: 555-9

Ammonia monooxygenase (amoA) phylogeny



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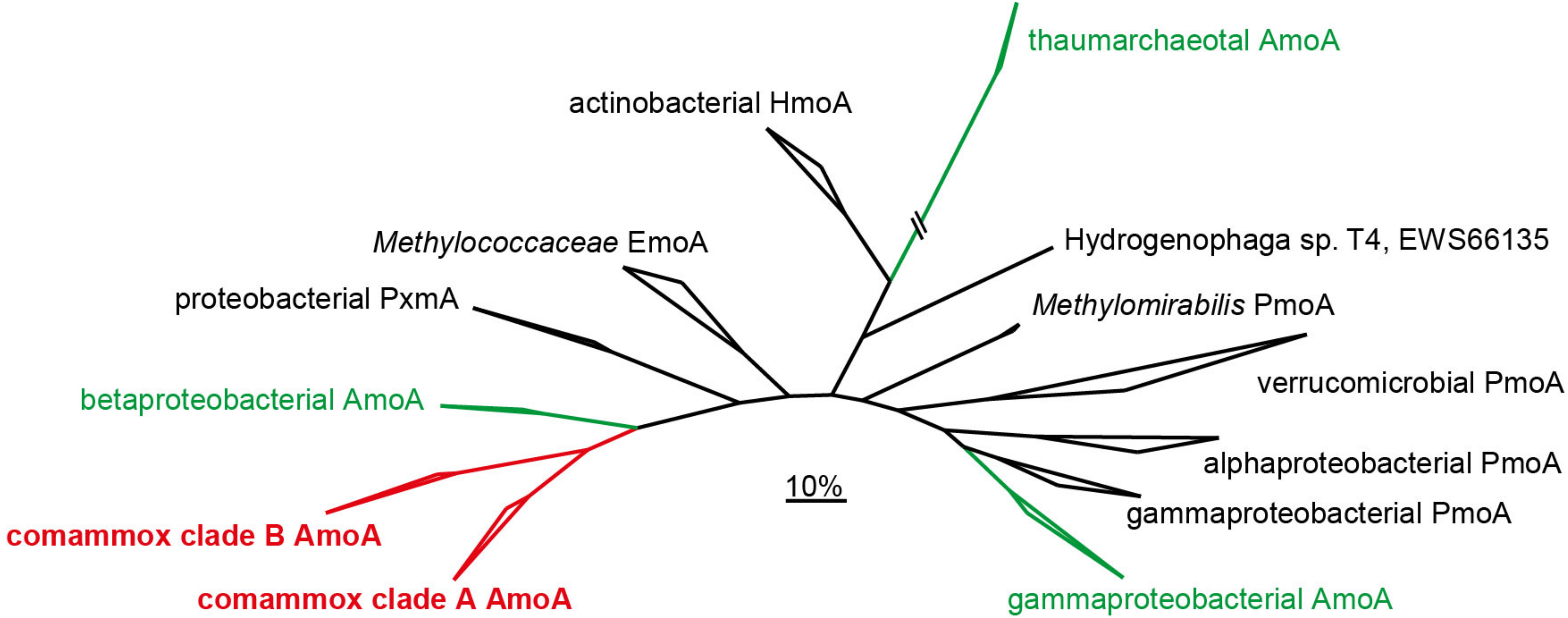
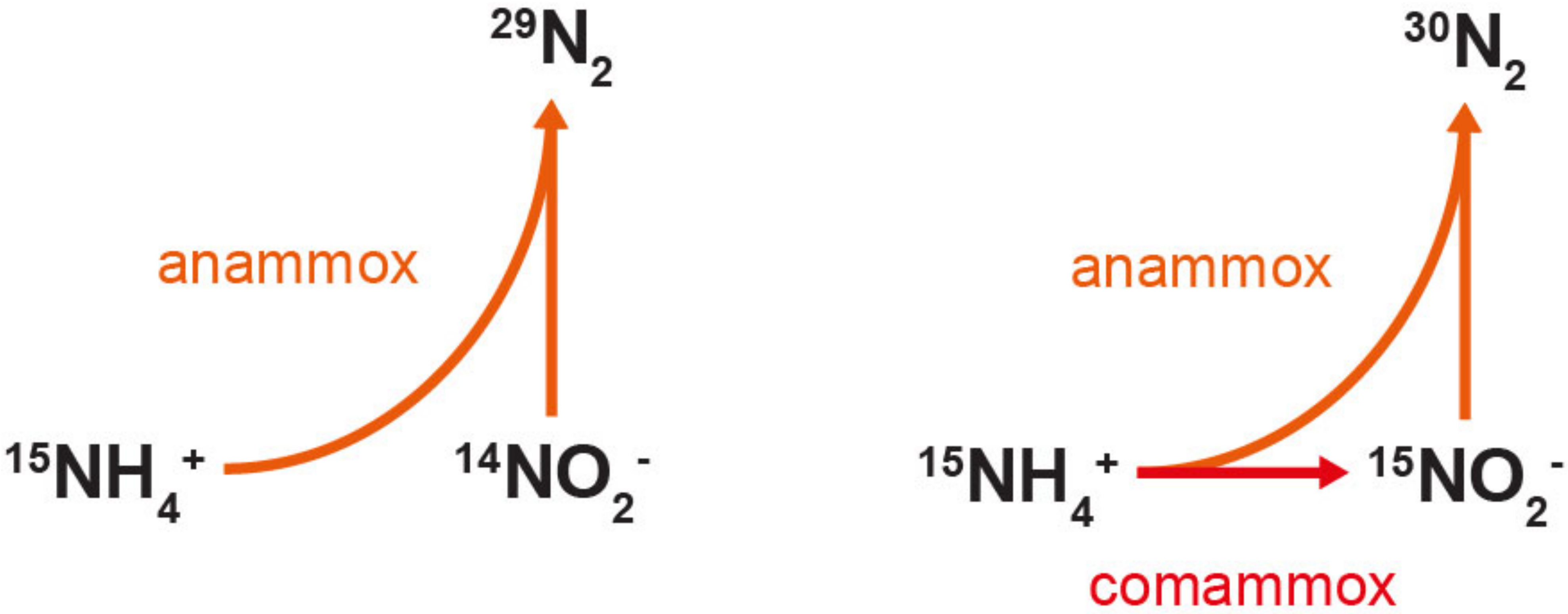


van Kessel *et al.* (2015) Nature 528: 555-9

Determining comammox activity via the anammox process



$^{29}N_2$

anammox

$^{15}NH_4^+$

$^{14}NO_2^-$

$^{30}N_2$

anammox

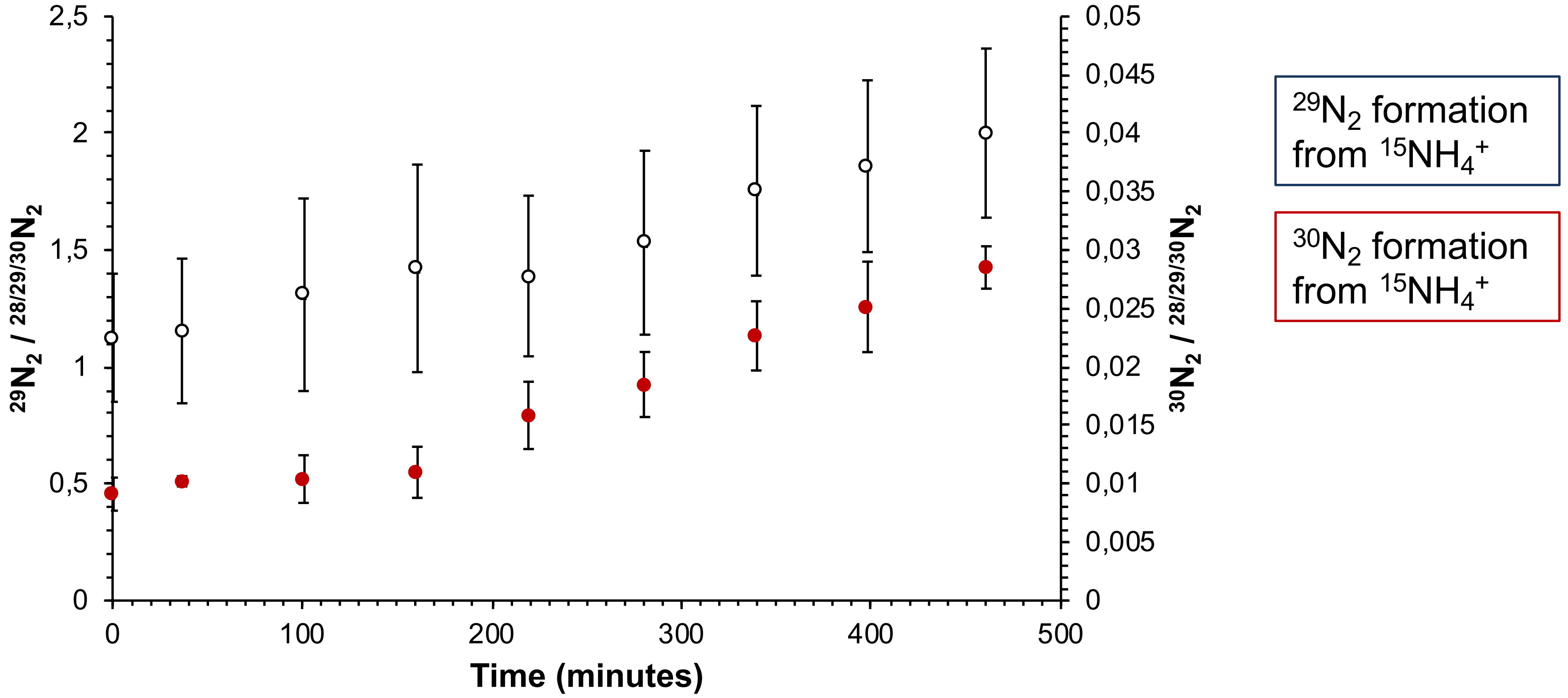
$^{15}NH_4^+$

$^{15}NO_2^-$

comammox

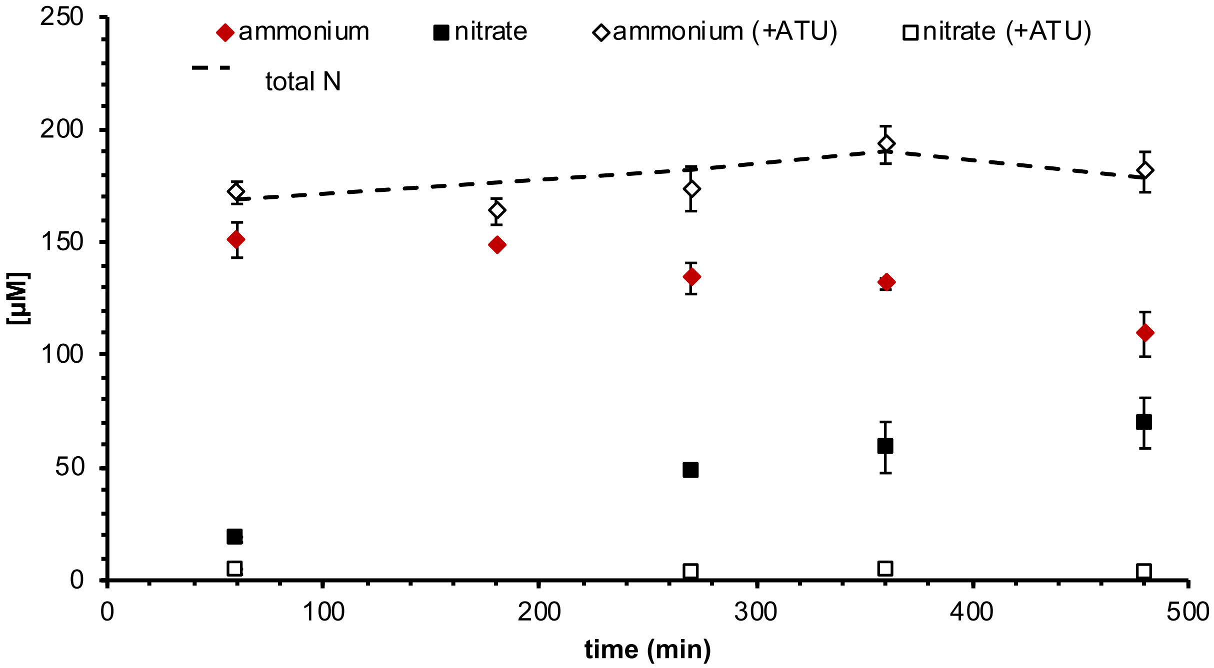
Anammox activity assays

- Formation of $^{30}N_2$ from $^{15}NH_4^+$ indicates ammonia oxidation



van Kessel *et al.* (2015) Nature 528: 555-9

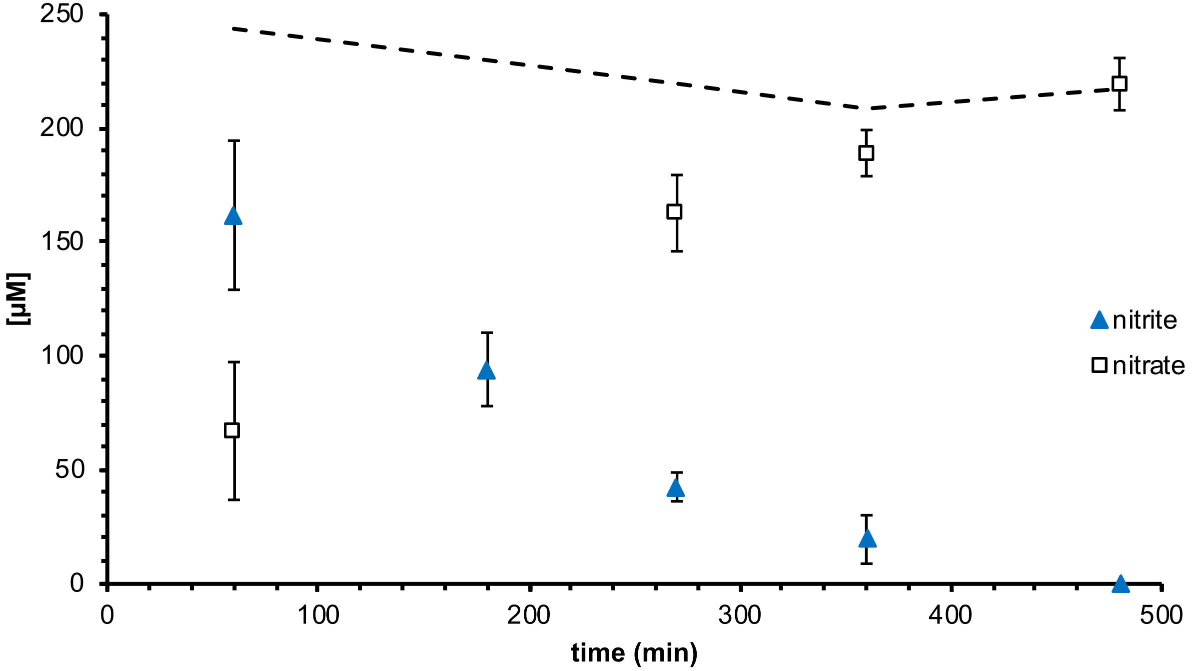
Aerobic batch incubation assays



NH_4^+ + ATU

NH_4^+ oxidation

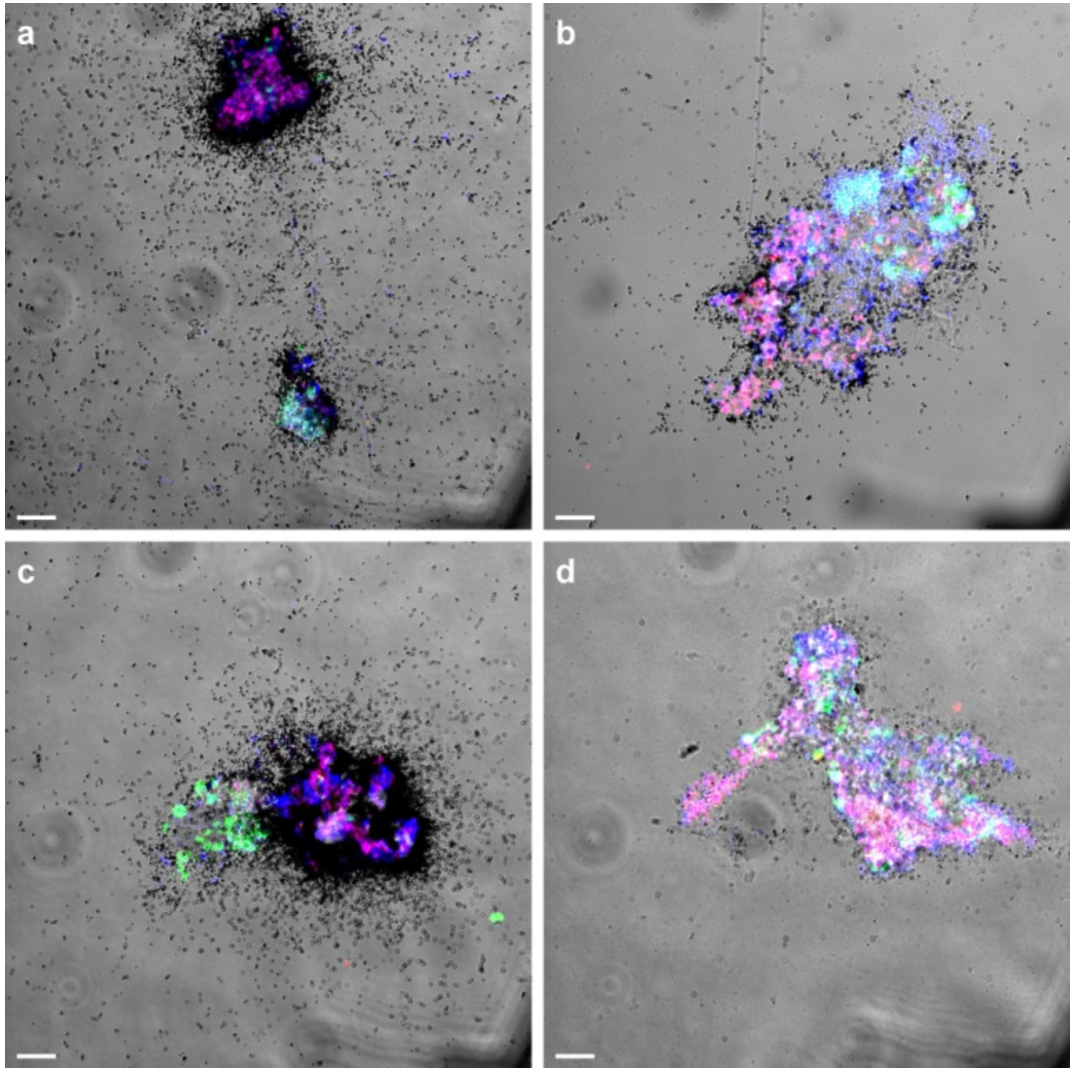
NO_2^- oxidation



van Kessel *et al.* (2015) Nature 528: 555-9

Ammonia-dependent carbon fixation

NH_4^+



NH_4^+ + ATU

NO_2^-

no substrate

red= *Nitrospira*; green = anammox; blue= all bacteria

van Kessel *et al.* (2015) Nature 528: 555-9

Conclusions I

- Novel *Nitrospira* spp. are complete nitrifiers
- Cooperation between anammox and comammox possible

Outline

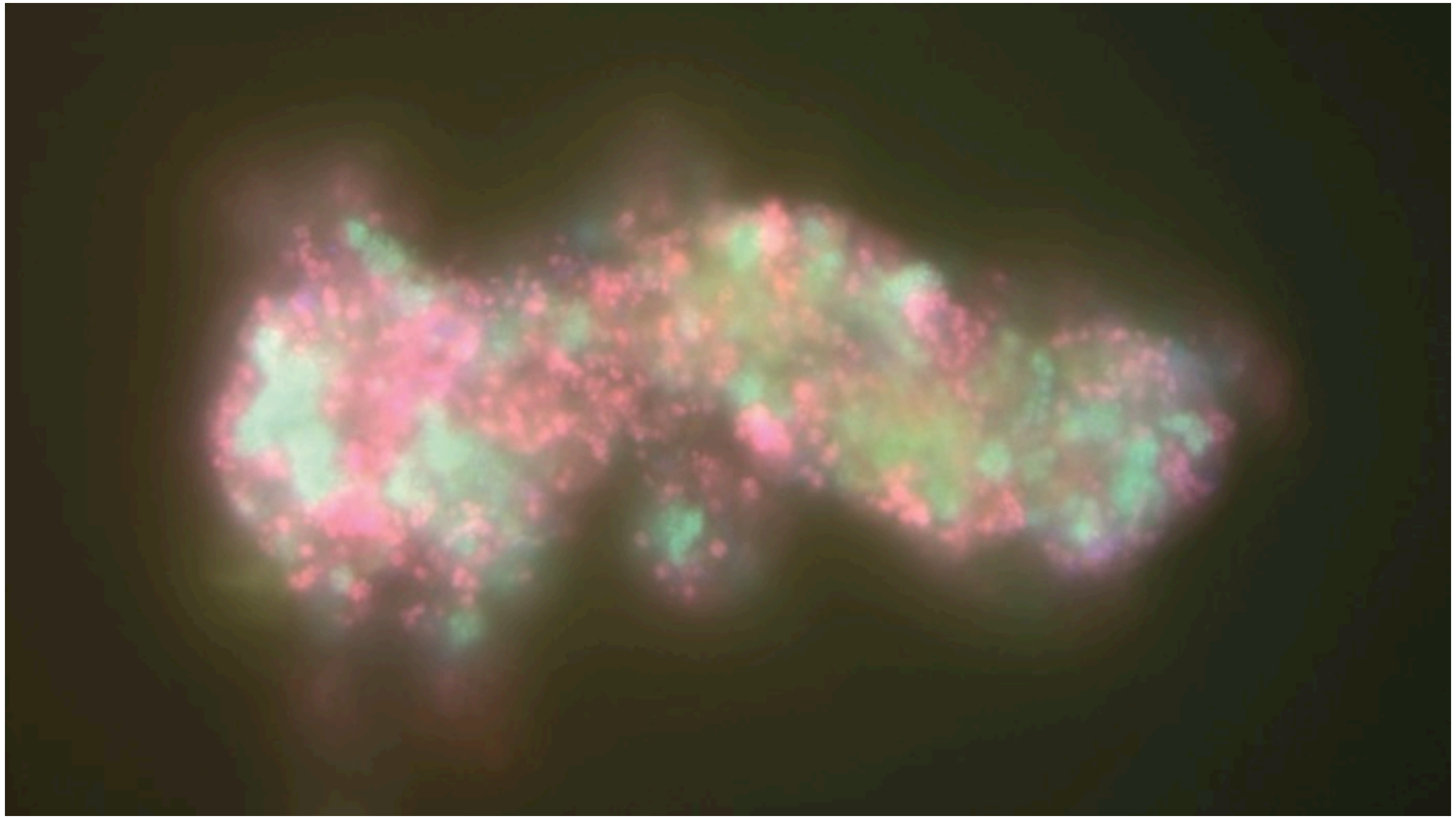
- Introduction
- Complete nitrification by *Nitrospira*
- **Novel physiologies of comammox *Nitrospira***
- *In situ* detection of ammonia-oxidizing bacteria
- Ammonia oxidation kinetics of comammox *Nitrospira*

Original comammox/anammox enrichment culture

- Inoculum:
  - Biofilm from aquaculture biofilter
- Medium:
  - **Aquaculture water**, supplemented with NH_4^+, NO_2^-, NO_3^-
  - No extra carbon source
- Hypoxic conditions ($\leq 3.1\ \mu M\ O_2$)

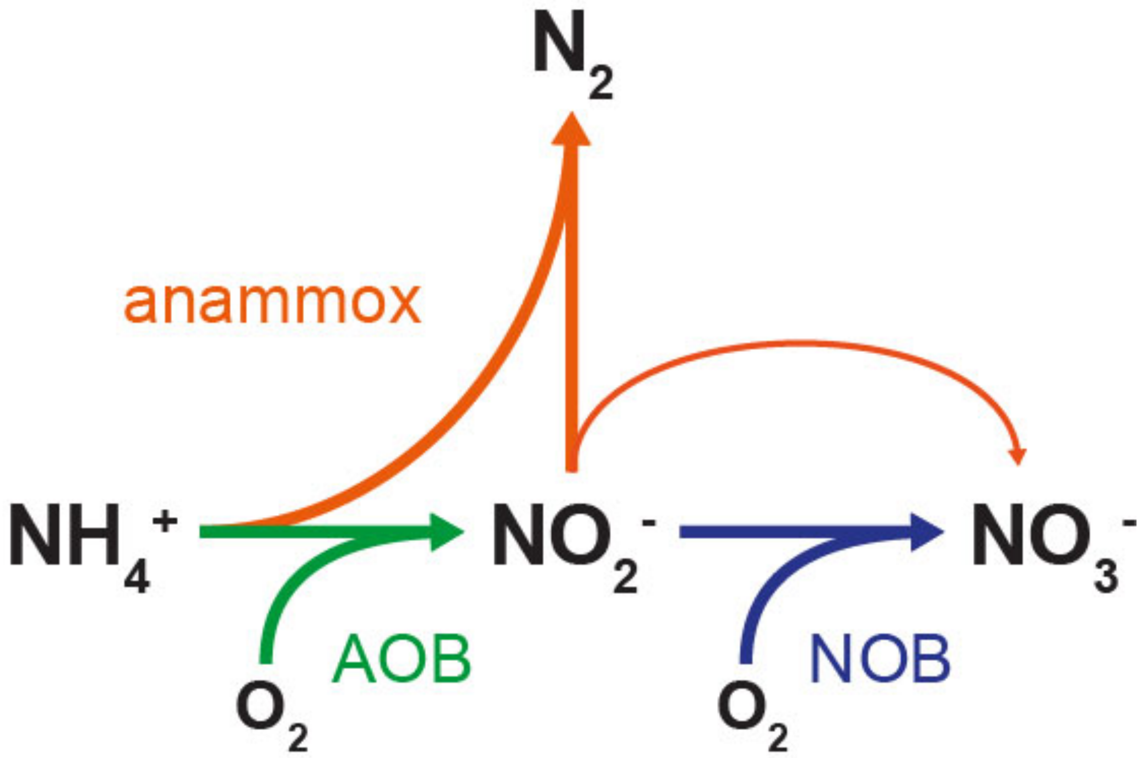
FISH on bioreactor enrichment

- Comammox *Nitrospira* are always present in flocs with anammox (*Brocadia*)
- Stable coculture



red = anammox; green = *Nitrospira*; blue= all bacteria

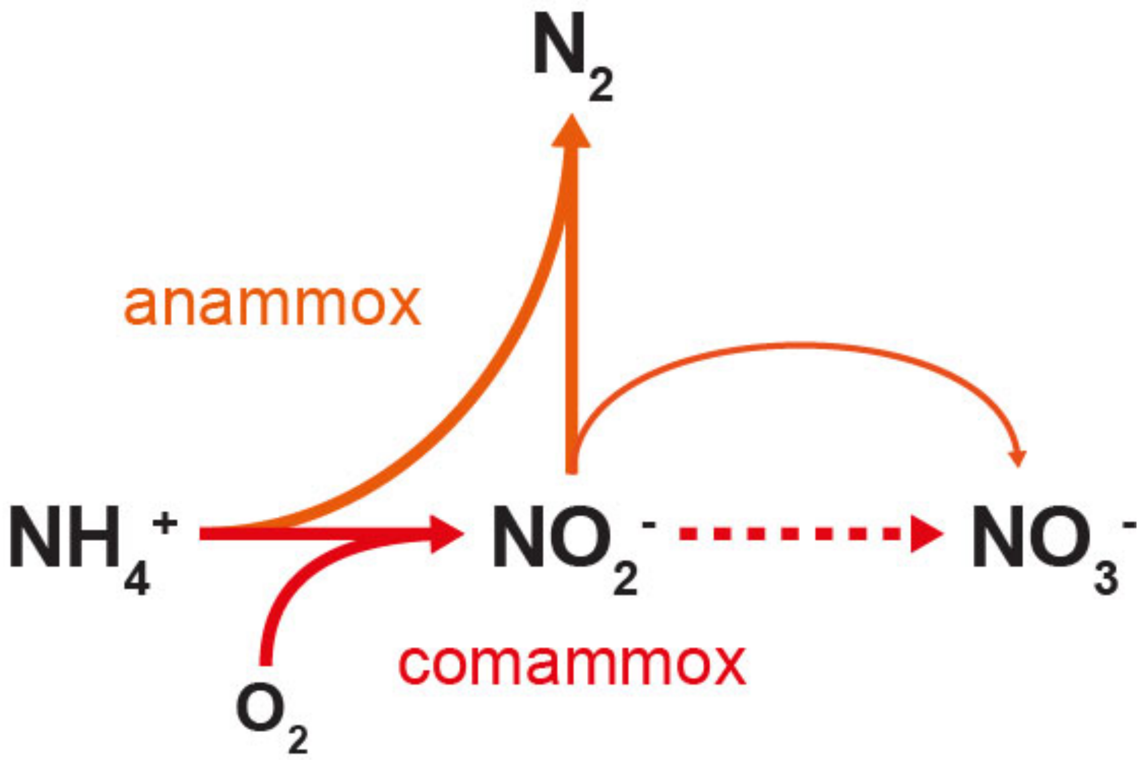
Interaction and competition between anammox and nitrifiers



Complete nitrification

$NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2H^+ \quad (\Delta G^{0\prime} = -348.9\ kJ \cdot mol^{-1})$

Alternative metabolisms of comammox under O_2 limitation

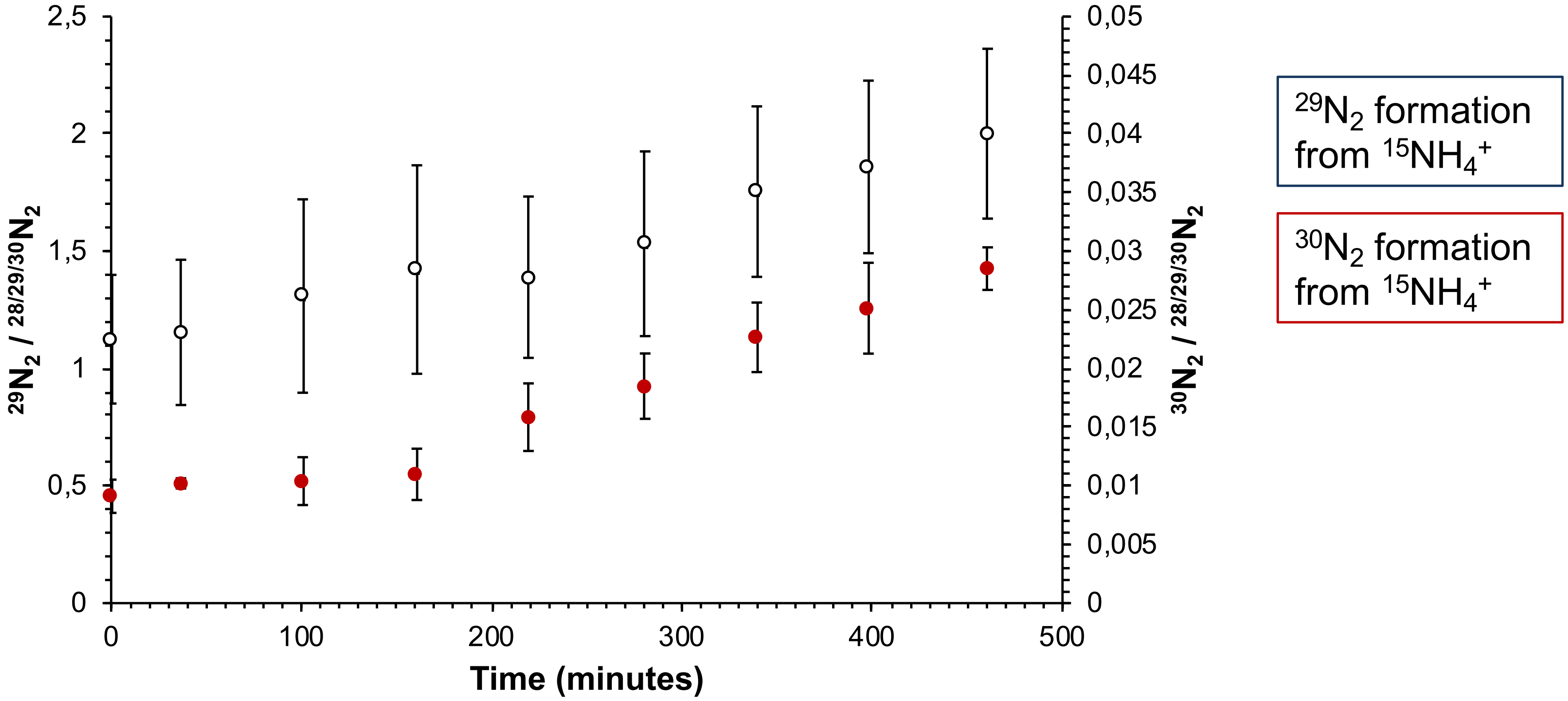


Ammonia oxidation:

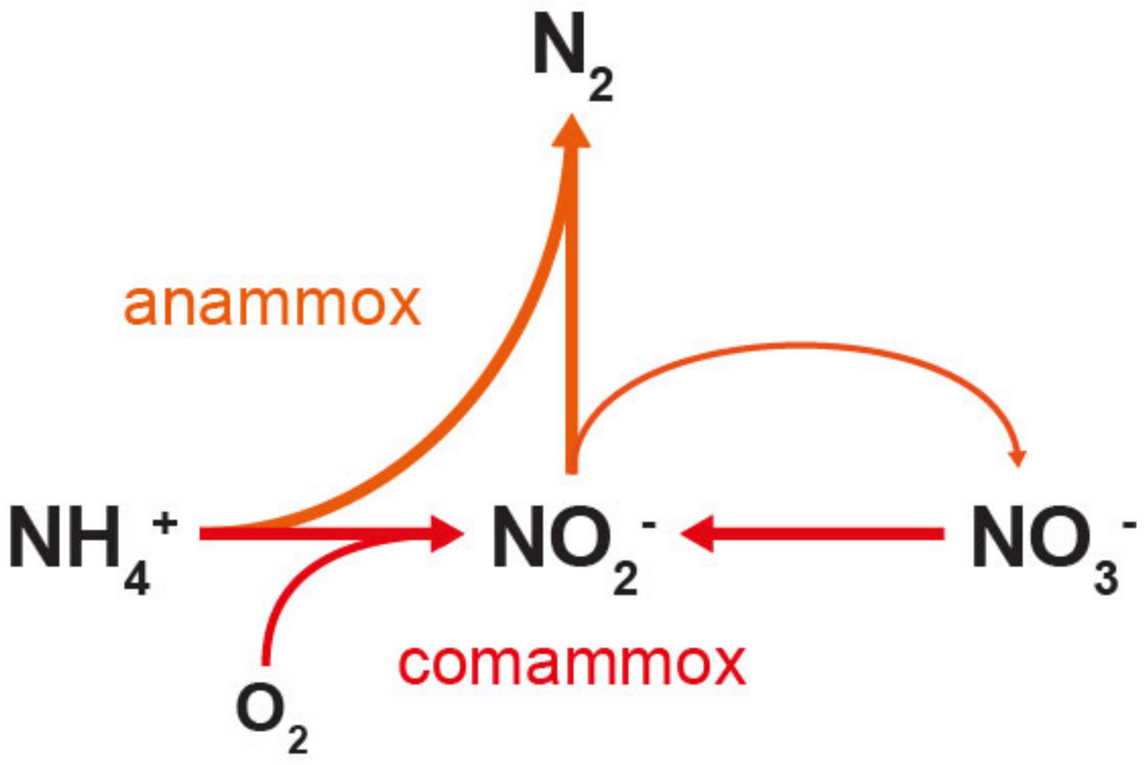
$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$ ($\Delta G^{0'}$= -274.7 kJ·mol^{-1})

Anammox activity assays

- Formation of $^{30}N_2$ from $^{15}NH_4^+$ indicates ammonia oxidation under hypoxic conditions



Alternative metabolisms of comammox under O_2 limitation

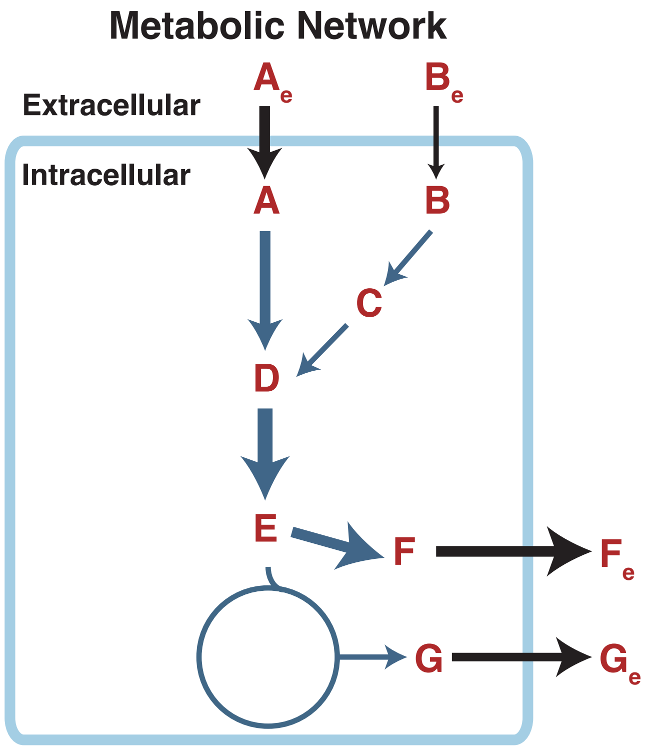


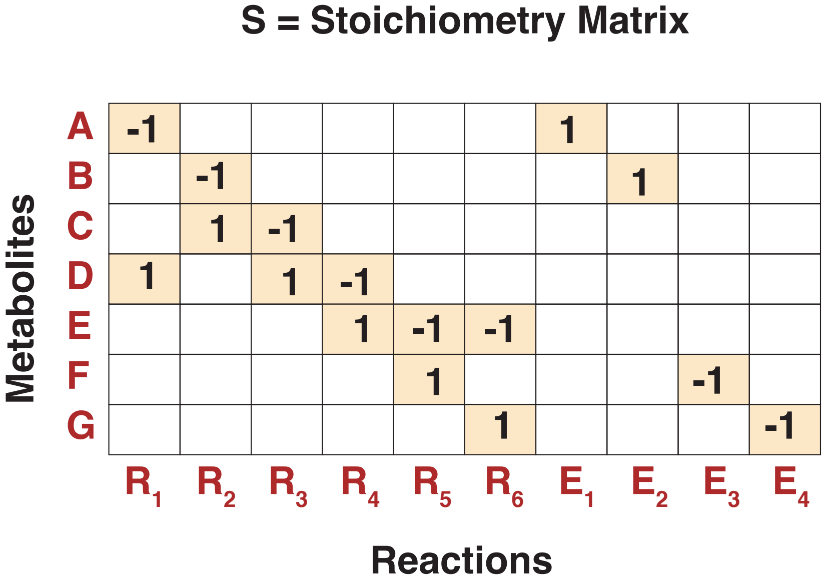
Nitrite comproportionation

$$NH_4^+ + NO_3^- + O_2 \quad \rightarrow \quad 2\ NO_2^- + H_2O + 2H^+ \qquad (\Delta G^{0'} = -200.6\ kJ\cdot mol^{-1})$$

Simulate phenotype – metabolic modelling

Christopher Lawson





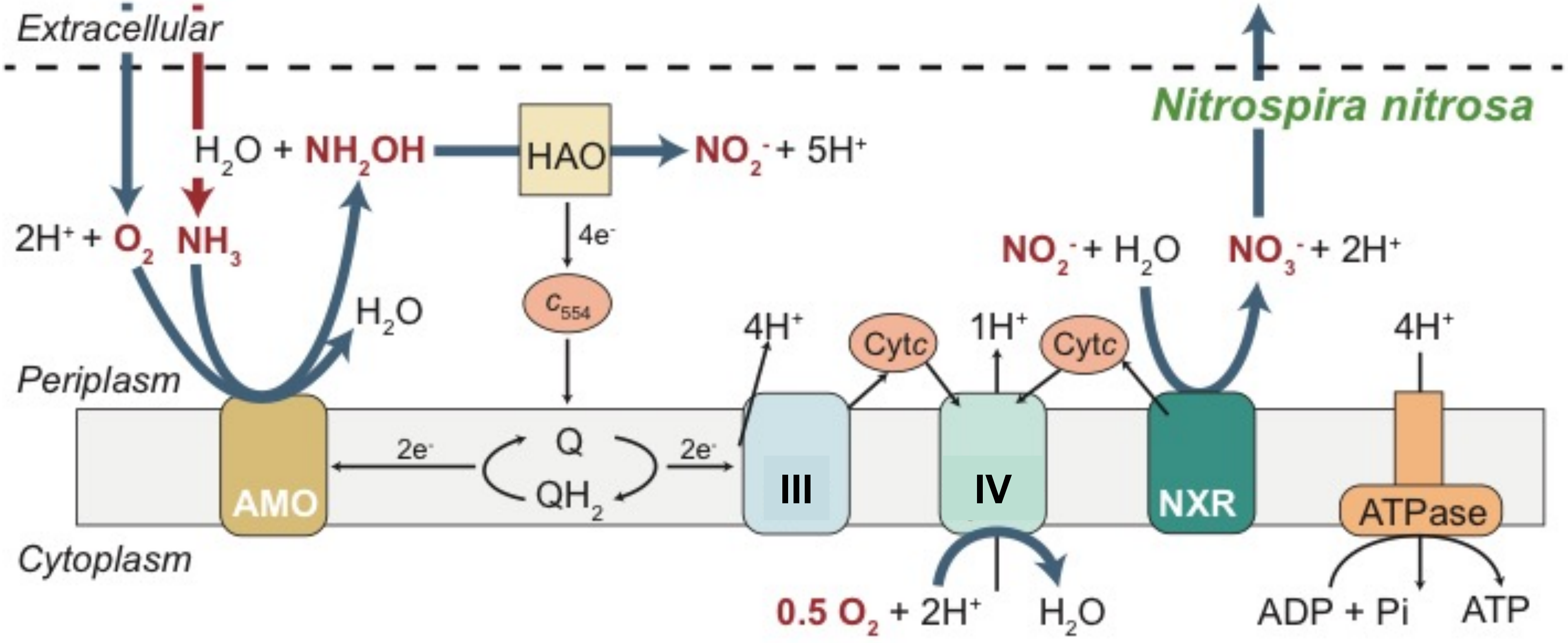
S = Stoichiometry Matrix

Metabolites	R_1	R_2	R_3	R_4	R_5	R_6	E_1	E_2	E_3	E_4
A	-1						1			
B		-1						1		
C		1	-1							
D	1		1	-1						
E				1	-1	-1				
F					1				-1	
G						1				-1

Reactions

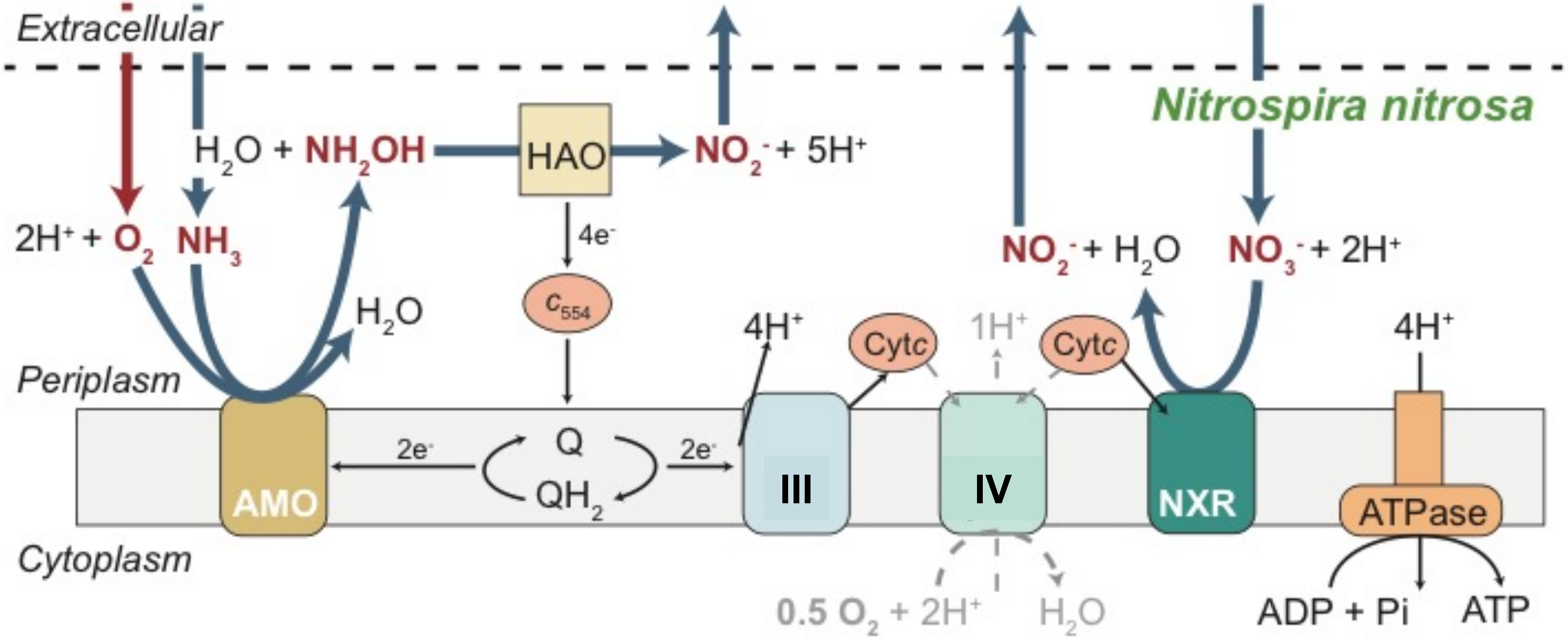
Aerobic, complete ammonia oxidation

NH_4^+ limiting



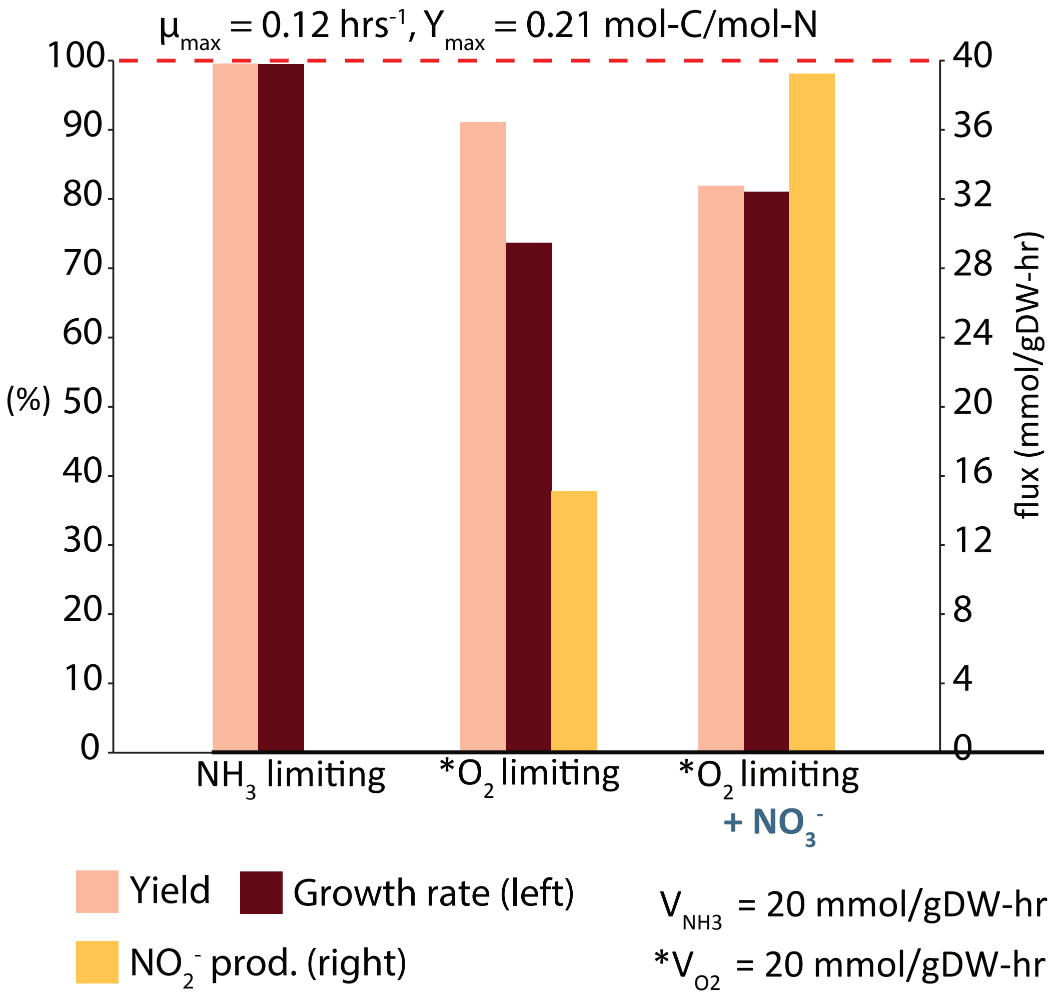
Nitrite comproportionation in the presence of nitrate

O_2 limiting + NO_3^-



→ Maximizes O_2 flux to AMO and NO_2^- production

How does O_2 control comammox activity?



V_{NH3} = 20 mmol/gDW-hr

*V_{O2} = 20 mmol/gDW-hr

NH_3 limiting:

$NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$

O_2 limiting:

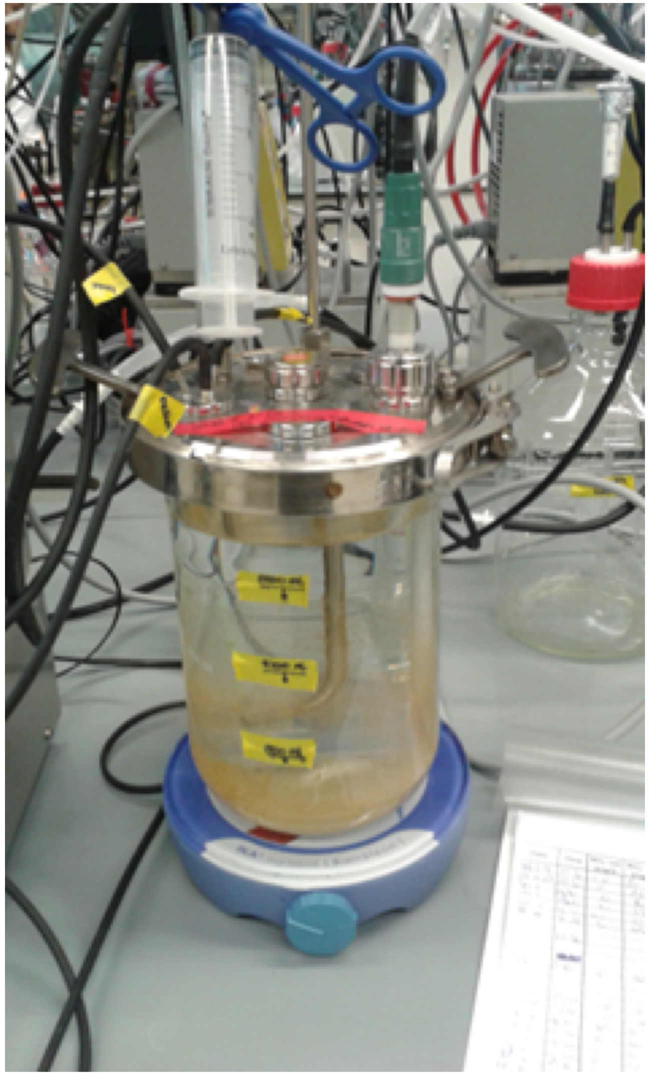
$NH_3 \rightarrow NO_2^-$

O_2 limiting + NO_3^-:

$NH_3 \rightarrow NO_2^- \leftarrow NO_3^-$

NO_2^- production and growth are maximized under O_2 limiting fluxes

Anammox/comammox coculture in synthetic medium



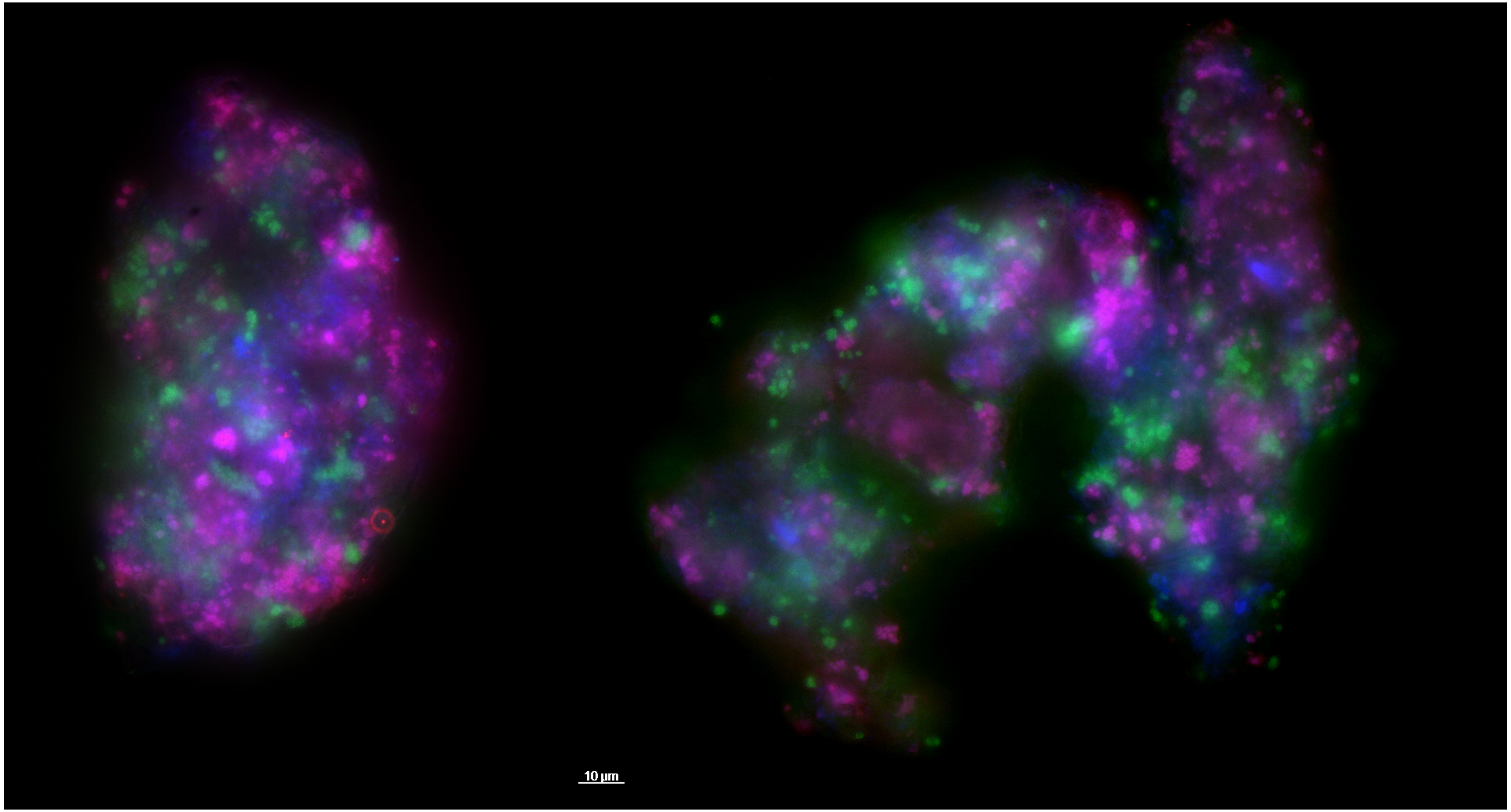
Maartje van Kessel

- **Mineral medium:**

– $[NH_4^+]$	100-200 µM
– $[NO_2^-]$	90-180 µM
– $[NO_3^-]$	250 µM
– Carbon source	CO_2

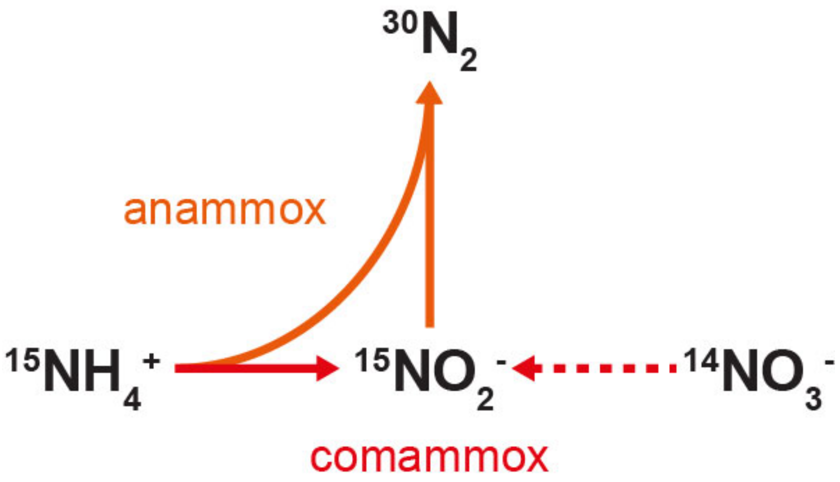
- **Hypoxic**

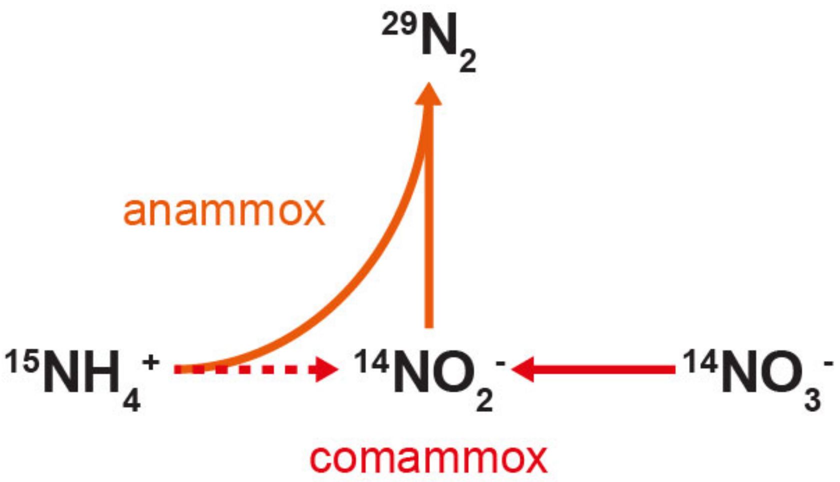
Stable anammox/comammox coculture



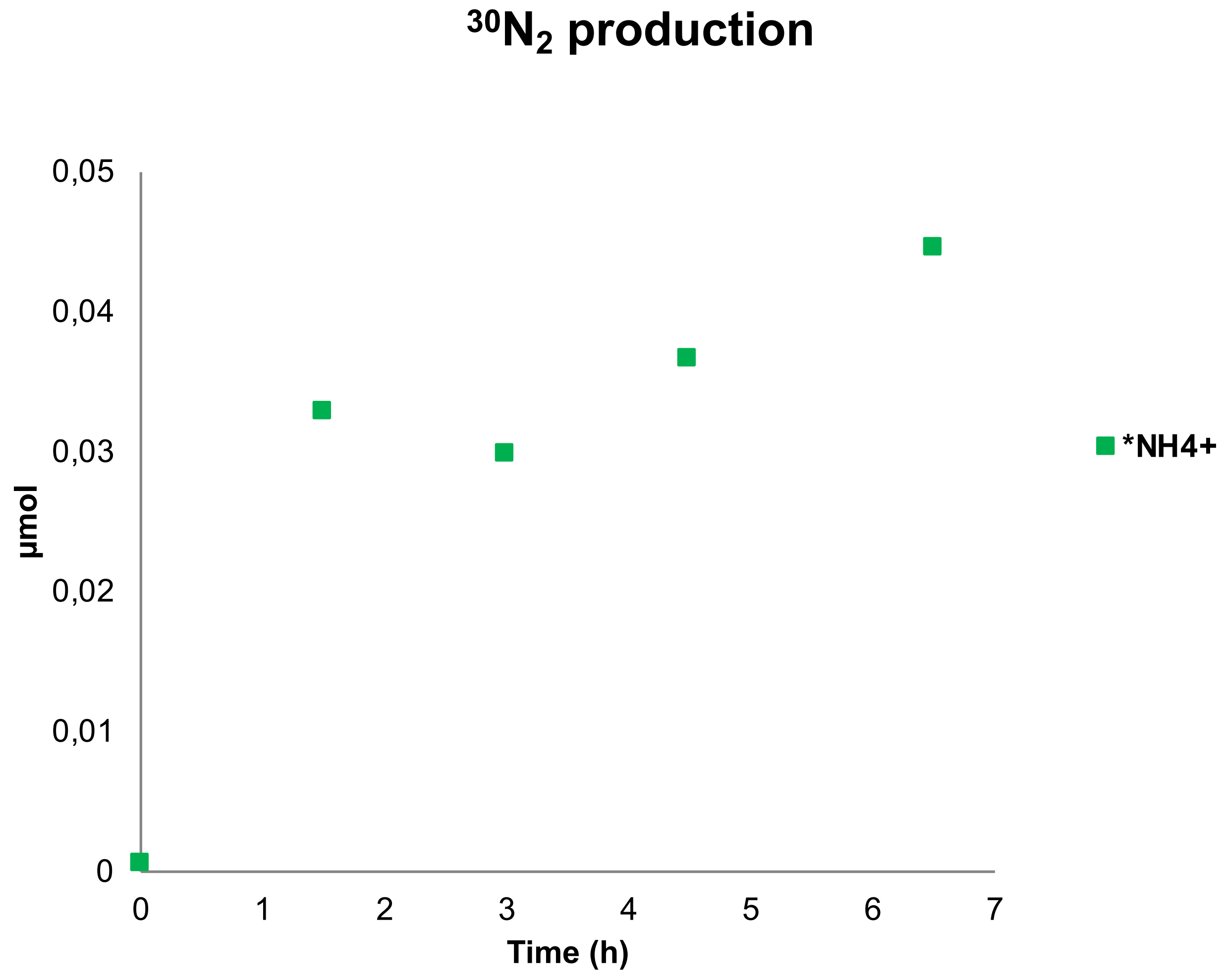
anammox, *Nitrospira*, all bacteria

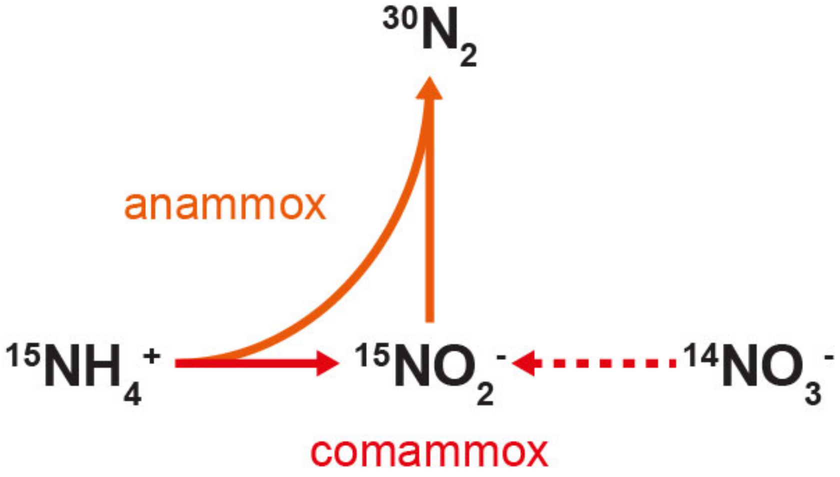
Determining comammox/anammox interactions



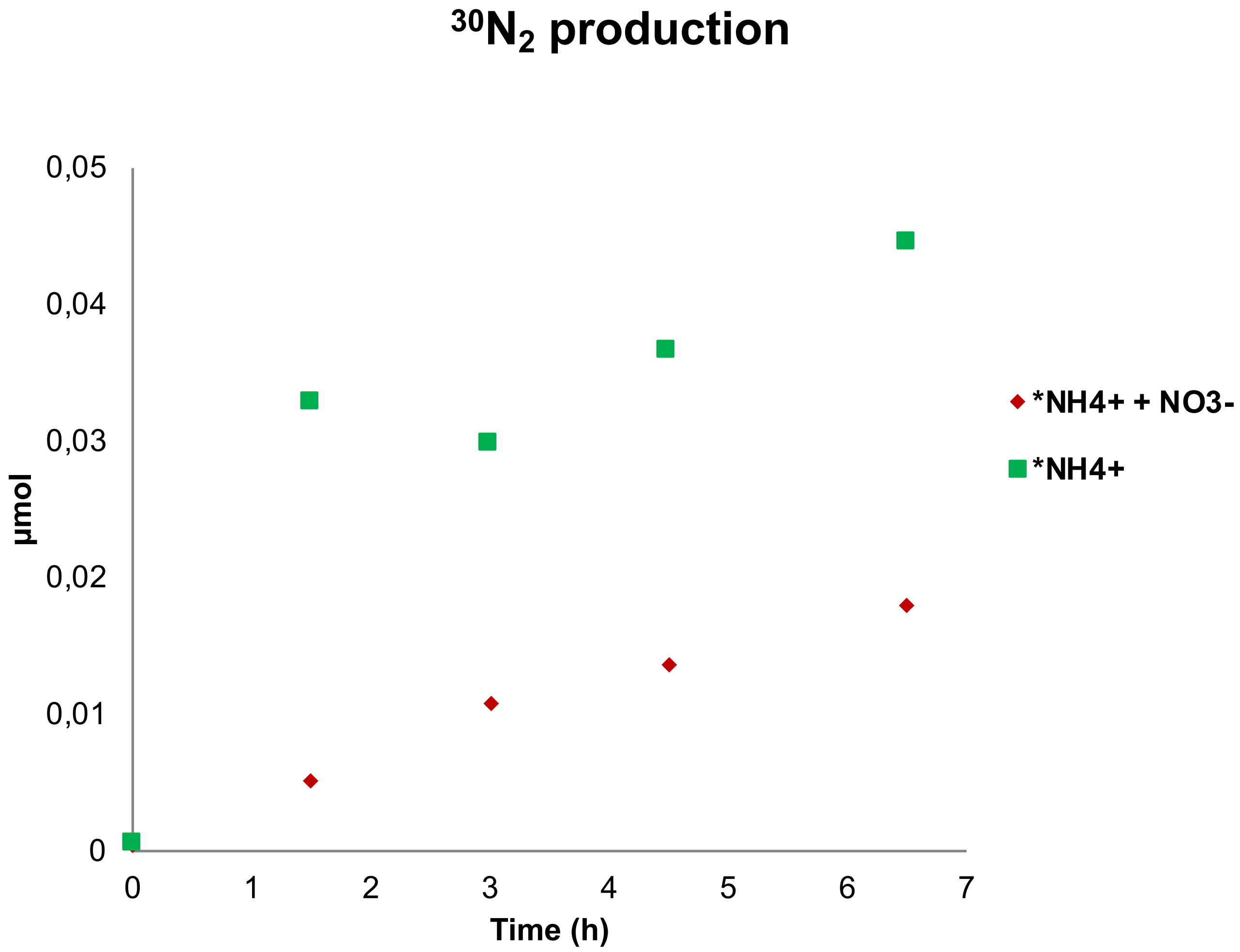


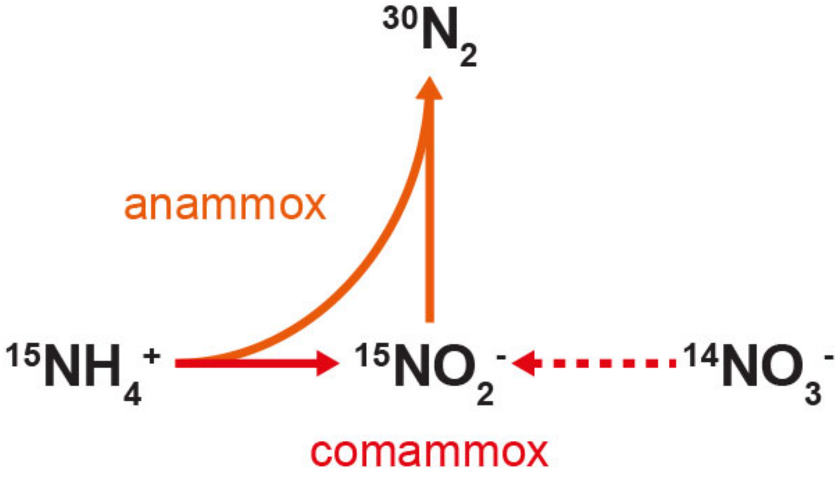
$^{30}N_2$ production indicates ammonia oxidation


$^{30}N_2$ production

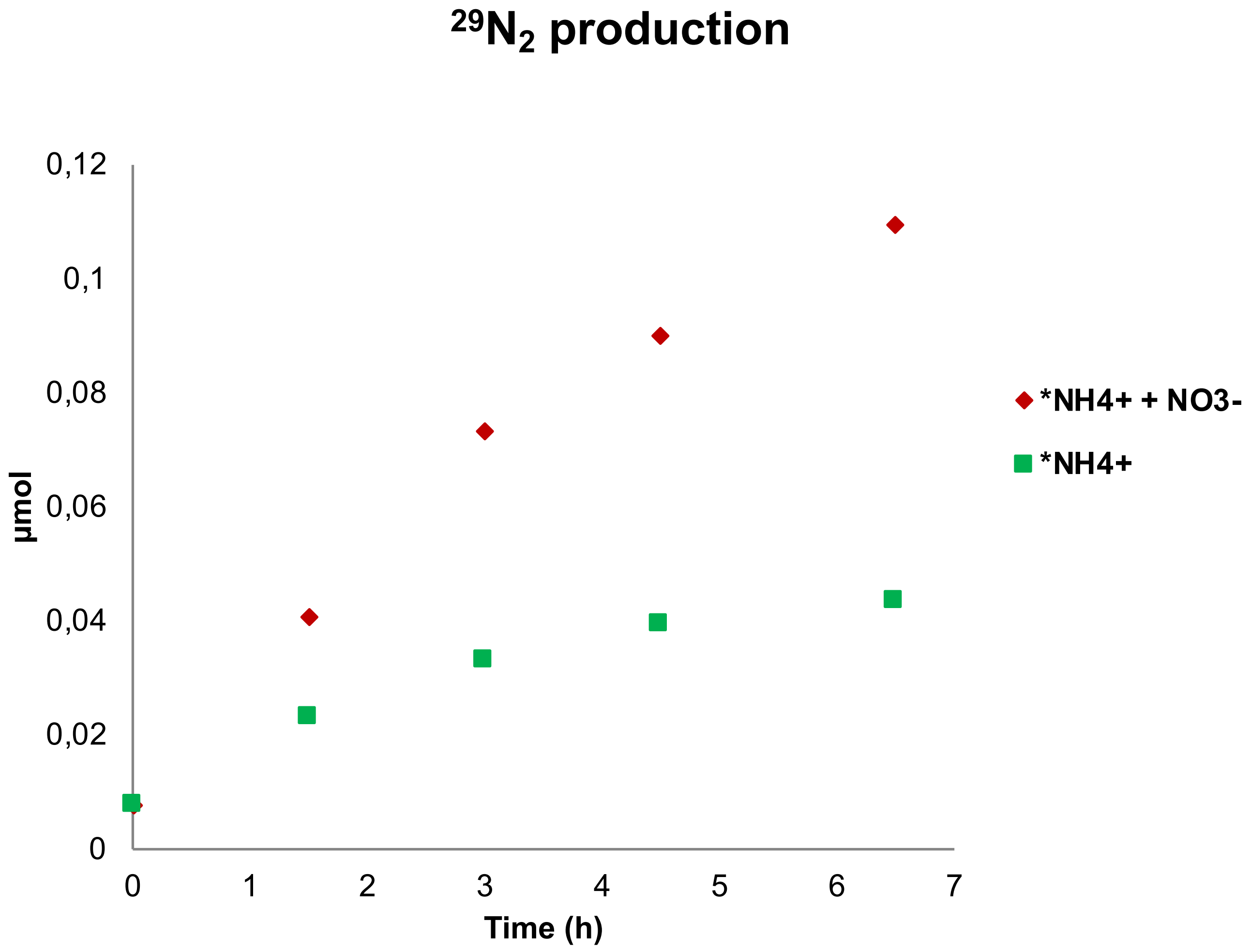


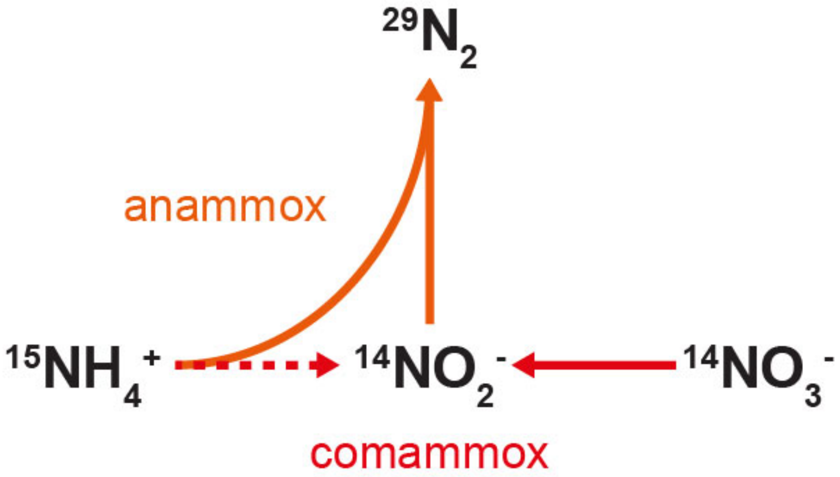
$^{30}N_2$ production indicates ammonia oxidation



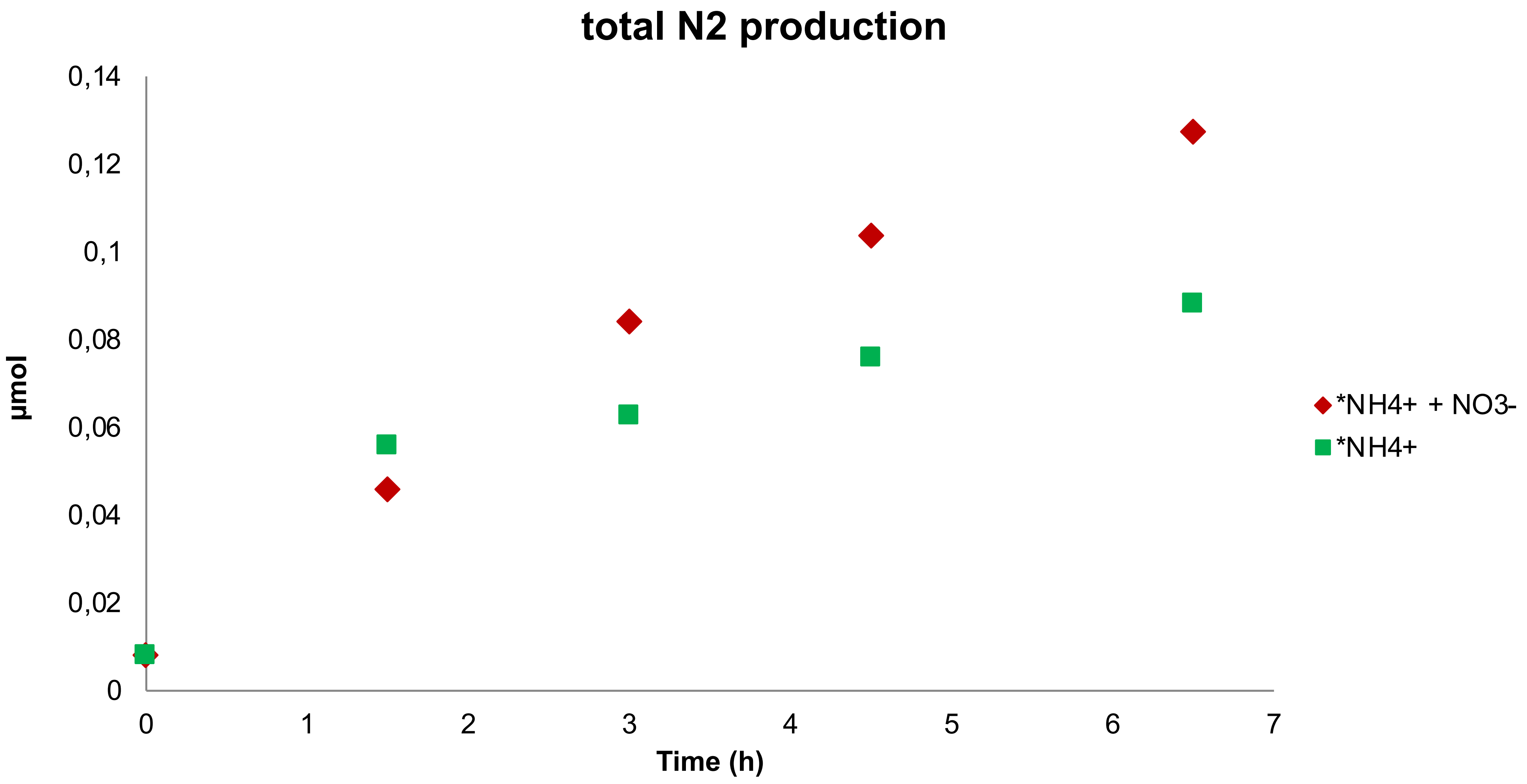


$^{29}N_2$ production indicates nitrate reduction



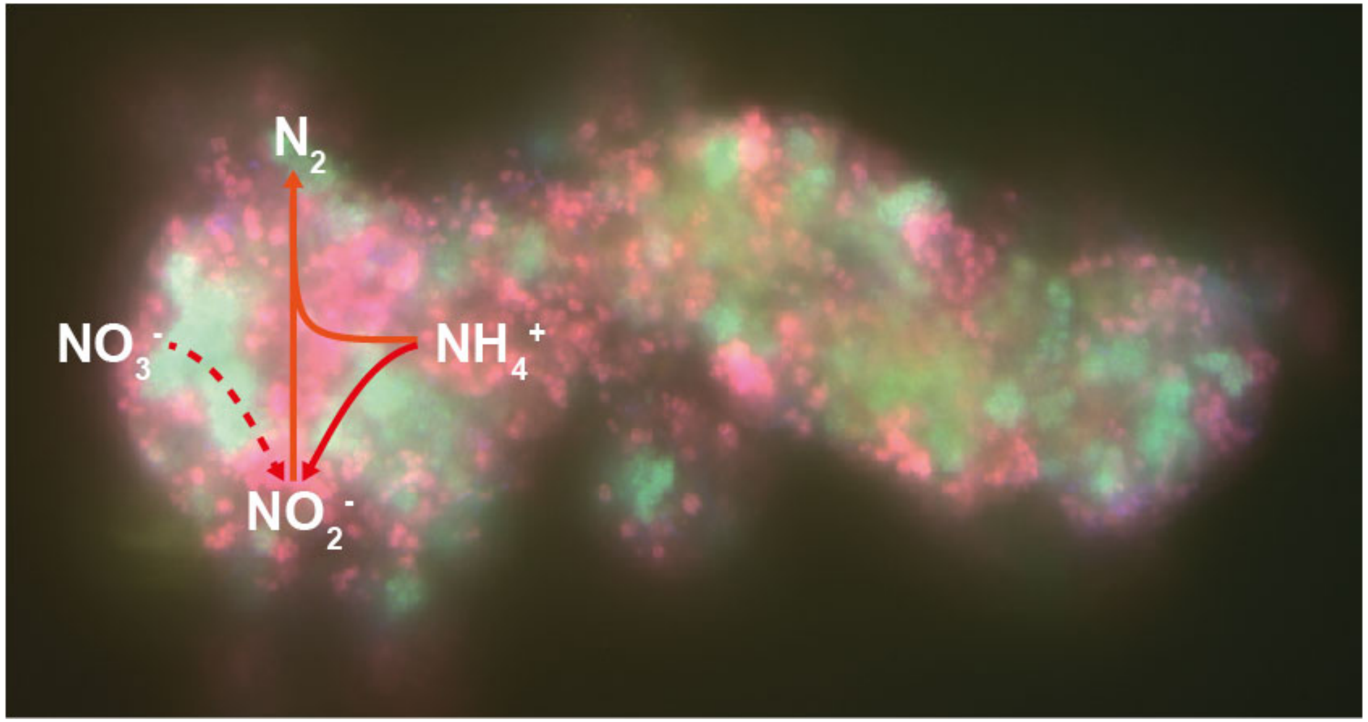


Higher anammox activity in the presence of nitrate



Conclusions II

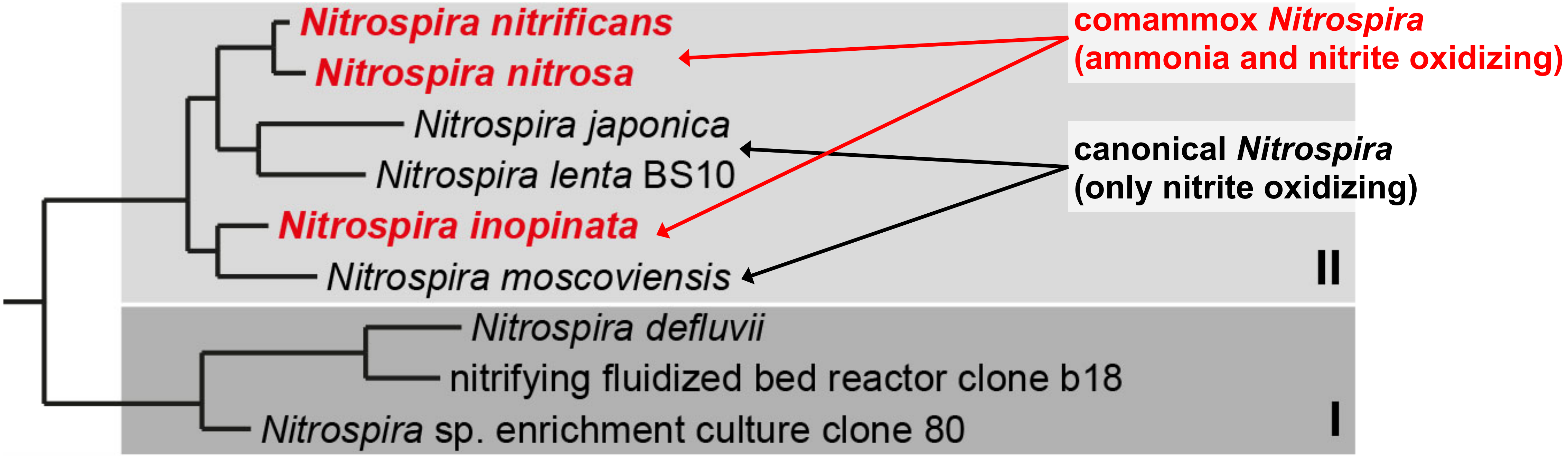
- Cooperation, not competition of anammox and comammox
- Comammox can supply anammox with nitrite
- Comammox performs nitrite comproportionation



Outline

- Introduction
- Complete nitrification by *Nitrospira*
- Novel physiologies of comammox *Nitrospira*
- ***In situ* detection of ammonia-oxidizing bacteria**
- Ammonia oxidation kinetics of comammox *Nitrospira*

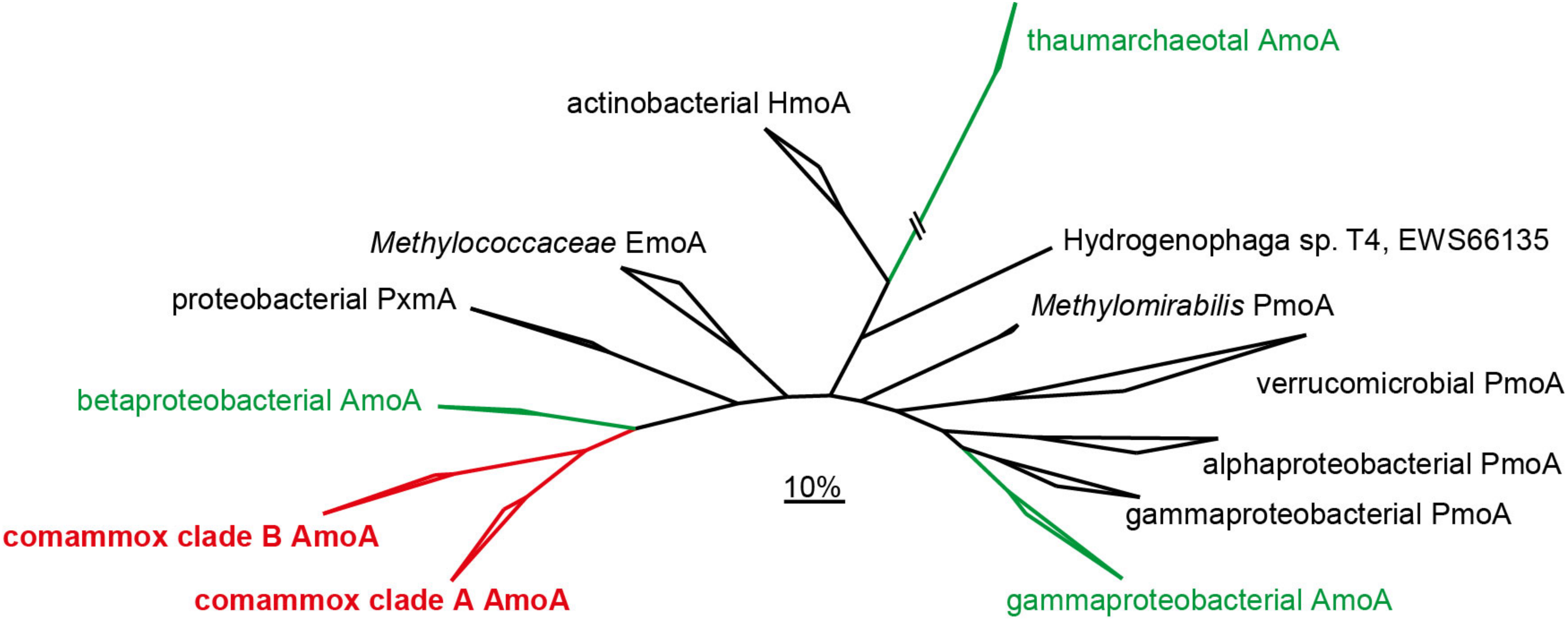
Comammox and nitrite-oxidizing *Nitrospira* are indistinguishable based on 16S rRNA



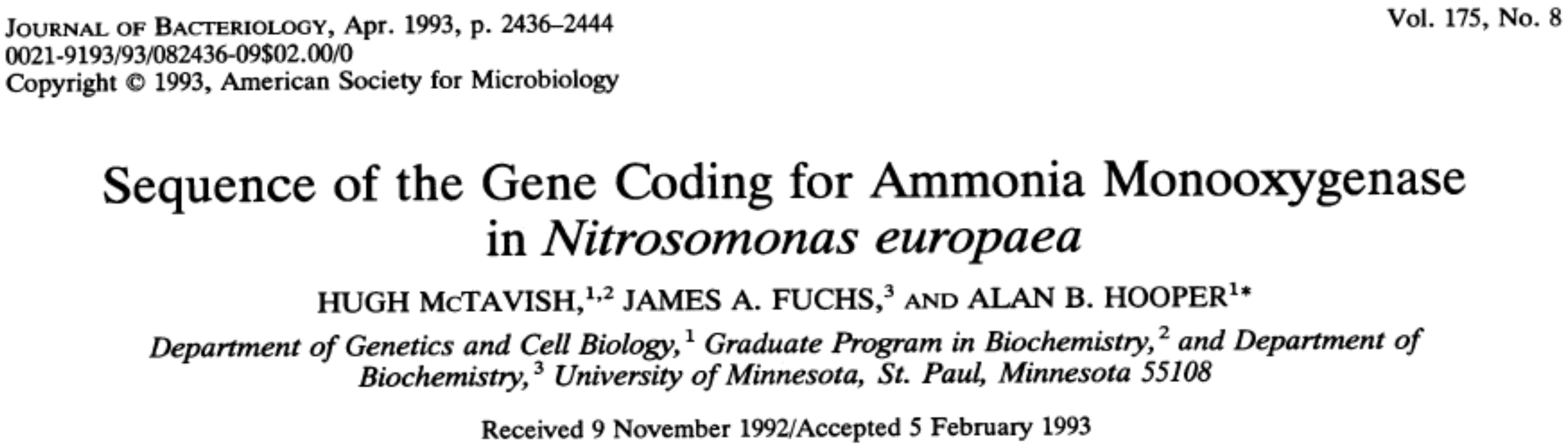
comammox *Nitrospira*
(ammonia and nitrite oxidizing)

canonical *Nitrospira*
(only nitrite oxidizing)

Presence of unique ammonia monooxygenase



AMO as functional marker



JOURNAL OF BACTERIOLOGY, Apr. 1993, p. 2436–2444
0021-9193/93/082436-09$02.00/0
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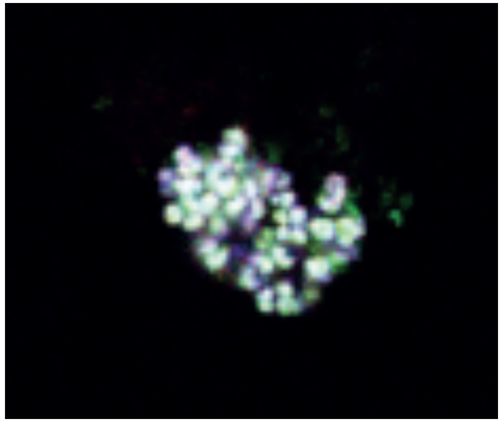
Vol. 175, No. 8

Sequence of the Gene Coding for Ammonia Monooxygenase in *Nitrosomonas europaea*

HUGH McTAVISH,[1,2] JAMES A. FUCHS,[3] AND ALAN B. HOOPER[1]*

Department of Genetics and Cell Biology,[1] Graduate Program in Biochemistry,[2] and Department of Biochemistry,[3] University of Minnesota, St. Paul, Minnesota 55108

Received 9 November 1992/Accepted 5 February 1993



van Kessel et al. (2015)

- AMO labeling with fluorescein thiocarbamoylpropargylamine (FTCP)

- phylogenetic staining with FISH

- Low staining efficiency
- Fluorescein isothiocyanate and propargylamine are highly toxic

ABPP-based AMO labeling

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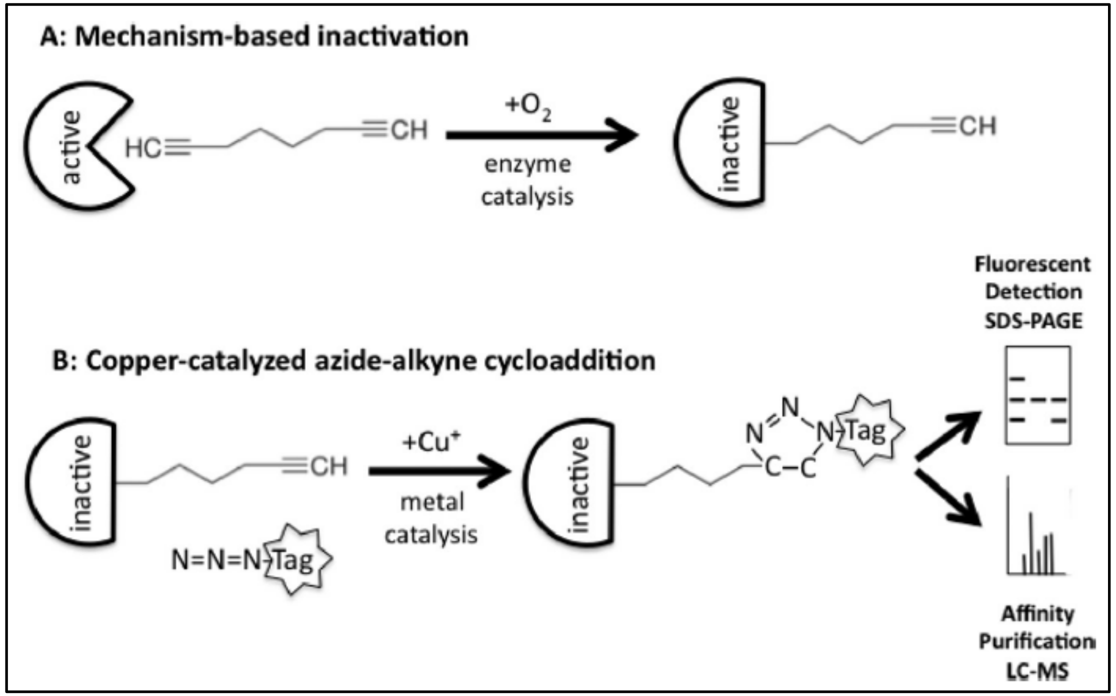
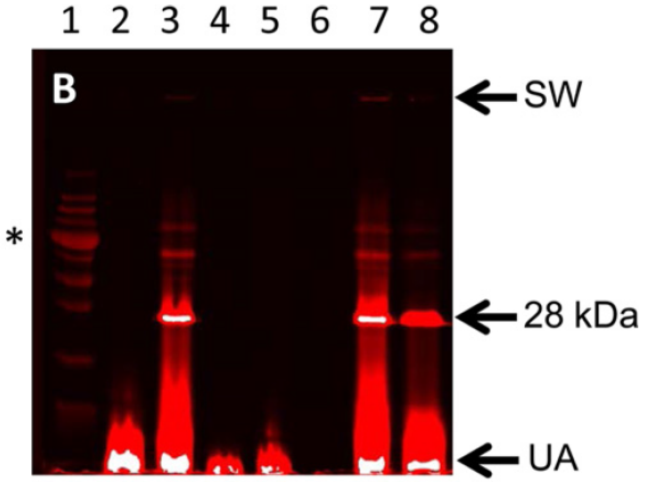
Activity-Based Protein Profiling of Ammonia Monooxygenase in *Nitrosomonas europaea*

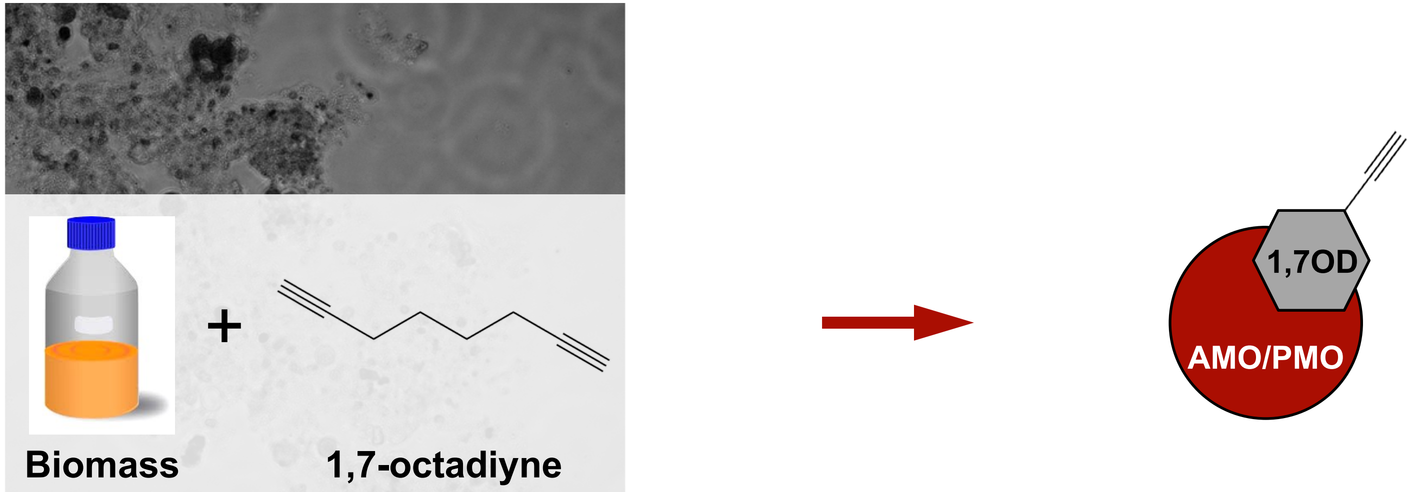
Kristen Bennett,[a] Natalie C. Sadler,[b] Aaron T. Wright,[b] Chris Yeager,[c] Michael R. Hyman[a]

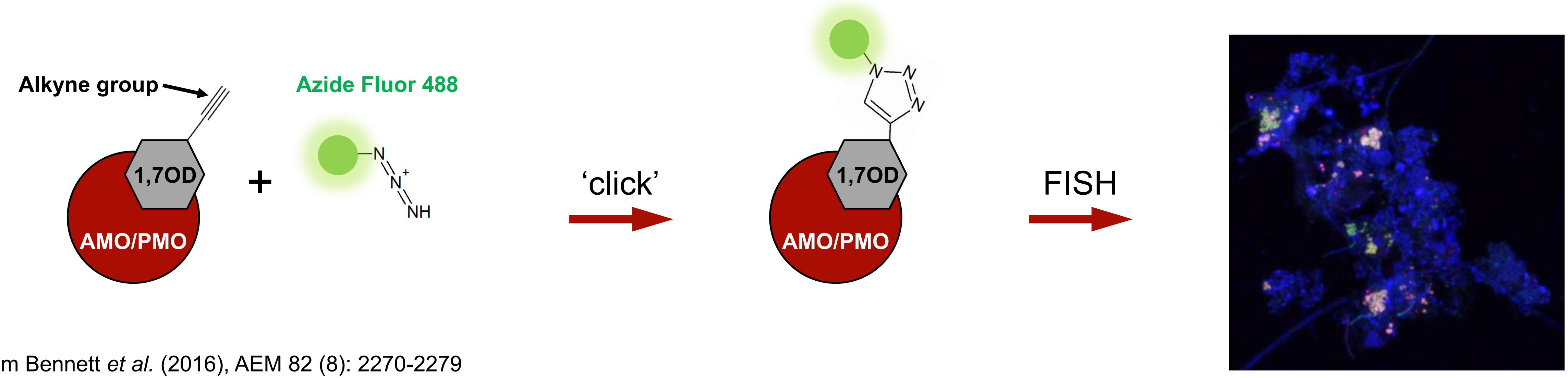




In situ detection of ammonia monooxygenase (AMO)

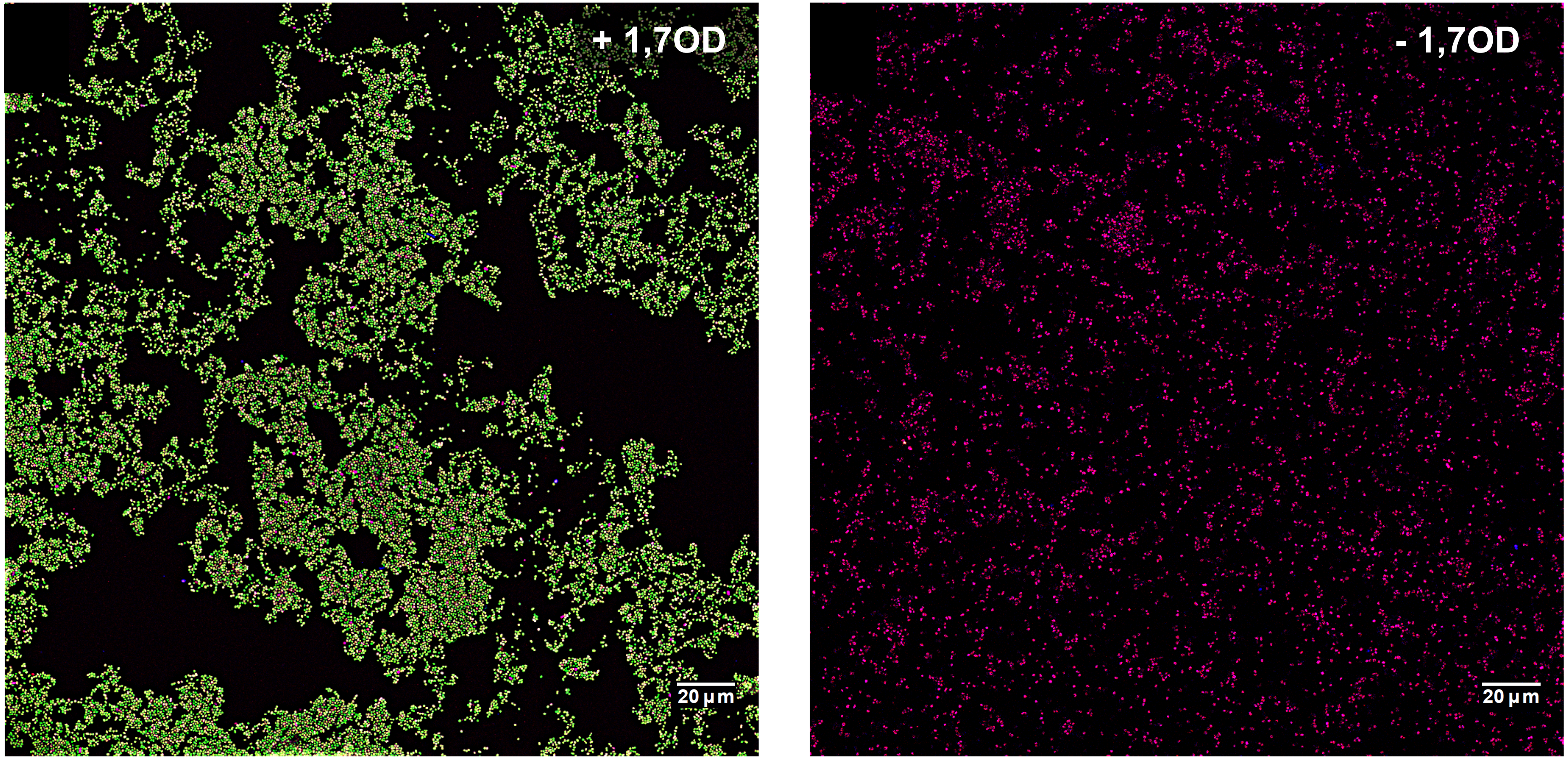
Dimitra Sakoula





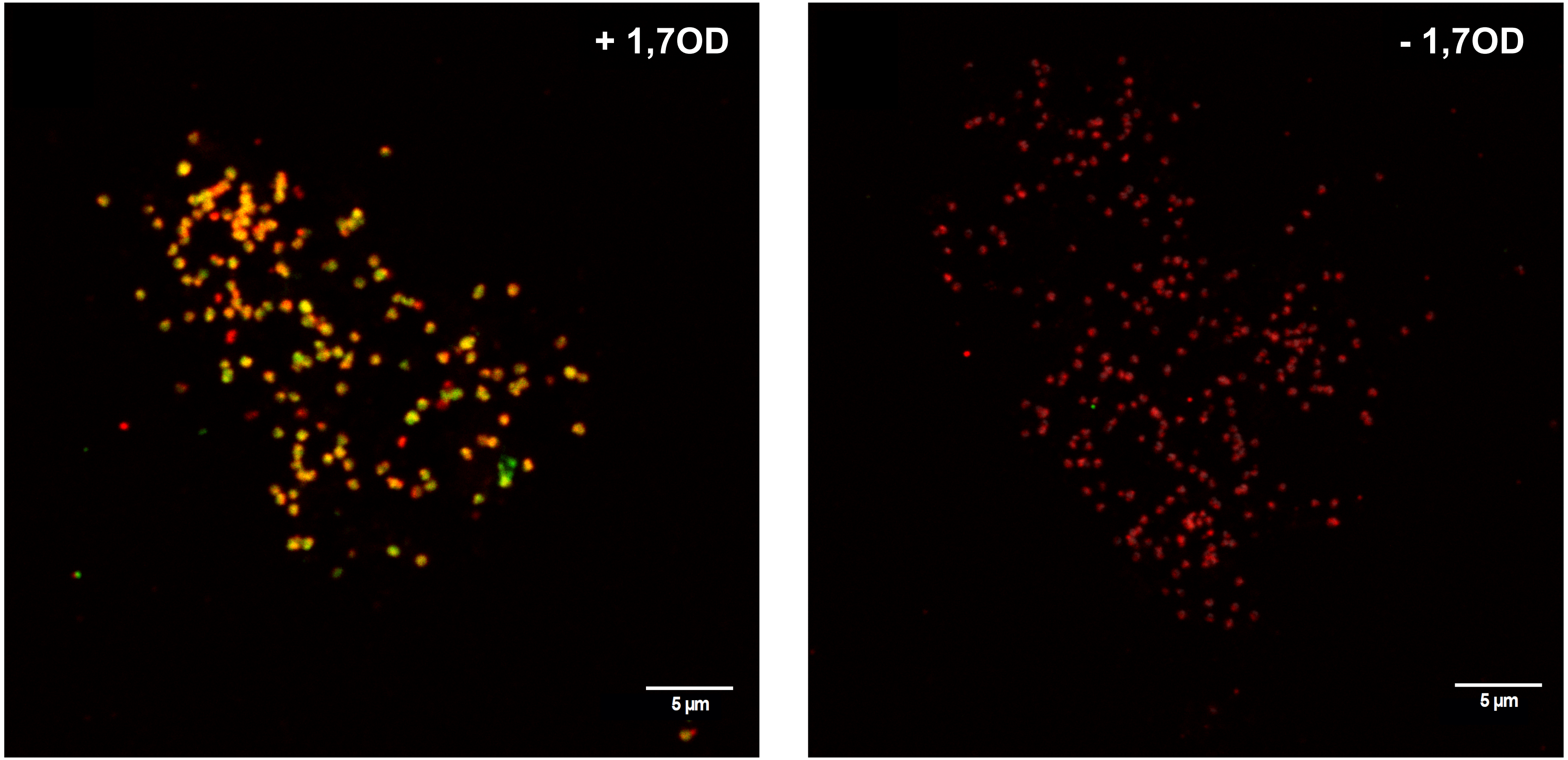
adapted from Bennett *et al.* (2016), AEM 82 (8): 2270-2279

AMO labeling of *Nitrosomonas europaea*



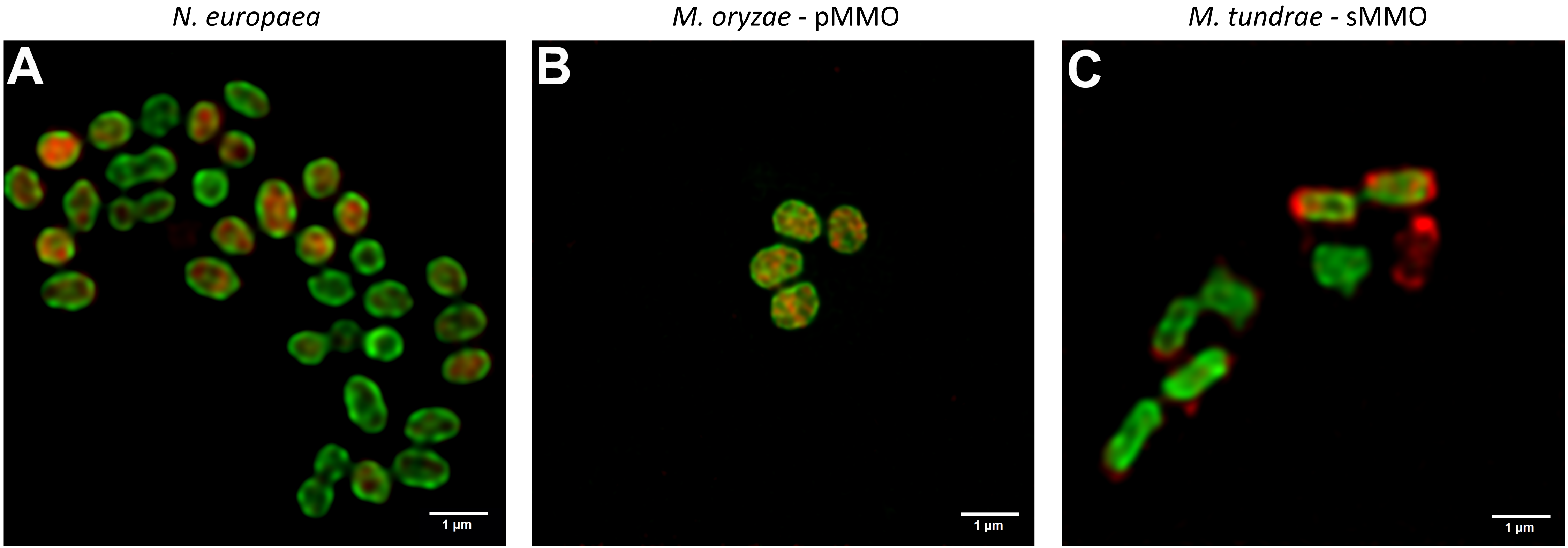
AMO, *Nitrosomonas*, all bacteria

AMO labeling of *Nitrospira inopinata*



AMO, *Nitrospira*

Subcellular localization of the AMO/MMO-derived signal



N. europaea

M. oryzae - pMMO

M. tundrae - sMMO

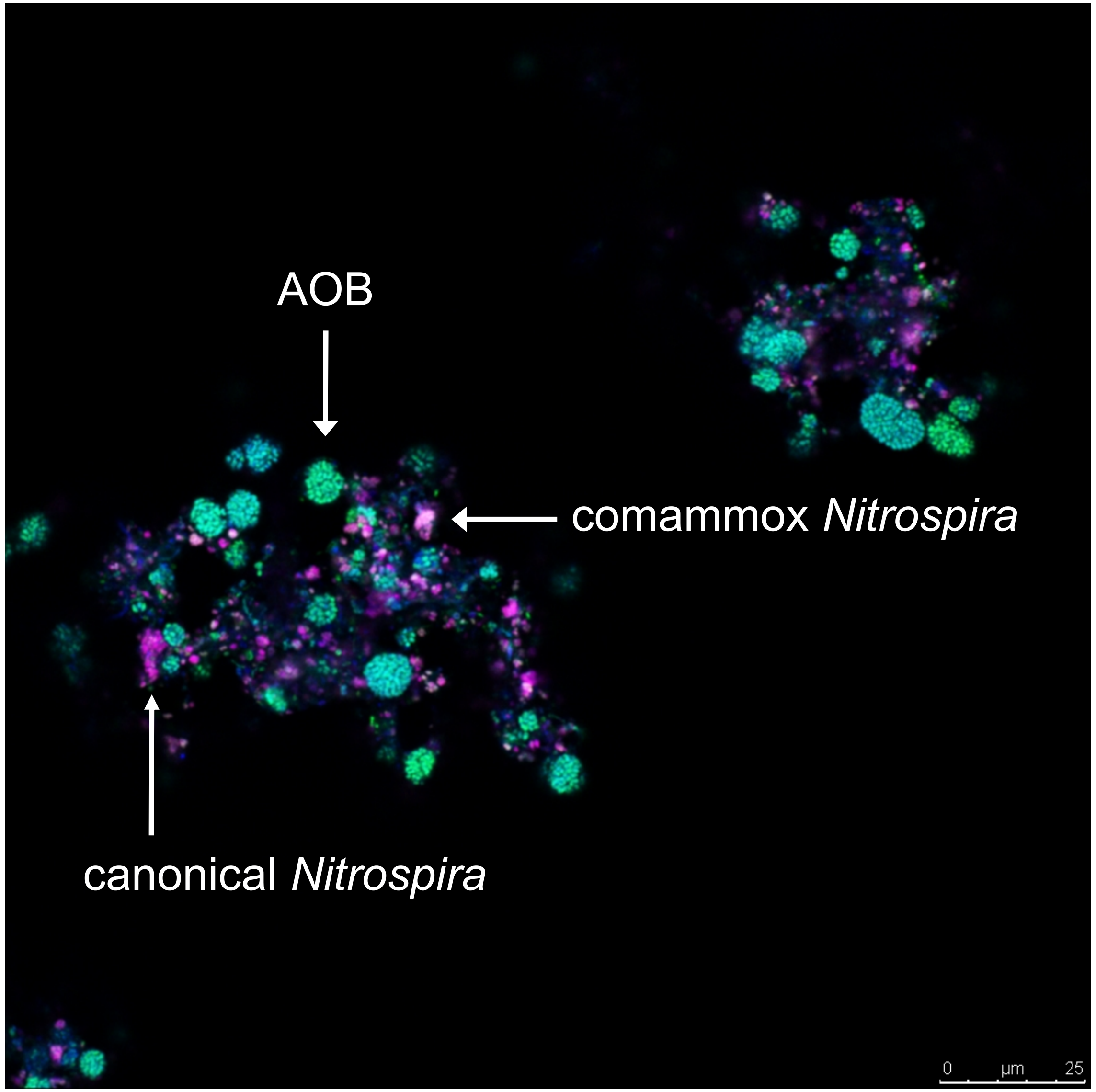
Intracytoplasmic membrane system

Intracytoplasmic membrane system

Cytoplasmic

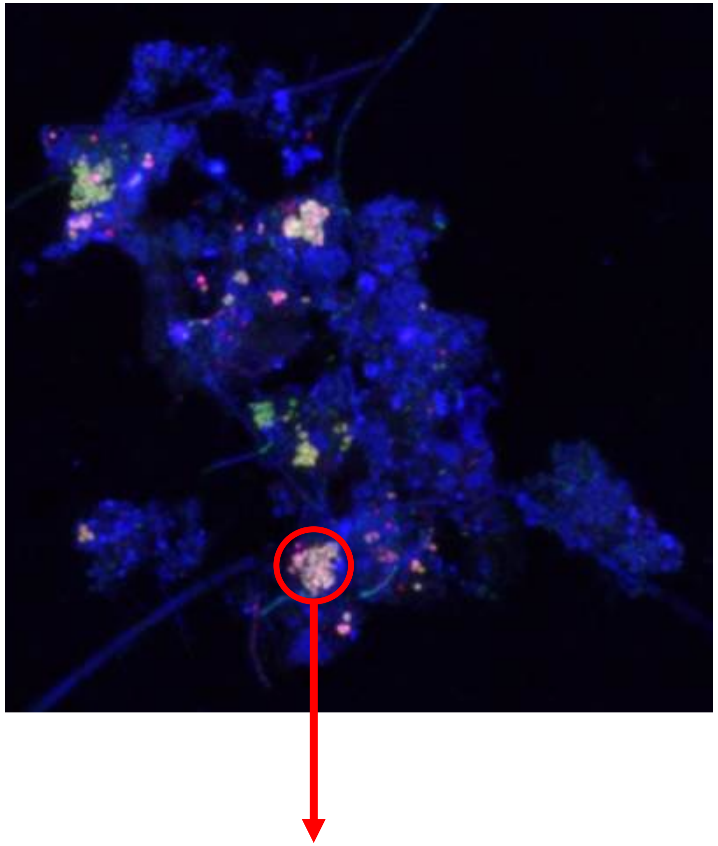
AMO/MMO-based staining, FISH-based staining

Linking function (AMO labeling) and identity (FISH)



AMO
Nitrospira
all bacteria

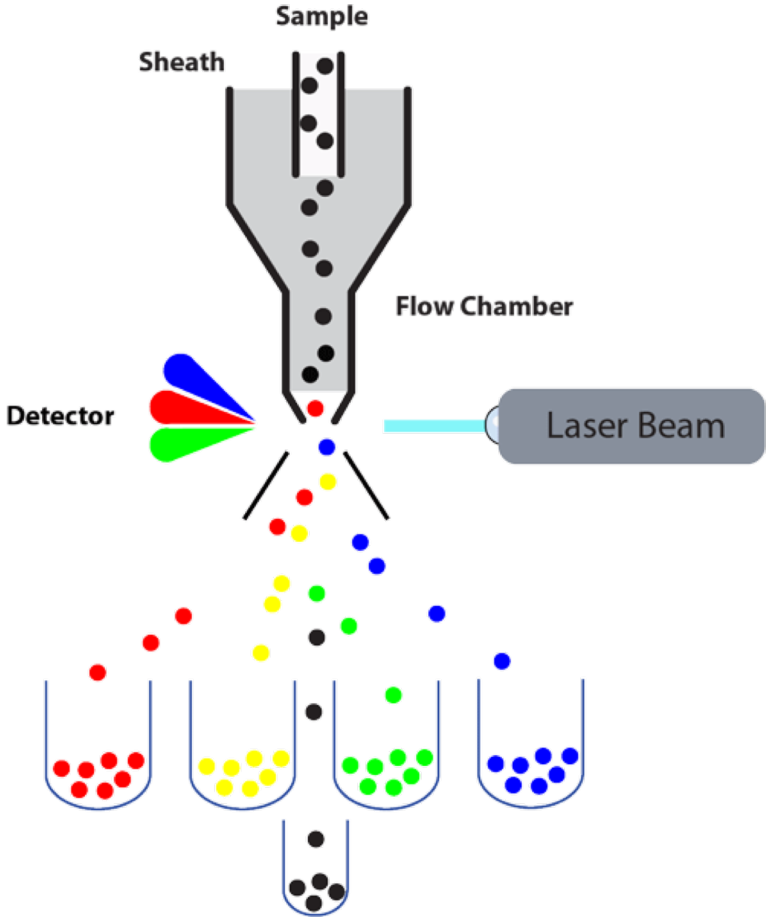
AMO labeling in combination with cell sorting



Targeted metagenomics

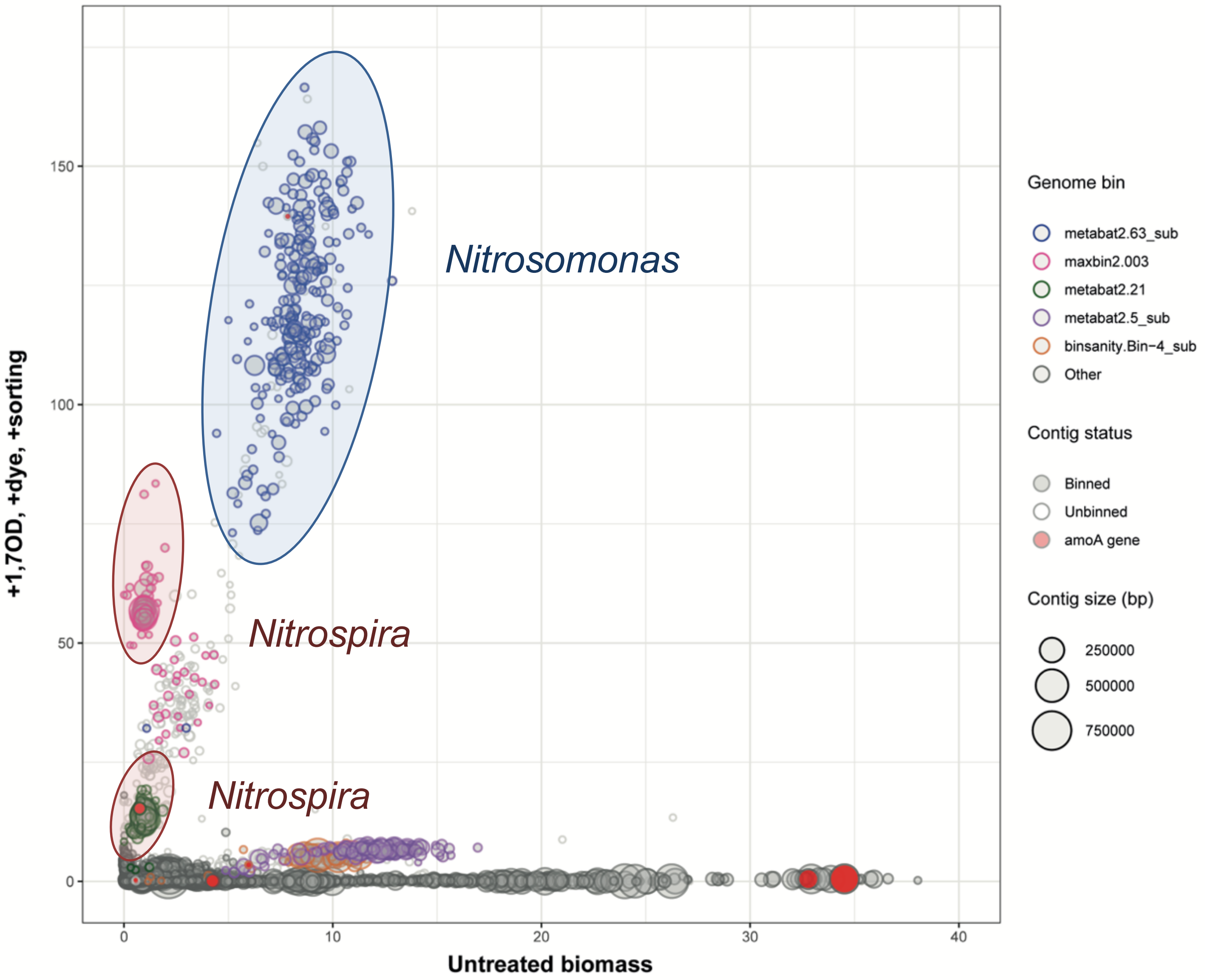
+

Fluorescence-activated cell sorting



https://www.tes.com/lessons/Wpg6sEfF7jdPgg/electrical-impedance

Targeted metagenomics – nitrifying enrichment culture

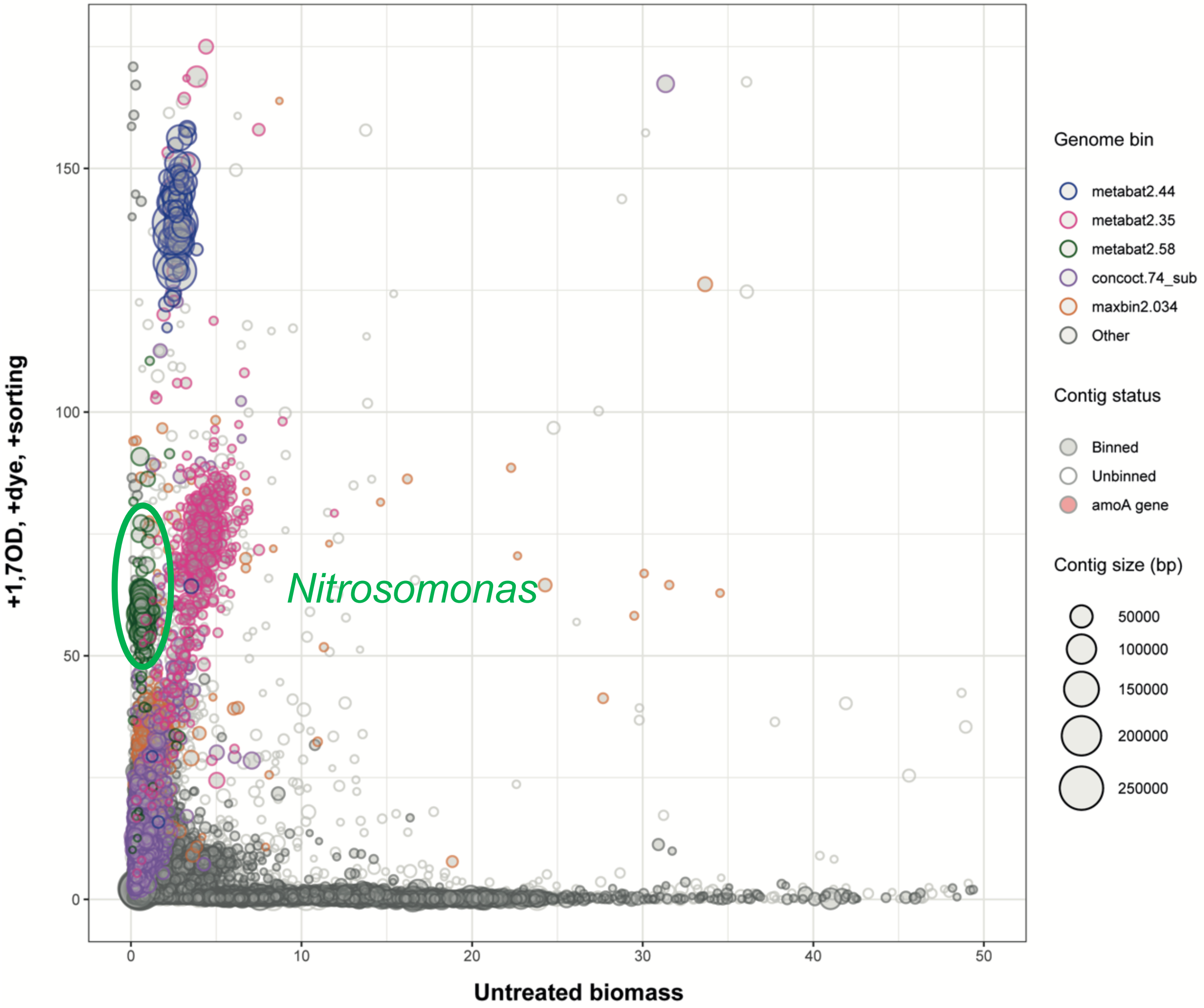


3 high quality MAGs

>92% completeness

<3.7% redundancy

Targeted metagenomics – activated sludge



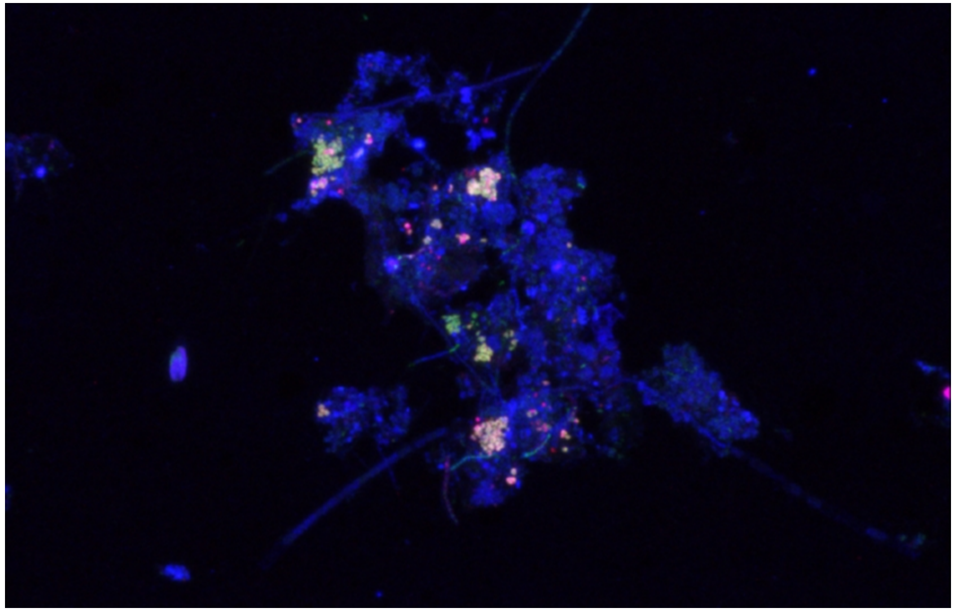
Very low abundance of ammonia oxidizers in original sample
(0.03% of total reads)

***Nitrosomonas* high quality MAG**
(188-fold enrichment)

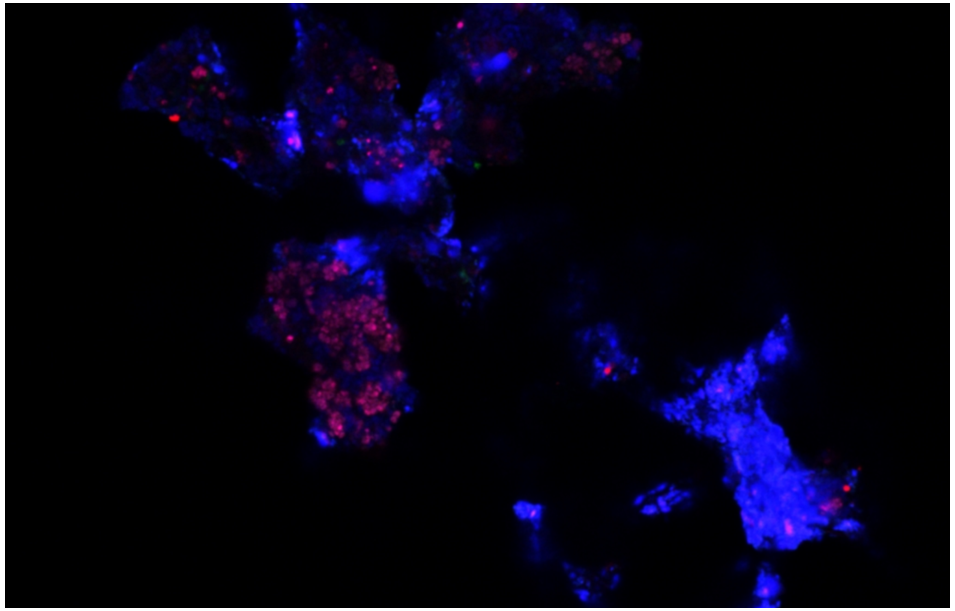
5 MAGs → *Competibacteraceae*

Conclusions III

- ABPP-based protocol allows
  - specific detection of AMO (and PMO) containing bacteria
  - phylogenetic identification in combination with FISH
  - targeted retrieval of enriched metagenomes



+ 1,7-Octadiyne



- 1,7-Octadiyne

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- Novel physiologies of comammox *Nitrospira*
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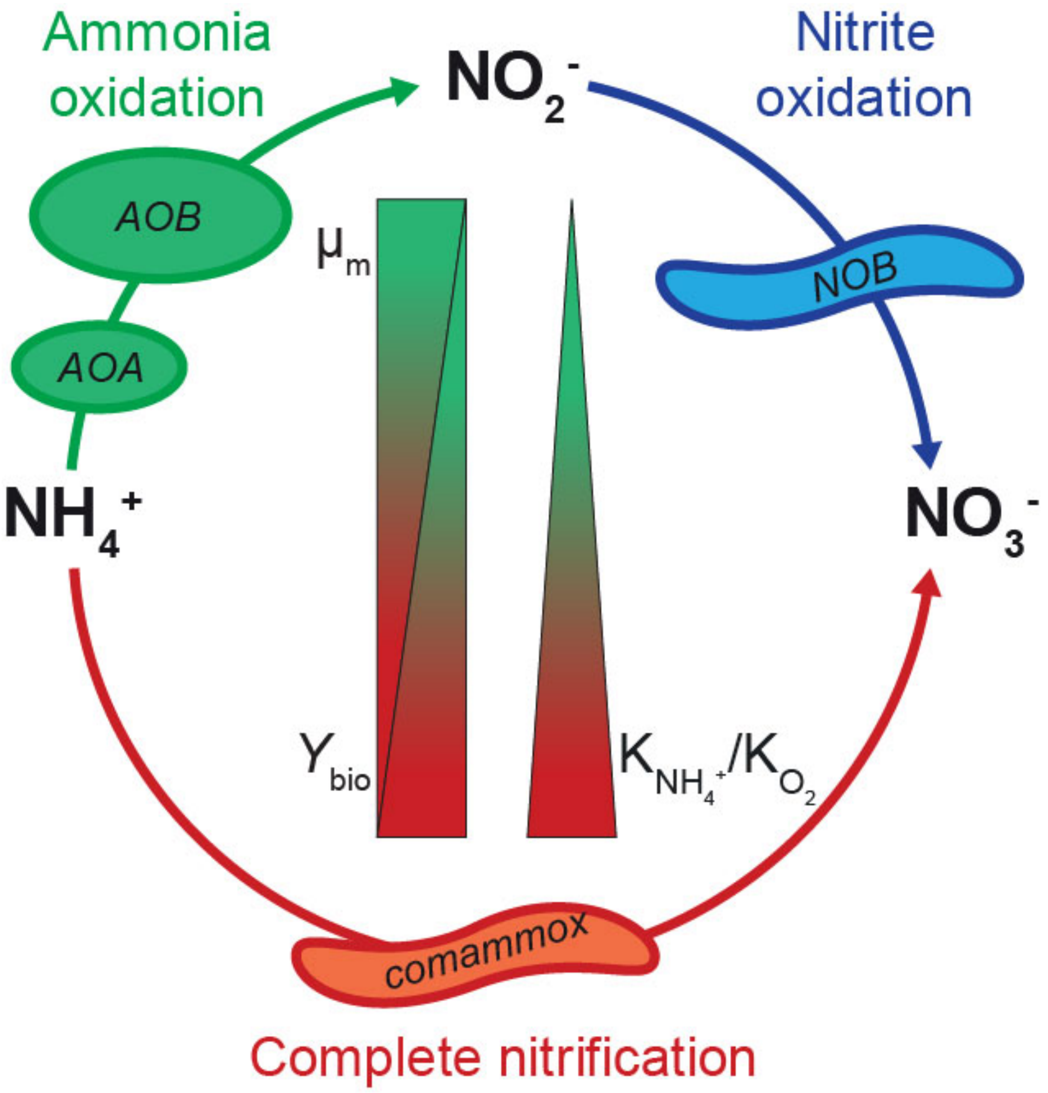
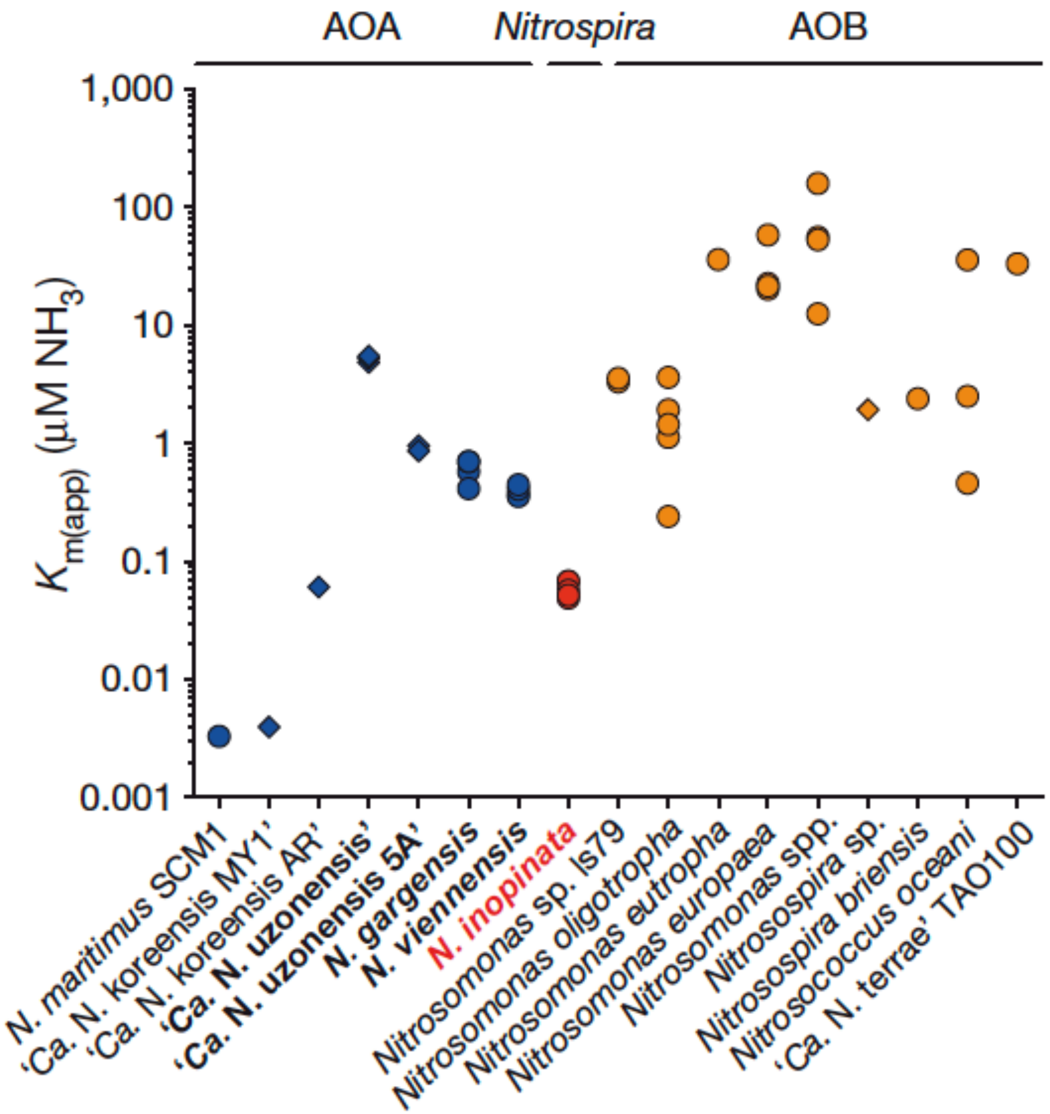
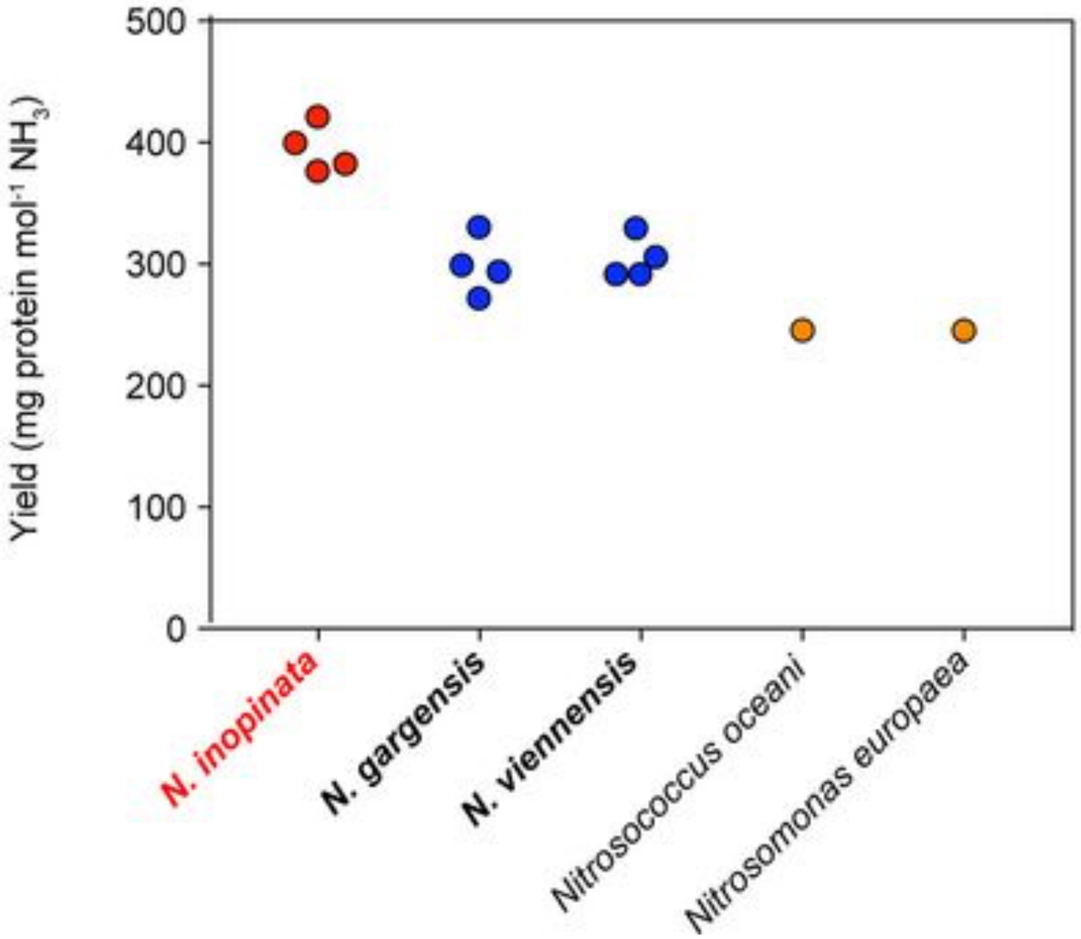
- **Ammonia oxidation kinetics of comammox *Nitrospira***

Niche adaptations of complete and canonical nitrifiers



Niche adaptation of complete nitrifiers





Kits *et al*., 2017

Bioreactor for the enrichment of comammox *Nitrospira*

Dimitra Sakoula

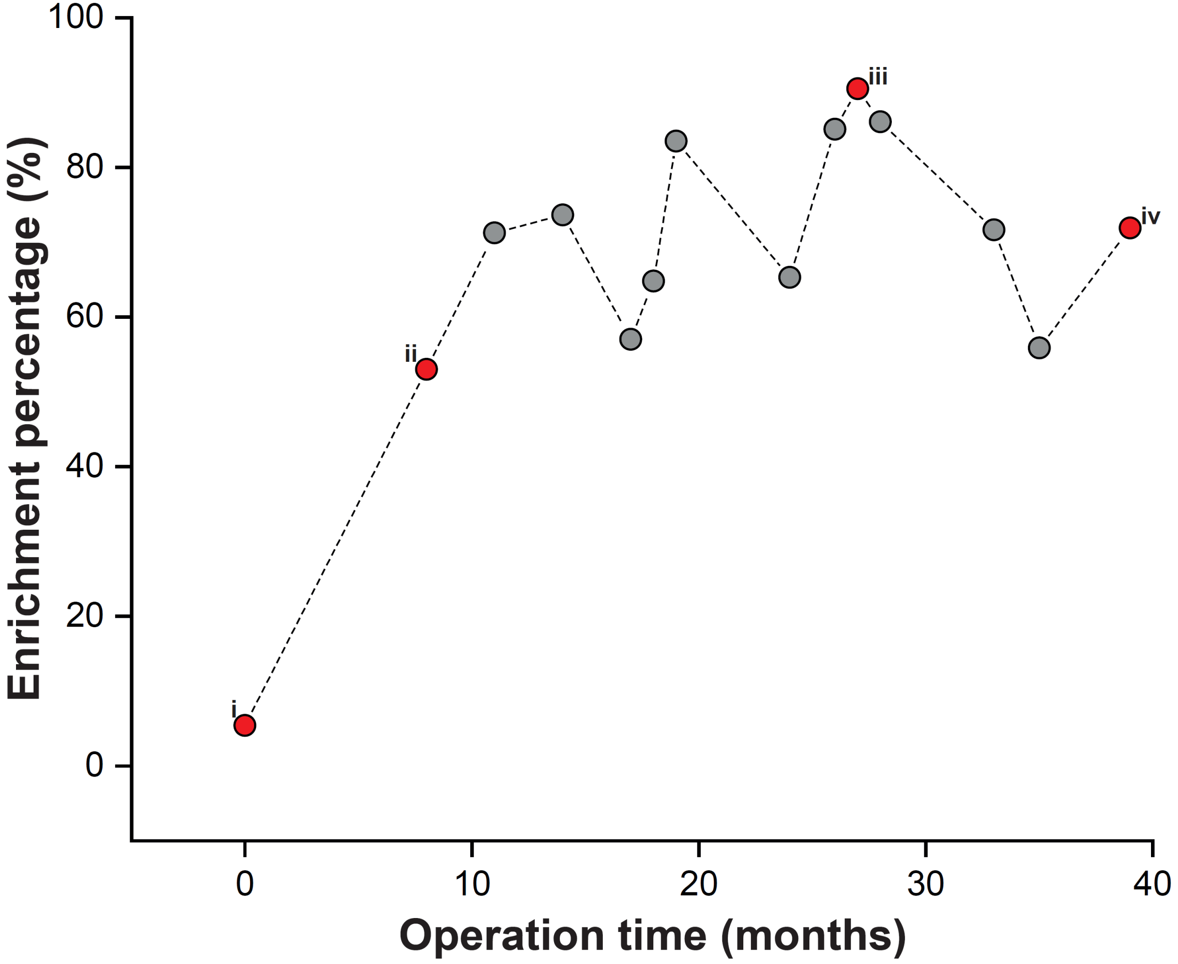
✓Inoculated with an **enrichment of *Ca.* N. nitrosa & *Ca.* N. nitrificans**
(as described in *van* Kessel *et al.*, 2015)

✓**Continuous flow membrane** bioreactor

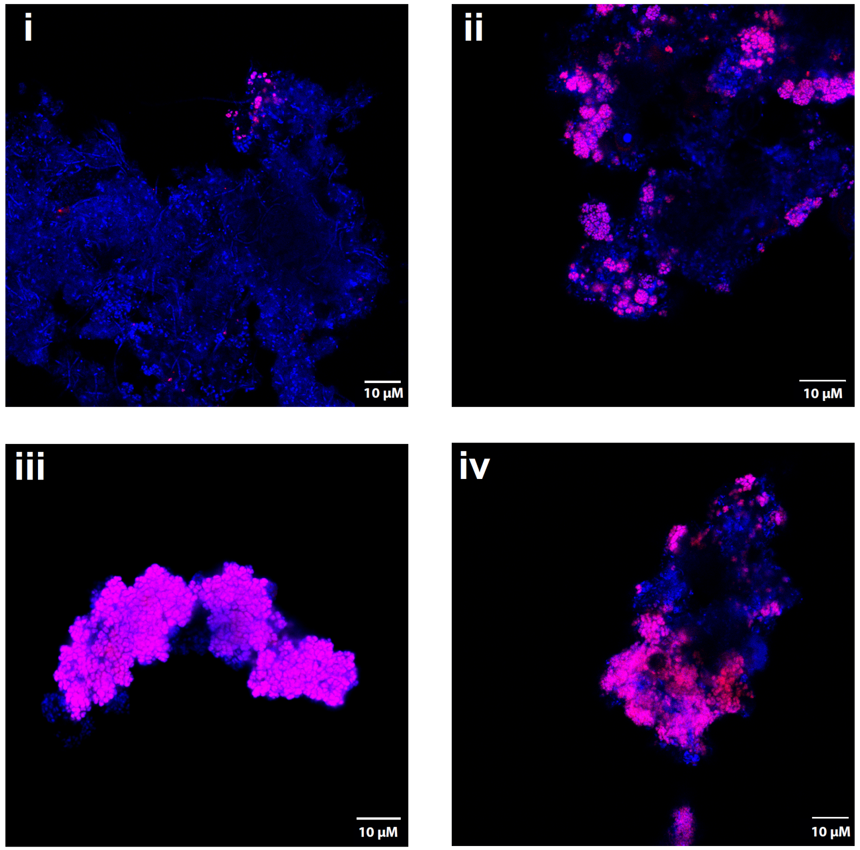
✓Supplied with **low concentrations of ammonium**
(80 µM - 2.5 mM NH_4^+/day)

Influent	NOB medium
pH	7.5
Exchange rate	20-30%
Stirring	200 rpm
Temperature	20-24 (RT)
Oxygen supply	5%

Enrichment of *Nitrospira* bacteria in the system



Nitrospira, general bacteria



~ 80% enrichment in *Nitrospira* bacteria

Absence of canonical ammonia oxidizers

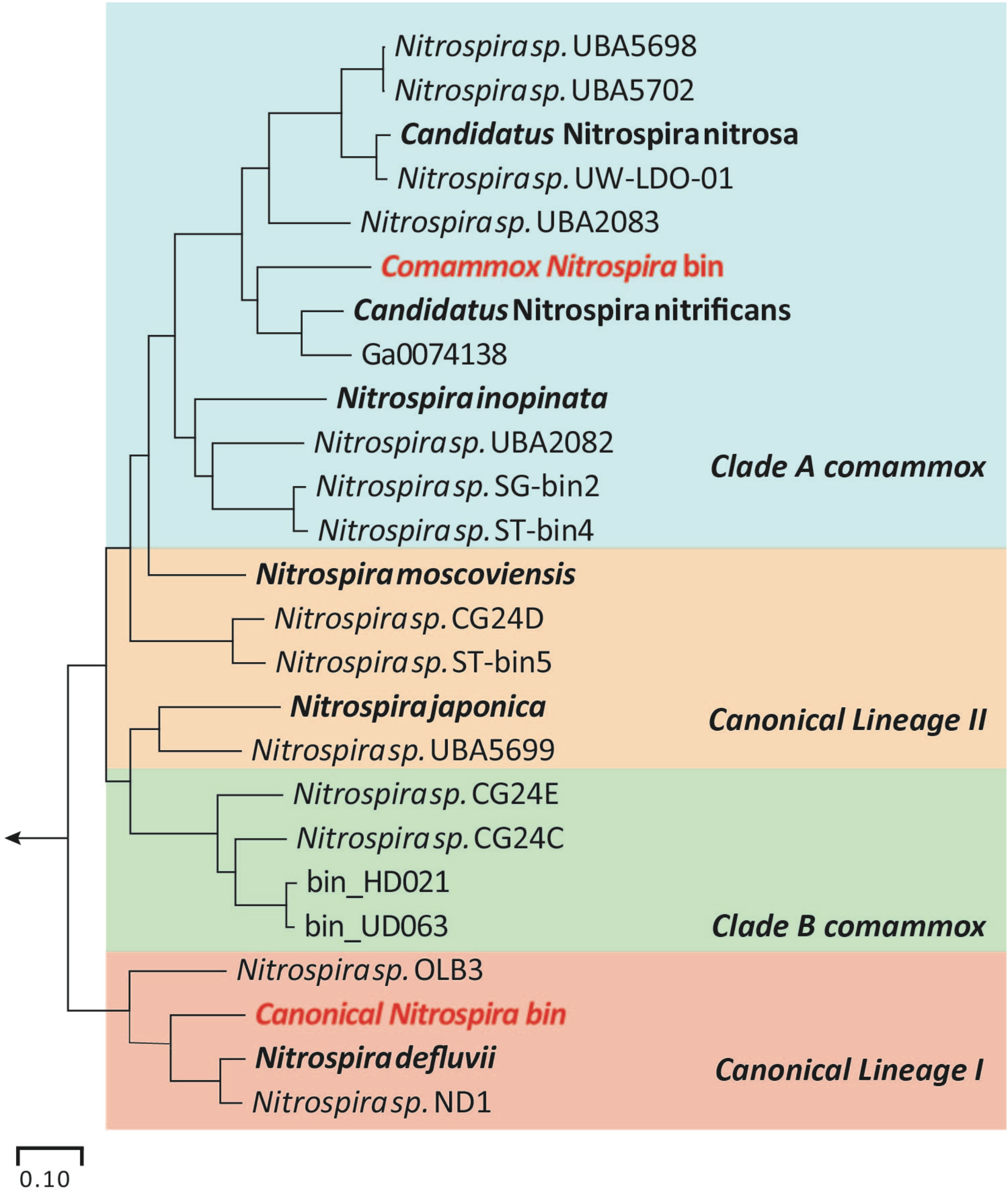
Enrichment of novel *Nitrospira*

- ✓ closed comammox *Nitrospira* genome
- ✓ high-quality draft canonical *Nitrospira* genome (5 contigs)

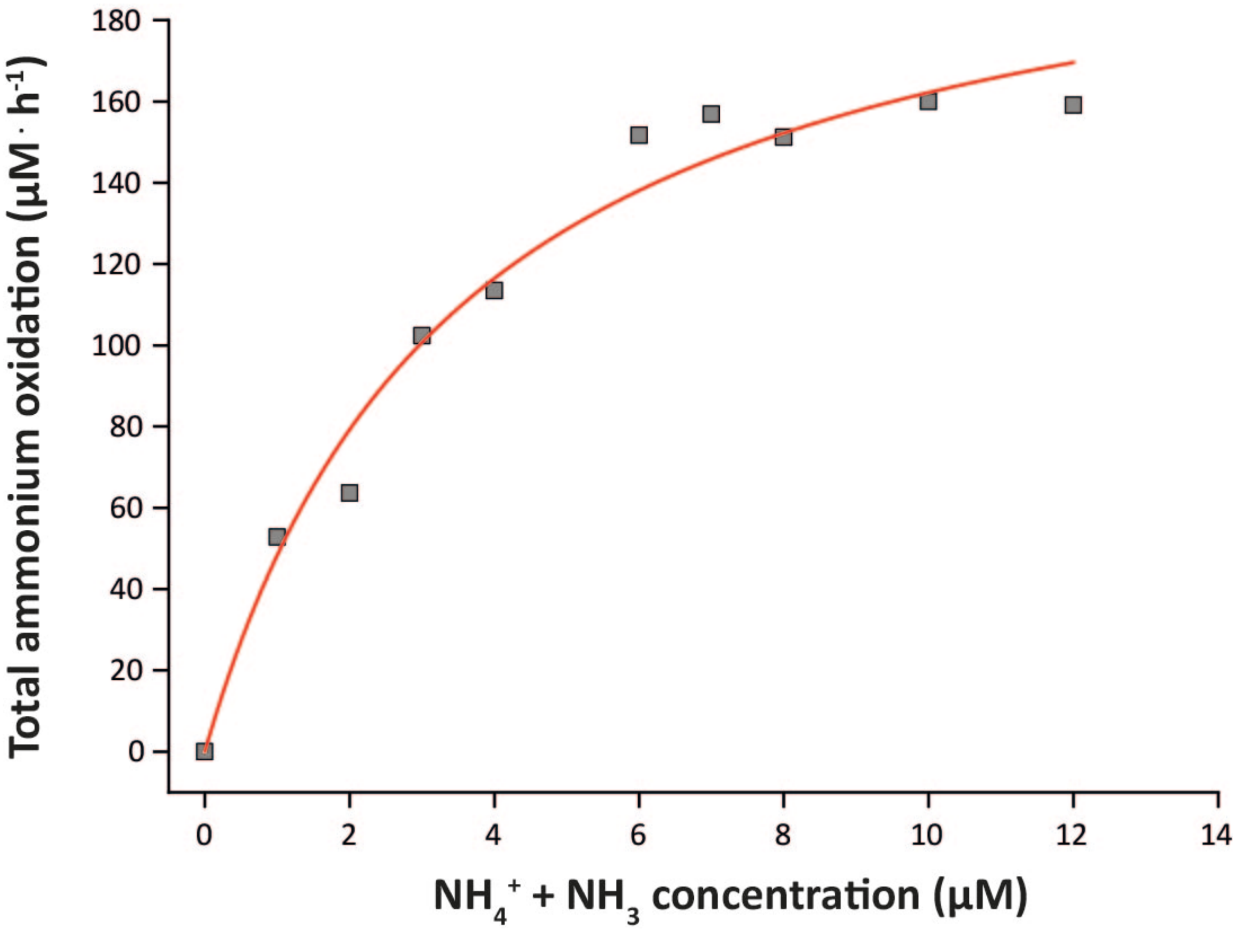
Average Nucleotide Identity (ANI)

Comammox *Nitrospira*	**77.5%**
Ca. N. nitrificans	
Canonical *Nitrospira*	**83.56%**
N. defluvii	

***Ca.* Nitrospira kreftii** (comammox)



Ammonia affinity



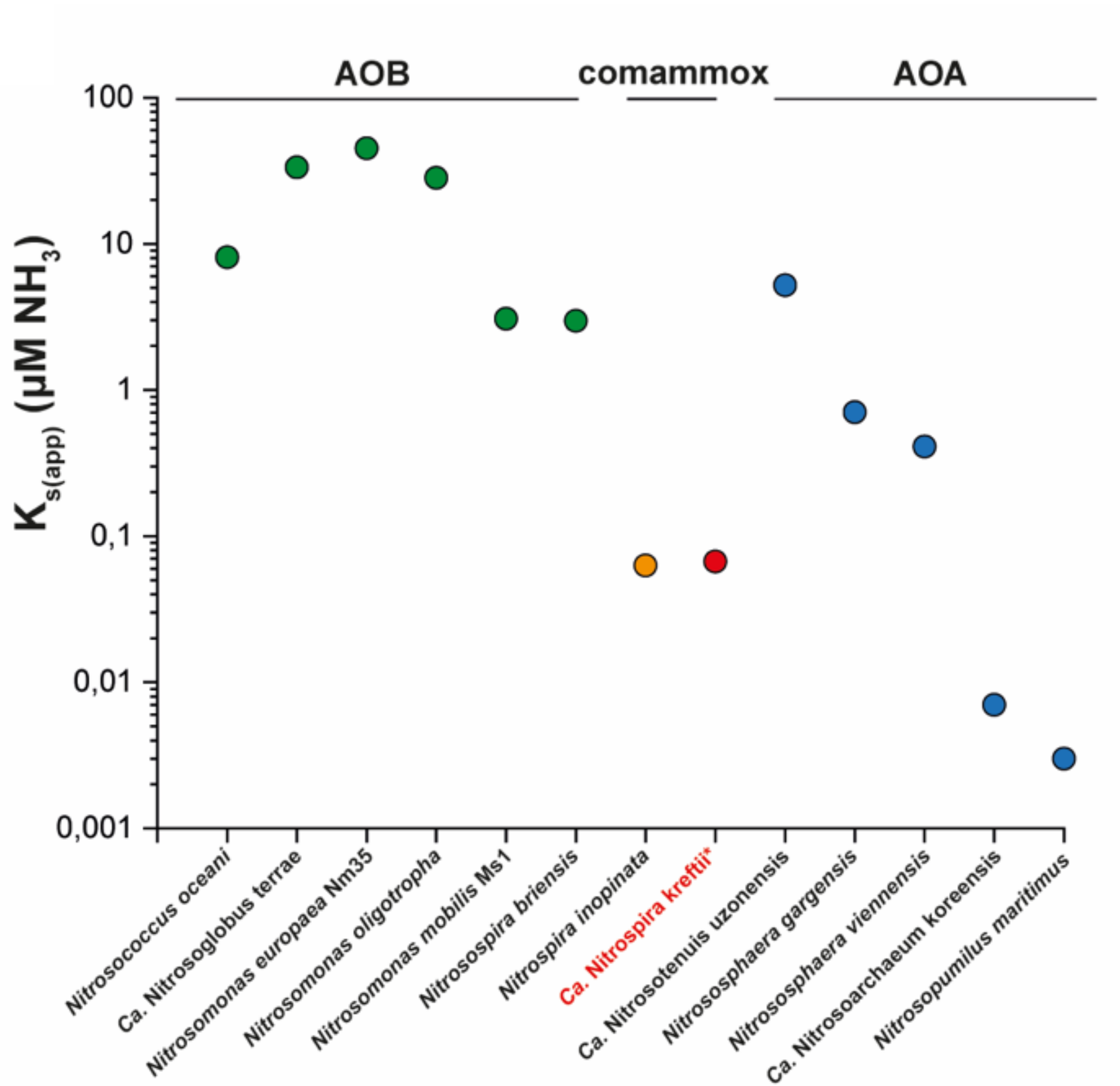
$K_{m(app)}$ = 3.8 ± 1.1 µM $NH_4^+ + NH_3$

~ 0.067 µM NH_3

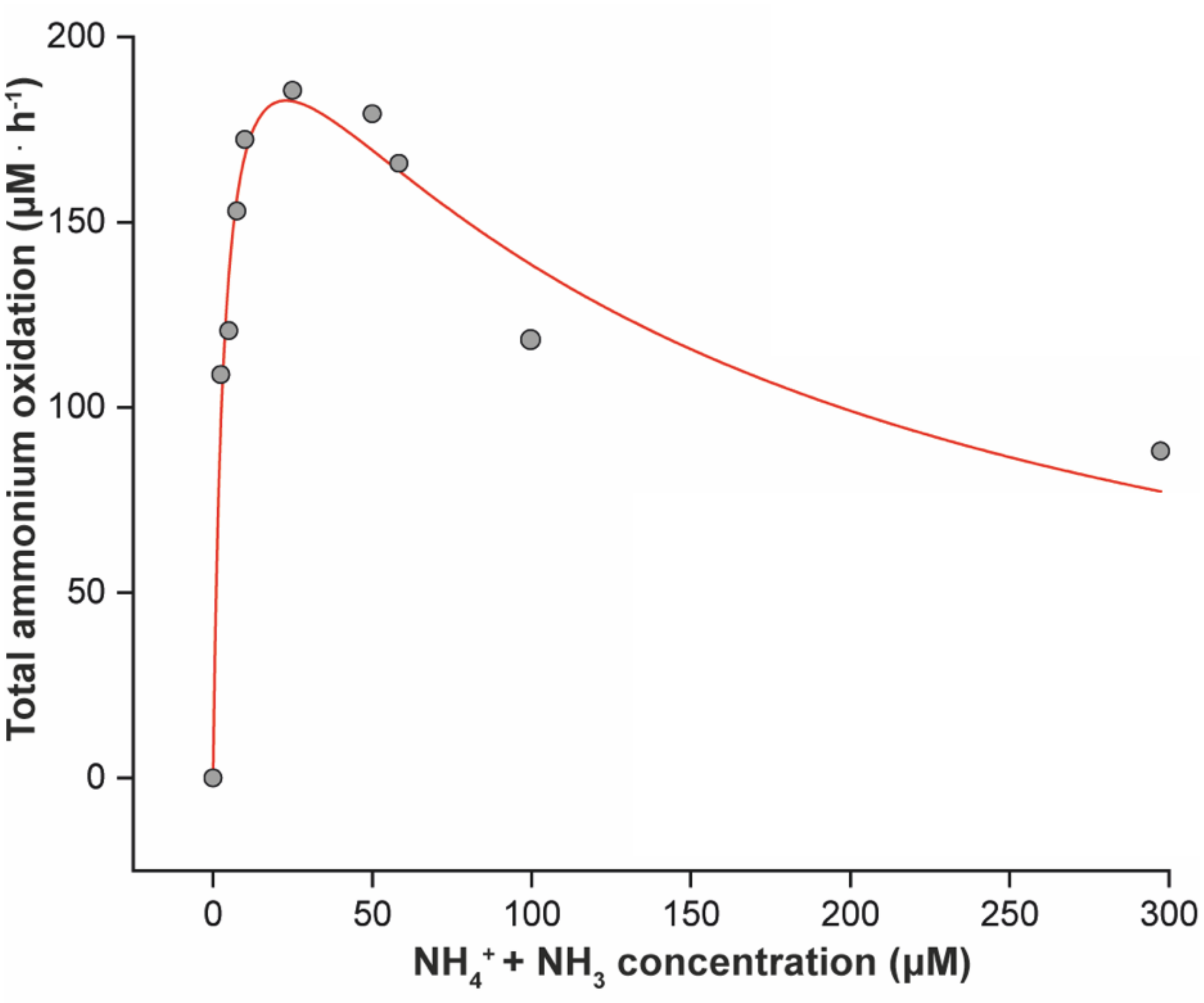
V_{max} = 242.7 ± 24.7 µM N h^{-1}

= 103.4 µmol N mg $protein^{-1}$ h^{-1}

Ammonia affinity

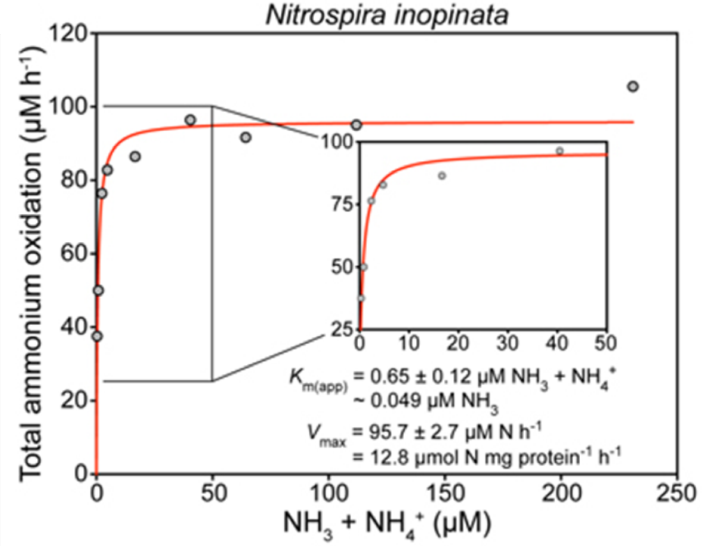


Surprising novel physiology – Ammonia inhibition



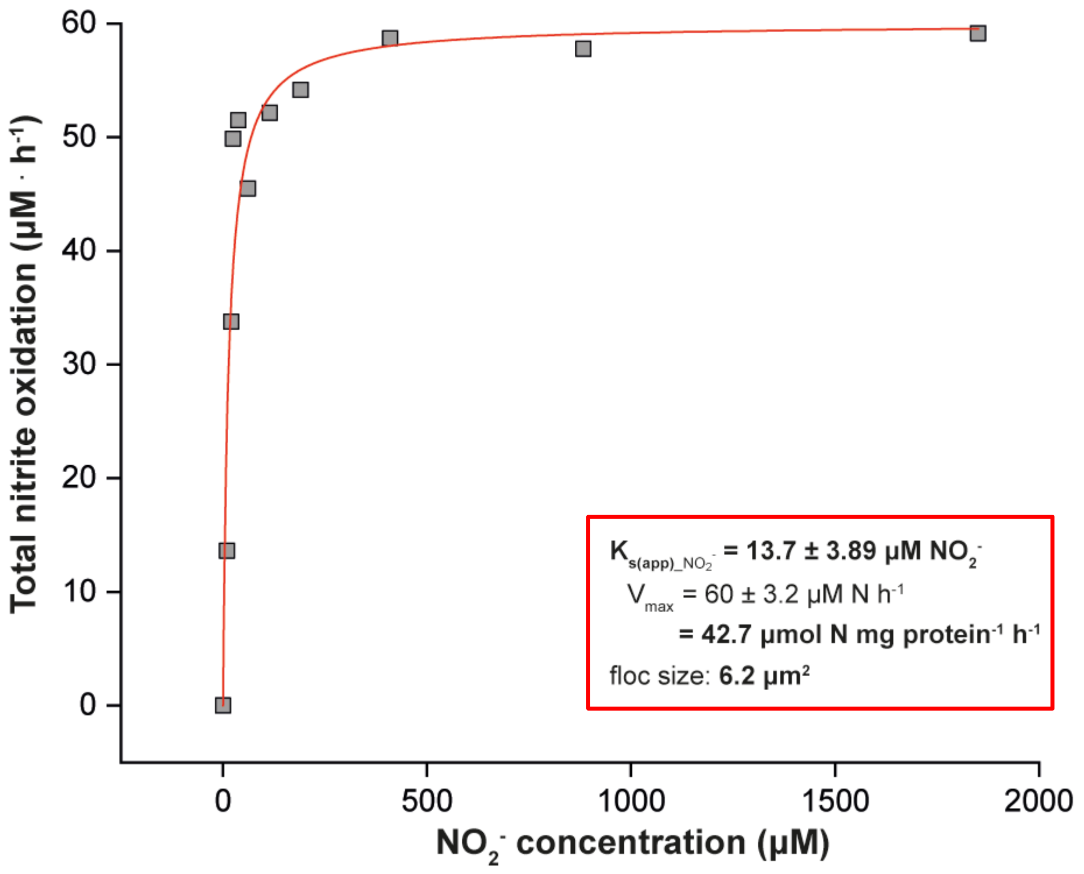
K_i = 139.9 ± 41.5 µM NH_4^+ + NH_3

~ 2.49 µM NH_3



Kits *et al.*, 2017

Surprising novel physiology – Nitrite affinity

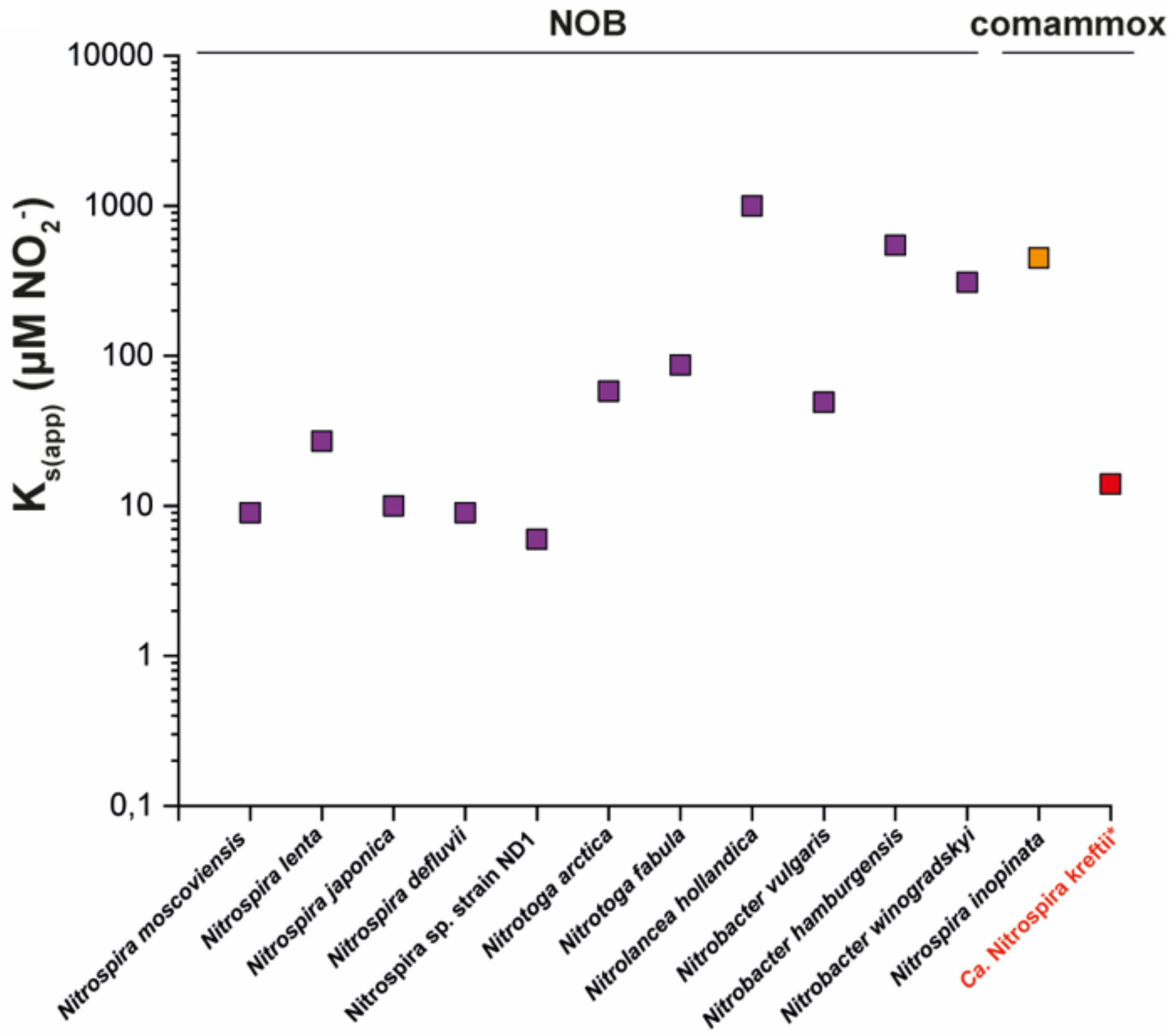


$K_{s(app)\_NO_2^-}$ = **13.7 ± 3.89 µM NO_2^-**

V_{max} = 60 ± 3.2 µM N h^{-1}

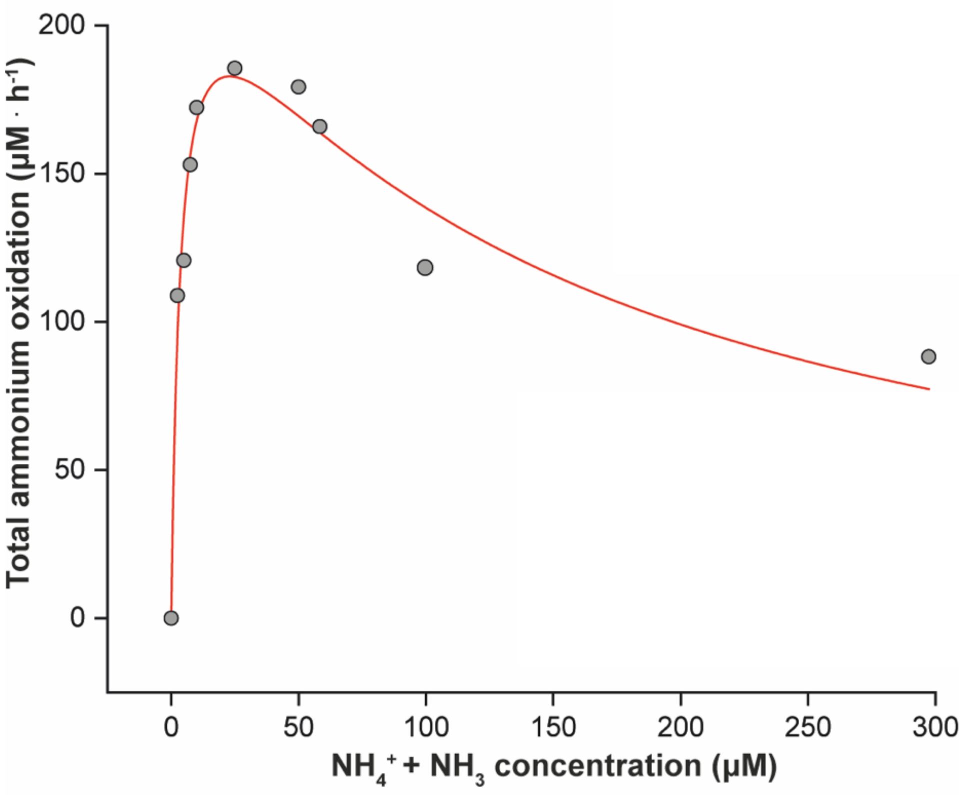
= 42.7 µmol N mg protein^{-1} h^{-1}

floc size: **6.2 µm²**



Conclusions IV

- Comammox is adapted to highly limited ammonia concentrations
- Ammonia oxidation partially inhibited at increasing ammonia concentrations



Acknowledgements

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Dimitra Sakoula
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VENI VIDI VICI NWO

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SIAM

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Mads Albersen
Per Halkjær Nielsen

WISCONSIN UNIVERSITY OF WISCONSIN–MADISON

Chris Lawson
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Holger Daims
Michael Wagner