

Closing the Nitrogen Cycle:

Utilizing Molecular Biological Tools to Address Nitrogen Transformation in Wastewater

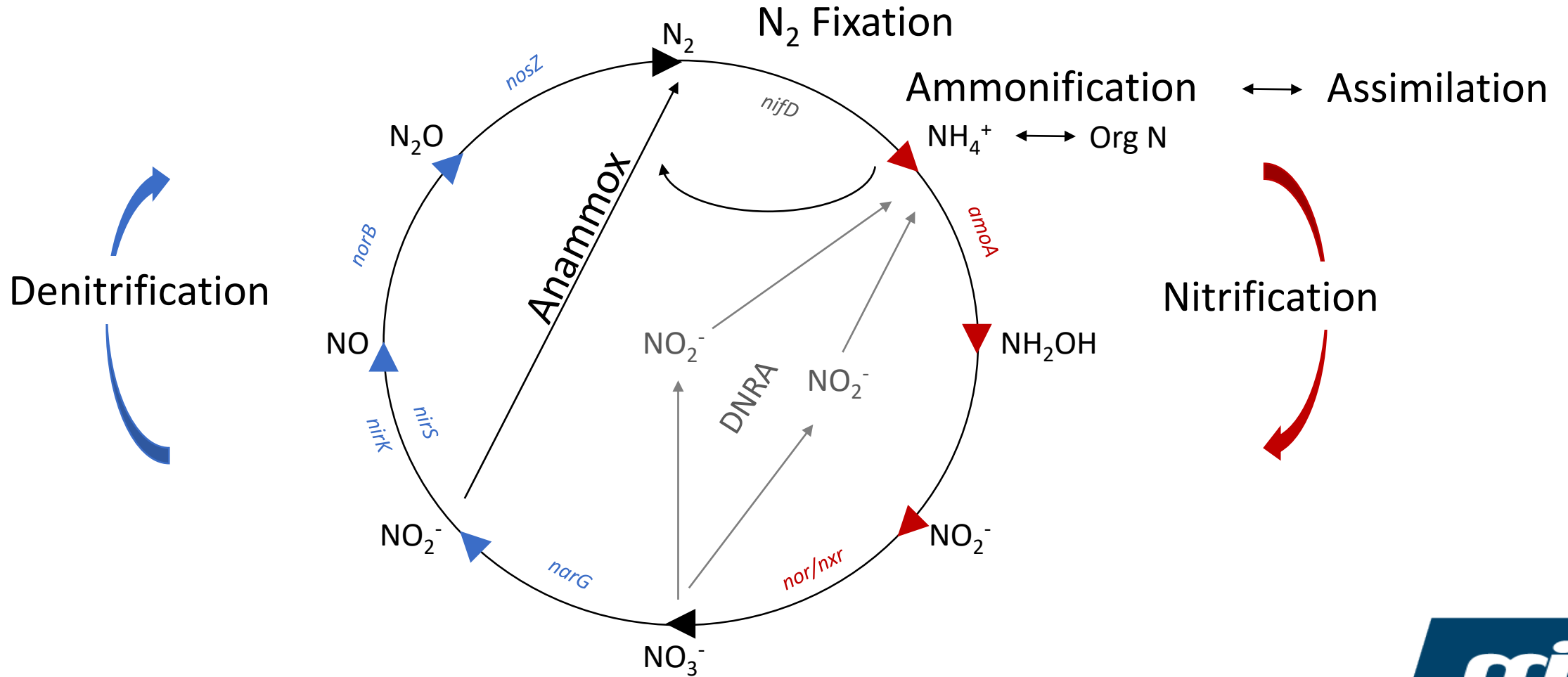


Introduction

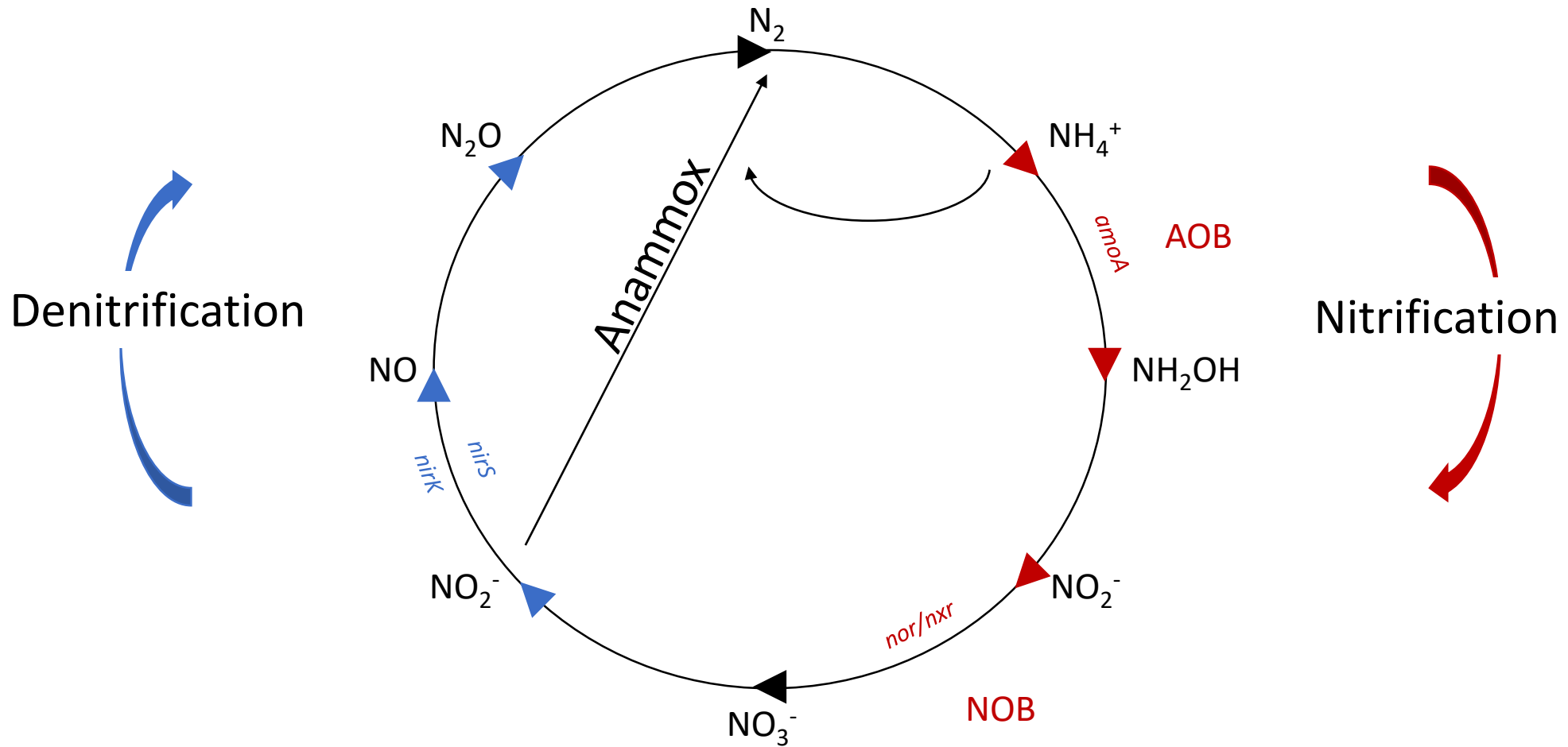
- The Nitrogen Cycle
 - Nitrification
 - Denitrification
 - Anammox
- Molecular Biological Tools (MBTs)
 - qPCR
 - Next generation sequencing (NGS)
 - metagenomics
- MBT Applications
- MI Microbiology Database



Nitrogen Cycle



Nitrogen Cycle



Why quantify nitrifiers?

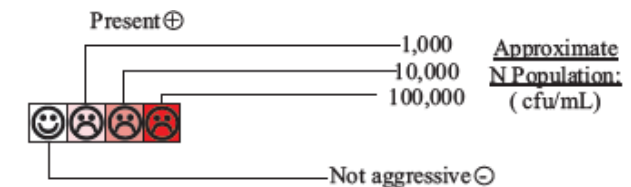
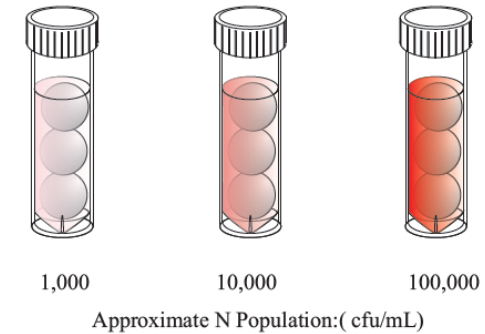


- The Ammonia Update (1999, 2009 Draft, 2013)
 - Nitrifiers are slower growers than heterotrophs
 - More sensitive to low temperatures, pH, and toxic compounds
- Identify minimum AOB and NOB concentrations for nitrification
 - Correlate AOB and NOB concentrations to operating parameters
 - Solids retention time (SRT), F/M ratio
 - DO concentration, temperature, alkalinity, pH

Estimate with cultivation approaches?

- Although **nitrification** is an important water and wastewater treatment process, isolating and **quantifying the related organisms is not a common practice**.
- Because nitrification yields little energy, these bacteria grow at a much slower rate than most heterotrophic bacteria, making them relatively **difficult to grow in the laboratory**.
- They also can be **tedious to identify** because of both their slow growth rate and their poor isolation and colony development on agar plates.

Cultivation Methods



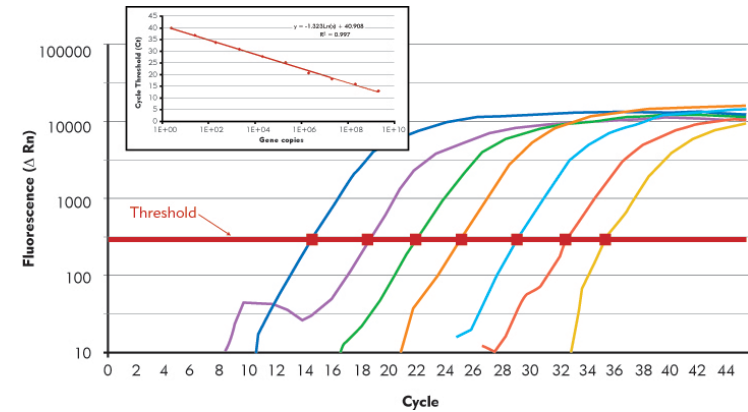
BART™ Test

Standard Methods for the Examination of Water and Wastewater

How to quantify - qPCR

Quantitative polymerase chain reaction (qPCR)

- DNA extracted directly from sample
- No need to grow the organisms
- Primers/probes specific to target gene
- Accurate quantification of a target gene
 - 16S rRNA genes (e.g. Nitrosomonas, Nitrobacter)
 - Functional genes (e.g. ammonia monooxygenase)



Nitrification

Ammonia Monooxygenase (MI Database)

- Average: $\sim 1.0E+05$ copies/mL
- Median: $\sim 7.0E+04$ copies/mL



Agreement between *amoA* Gene-Specific Quantitative PCR and Fluorescence *In Situ* Hybridization in the Measurement of Ammonia-Oxidizing Bacteria in Activated Sludge

J. D. C. Baptista,^{*} M. Lunn,^{*} R. J. Davenport,^{*} D. L. Swan,^{*} L. F. Read,^{*} M. R. Brown,^{*} C. Morals,^{*} T. P. Curtis^{*}
School of Civil Engineering and Geosciences, Newcastle University, Newcastle upon Tyne, United Kingdom; Department of Statistics, University of Oxford, Oxford, United Kingdom^{*}

Environmental Microbiology (2009) 11(9), 2310–2328

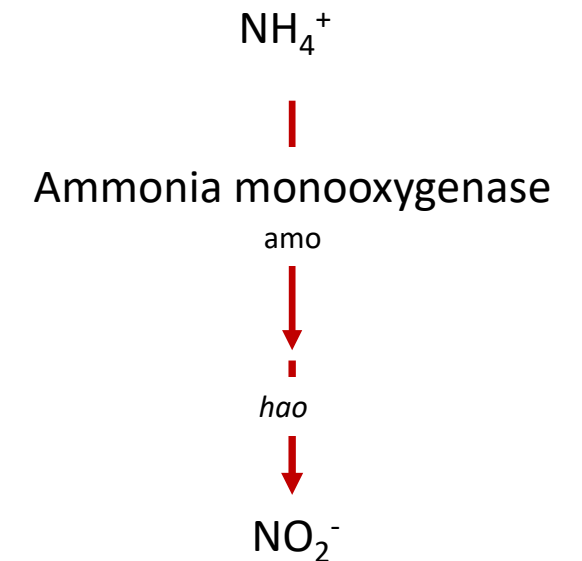
doi:10.1111/j.1462-2920.2009.01958.x

Ammonia-oxidizing communities in a highly aerated full-scale activated sludge bioreactor: betaproteobacterial dynamics and low relative abundance of Crenarchaea

George F. Wells,^{1†} Hee-Deung Park,^{1,4†}
Chok-Hang Yeung,¹ Brad Eggleston,²
Christopher A. Francis^{3*} and Craig S. Criddle^{1*}

10^4 to 10^9 copies/mL

10^5 to 10^6 copies/mL



Nitrification

Nitrite Oxidoreductase (MI Database)

- Average: $\sim 2.0E+04$ copies/mL
- Median: $\sim 1.0E+03$ copies/mL

Appl Microbiol Biotechnol (2007) 75:211–221
DOI 10.1007/s00253-006-0805-8

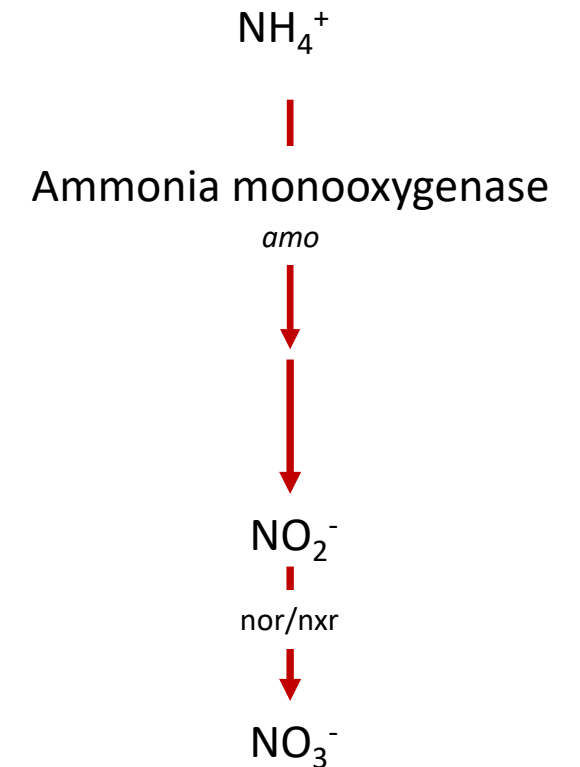
ENVIRONMENTAL BIOTECHNOLOGY

Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge

Joke Geets • Michaël de Cooman • Lieven Wittebolle •
Kim Heylen • Bram Vanparys • Paul De Vos •
Willy Verstraete • Nico Boon

ND to 10^6 copies/mL

nxrB



Nitrification

Nitrite Oxidizing Bacteria (MI Database)

- Average: $\sim 2.0E+08$ cells/mL
- Median: $\sim 1.4E+04$ copies/mL

Appl Microbiol Biotechnol (2007) 75:211–221
DOI 10.1007/s00253-006-0805-8

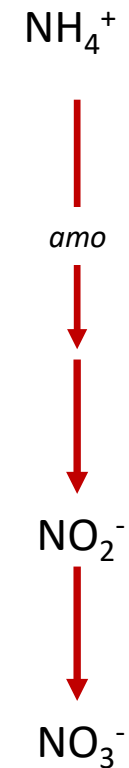
ENVIRONMENTAL BIOTECHNOLOGY

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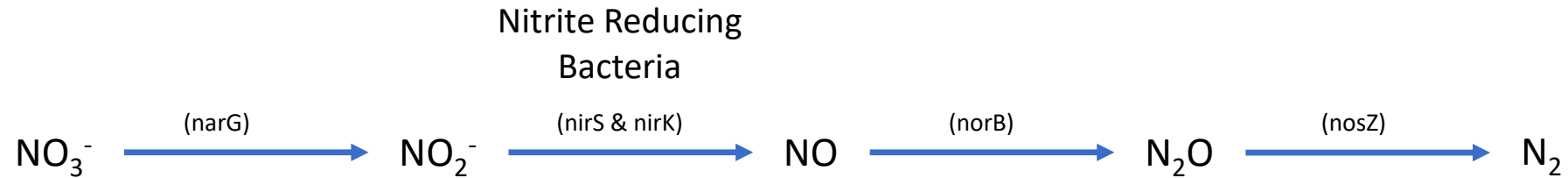
Joke Geets • Michaël de Cooman • Lieven Wittebolle •
Kim Heylen • Bram Vanparrys • Paul De Vos •
Willy Verstraete • Nico Boon

10^2 to 10^7 copies/mL
Nitrospira 16S rRNA genes

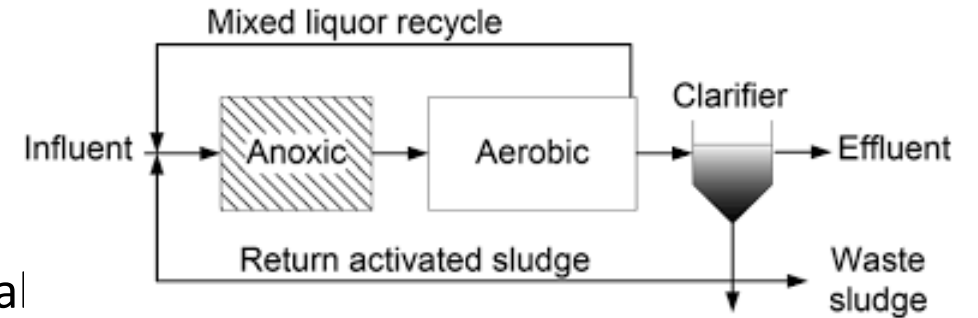
Nitrospira



Why quantify denitrifiers?



- Nitrogen limits and monitoring requirements
- Total nitrogen (TN) removal
- Biological phosphorus removal depends on nitrate removal
- Identify minimum concentrations for denitrification
- Correlate concentrations to operating parameters
- Optimize operating parameters (internal recycle, aeration)



Modified Ludzack-Ettinger (MLE)

Denitrification

Nitrite Reductases *nirK* and *nirS* (MI Database)

- Average: $\sim 2.0 \times 10^7$ copies/mL
- Median: $\sim 1.0 \times 10^5$ copies/mL

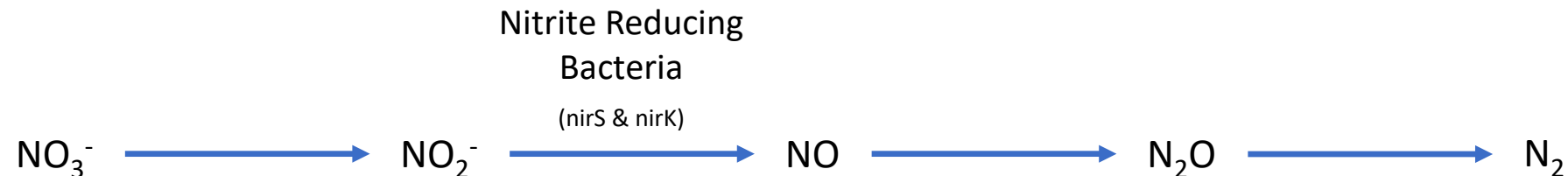
Appl Microbiol Biotechnol (2007) 75:211–221
DOI 10.1007/s00253-006-0805-8

ENVIRONMENTAL BIOTECHNOLOGY

Real-time PCR assay for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge

10^3 to 10^7 copies/mL

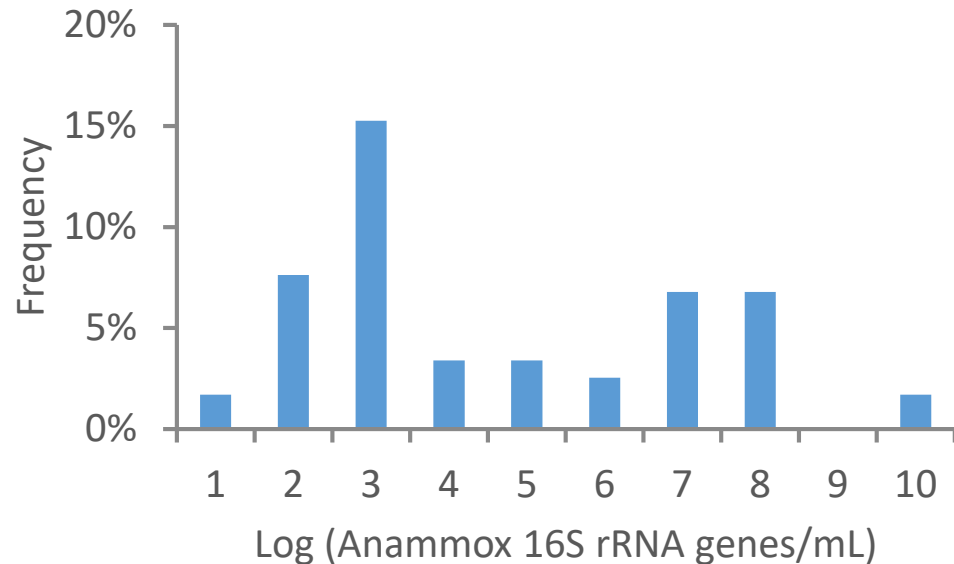
Joke Geets · Michaël de Cooman · Lieven Wittebolle ·
Kim Heylen · Bram Vanparys · Paul De Vos ·
Willy Verstraete · Nico Boon



Anammox

Anammox 16S rRNA (MI Database)

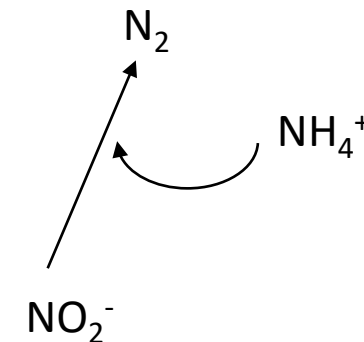
- Average: $\sim 2.0E+08$ copies/mL
- Median: $\sim 1.0E+03$ copies/mL
- Detection: $\sim 50\%$



Identification and quantification of anammox bacteria in eight nitrogen removal reactors

Bao-lan Hu^{a,b}, Ping Zheng^a, Chong-jian Tang^a, Jian-wei Chen^a, Erwin van der Biezen^{b,1}, Lei Zhang^a, Bing-jie Ni^c, Mike S.M. Jetten^{b,d}, Jia Yan^b, Han-Qing Yu^c, Boran Kartal^{b,*}

10^7 to 10^9 copies/mL



Additional qPCR Applications

- Denitrification w/ external carbon addition
 - Denitrifiers
 - Methanotrophs
 - Alcohol dehydrogenase
 - Glycerol utilizing bacteria
- Anaerobic Digesters
 - Fermenters (acid formers)
 - Methanogens



<https://www.waterworld.com/>



<https://www.westlafayette.in.gov/>

Additional qPCR Applications

Microbial Source Tracking (MST)

- General *Bacteroides*
- Human
- Dog
- Cattle
- Canada Goose
- Seagull

Identify fecal pollution sources



Additional Issues

Sludge Bulking or Foaming

- *Sphaerotilus natans*
- *Microthrix*
- *Nocardia*
- *Thiothrix*
- *Gordonia*

Tools for Metagenomic Analysis at Wastewater Treatment Plants: Application to a Foaming Episode

Gretchen E. Rosso, Jeffrey A. Muday, and James F. Curran*†

WATER ENVIRONMENT RESEARCH • March 2018

Additional Applications of MBTs

Biological Phosphorus Removal

- Phosphorus accumulating organisms (PAOs)
 - *Candidatus Accumulibacter*
 - *Tetrasphaera*
- Glycogen-accumulating organisms (GAOs)
 - *Competibacter*

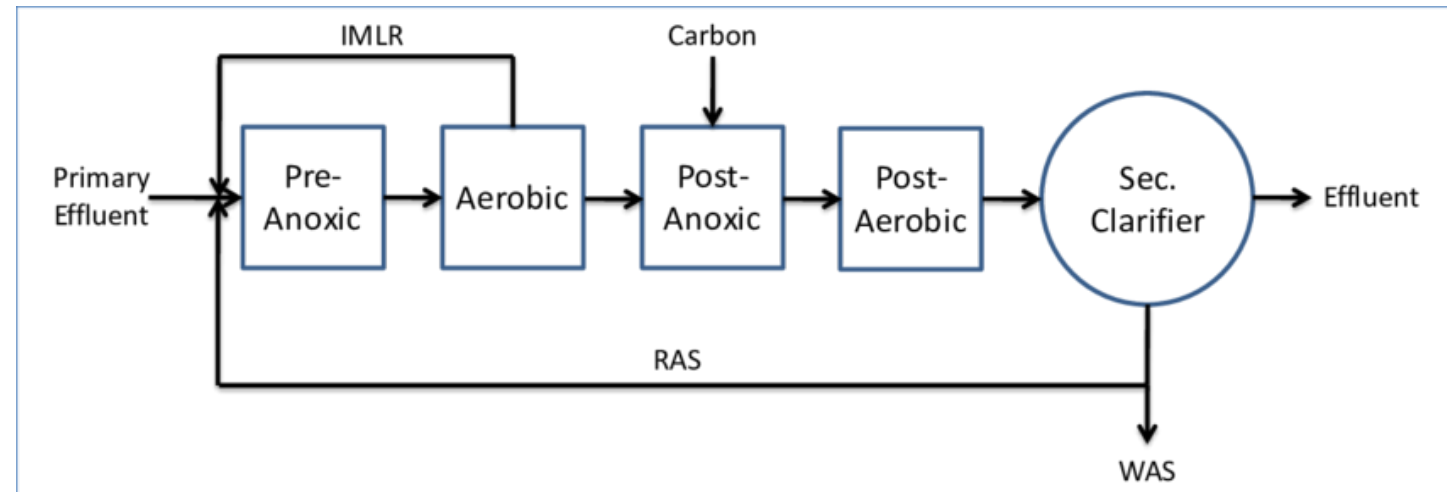
 The ISME Journal (2012) 6, 1094–1106
© 2012 International Society for Microbial Ecology. All rights reserved 1751-7362/12
www.nature.com/ismej

ORIGINAL ARTICLE

A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal

Mads Albertsen, Lea Benedicte Skov Hansen, Aaron Marc Saunders, Per Halkjær Nielsen and Kåre Lehmann Nielsen

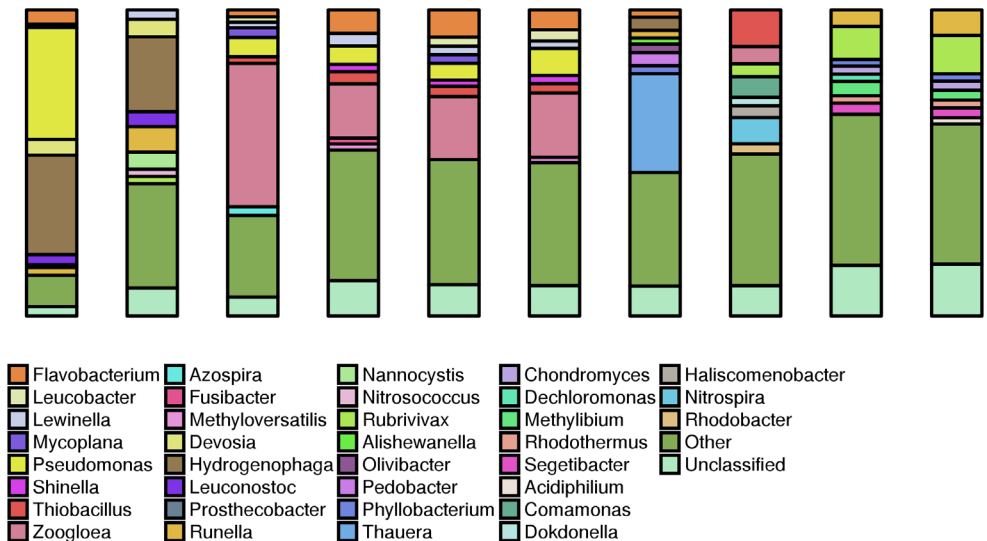
Four Stage Bardenpho



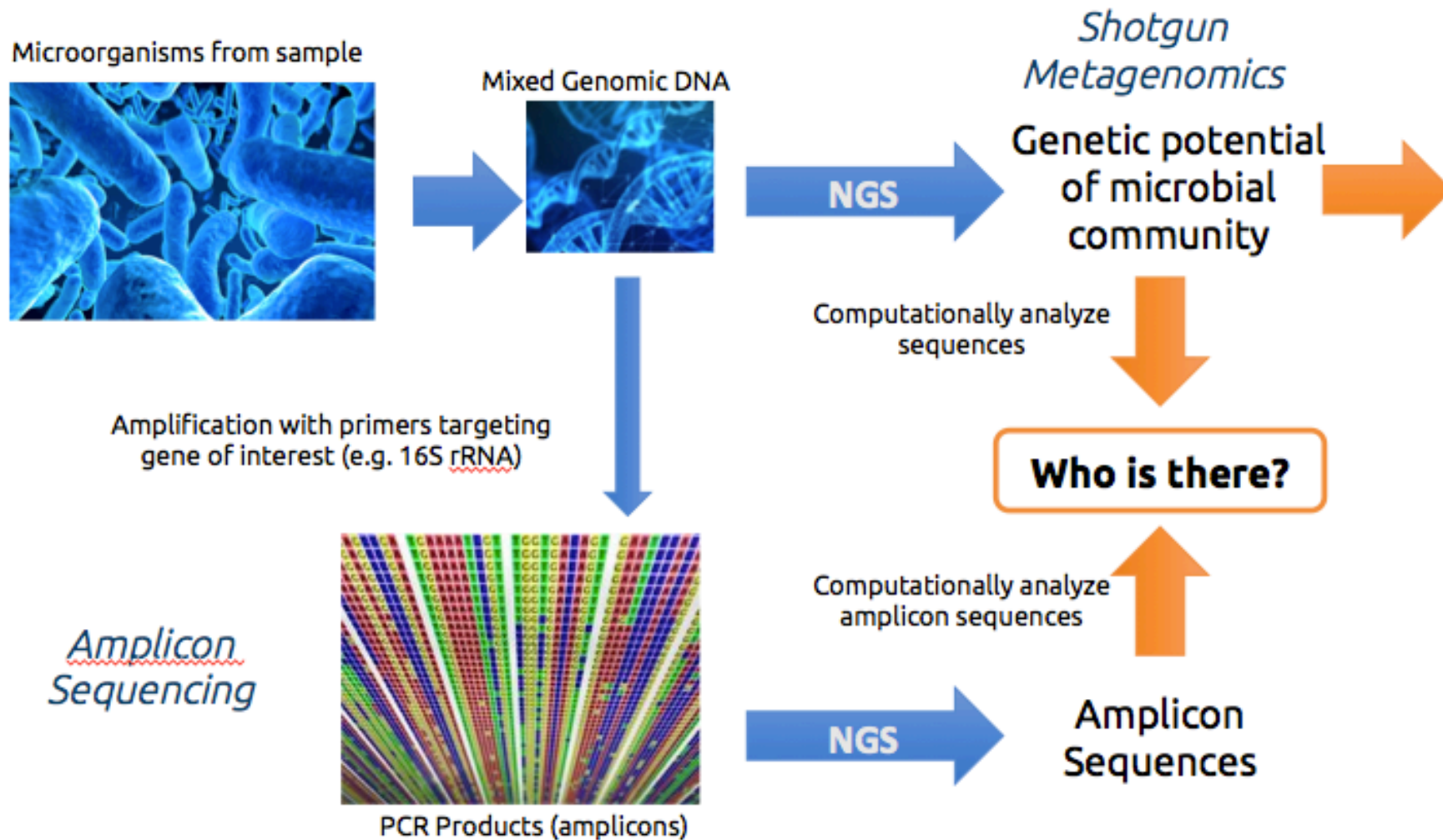
Metagenomics (Sequencing)

Who is there?

- Profile of microbial community composition
- Broad range (not specific)
- Relative abundance (not quantitative)
- Data intensive but informative



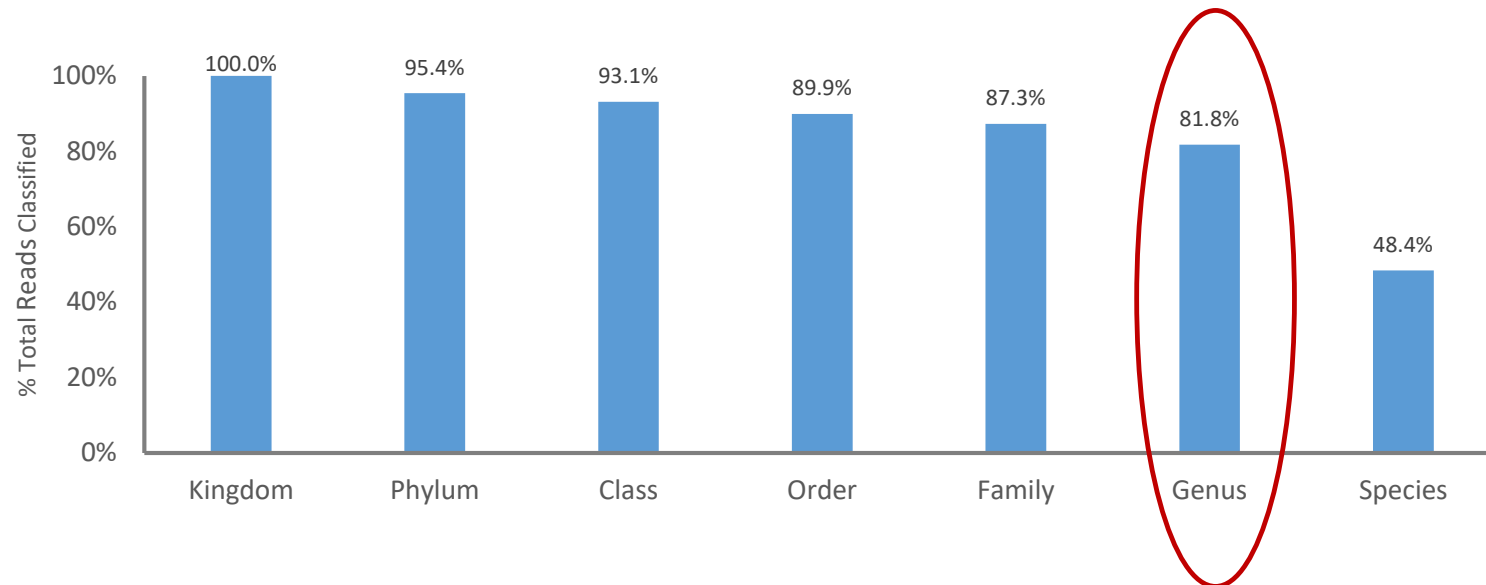
Metagenomics Terminology



(Adapted from Frank Löffler)

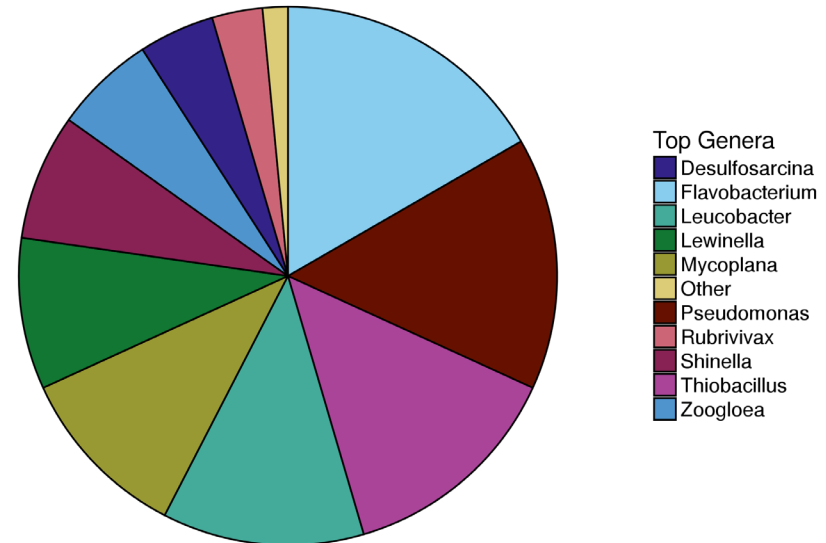
NGS Results

Sample ID	Reads Passing Quality Filtering	% Reads Classified to Genus	Shannon	Simpson	Chao1 Predicted Genera	Total Genera Observed
Sample 1	343,910	81.8%	3.9	0.95	620	566
Sample 2	333,779	84.8%	3.6	0.91	620	552
Sample 3	326,837	80.5%	4	0.96	630	565
Sample 4	393,302	82.0%	4	0.95	620	571
Sample 5	512,991	90.0%	2.8	0.88	580	490



Top Genus Classification Results

	Classification	Number of Reads	% Total Reads	Description
Bulking	Zoogloea	104,259	20.6	Floc forming obligate aerobes found in activated sludge
	Flavobacterium	44,946	8.9	Degrades biopolymers such as chitin and cellulose. This genus is aerobic and is widely distributed in soil and water.
	Pseudomonas	27,626	5.5	Genus of metabolically versatile, chemoorganotrophic aerobes. Generally, oxygen is the terminal electron acceptor, though nitrate reduction or complete denitrification may also be used under anaerobic conditions by many species. Overall, growth conditions vary greatly between species. Pseudomonas is ubiquitous in nature but may be less tolerant of acidic environments.
	Thiobacillus	16,734	3.3	Genus of sulfur oxidizing to sulfate. Thiobacillus genera are facultative chemoorganotrophically species are facultative de
	Leucobacter	14,847	2.9	Genus of chemoorganotrophic diverse and have been isolated from activated sludge.
Filamentous	Mycoplana	14,319	2.8	Genus of aerobic bacteria that oxidatively from sugars, ammonium salts, urea and filament forming.
	Lewinella	14,060	2.8	Genus of sheathed aerobic species-dependent.
	Shinella	10,511	2.1	Aerobic genus of non-specific and carbon sources.



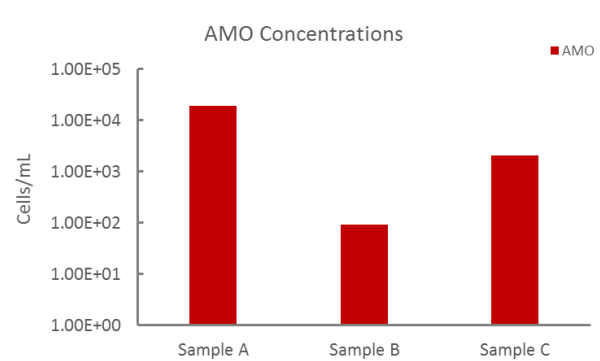
Genus Classification Results

	Classification	Number of Reads	% Total Reads	Description
Nitrifying	Nitospira	1,044	0.2	Nitospira species are nitrite-oxidizing bacteria
	Nitrosococcus	320	0.1	Genus of strictly aerobic ammonia-oxidizing bacteria (AOB) performing chemolithotrophic growth.
	Nitrobacter	9	0.0	Genus of nitrifying lithotrophic or chemoorganotrophic bacteria possessing aerobic and anaerobic respiration capabilities. Best known as a member of the nitrite oxidizing bacteria (NOB).
Denitrifying	Thauera	612	0.1	Thauera is a genus of denitrifying bacteria. Some strains are capable of anoxically degrading toluene via the benzyl succinate synthase gene (<i>bssA</i>).
PAO	Tetrasphaera	1	0.0	Aerobic genus of bacteria considered to be polyphosphate accumulating organisms (PAOs)
Foaming	Gordonia	505	0.1	Genus of aerobic actinomycetes. Some species are strongly associated with foaming problems in wastewater treatment. Highly versatile and have been shown to degrade xenobiotics, environmental pollutants, and slowly biodegradable natural polymers. Gordonia form sessile communities resulting in biofilms. A nocardioform actinomycete with mycelial growth fragmentation into rod or coccoid elements.

MBT Summary

qPCR

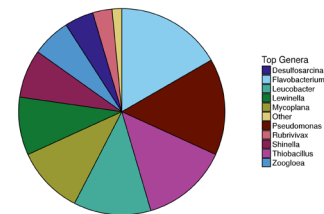
- What are the concentrations of nitrifiers, denitrifiers, etc.?
- Quantitative
- Absolute concentration (cells/mL)
- Target specific
- Functional genes or taxonomic
- Small, specific dataset



Next Generation Sequencing (NGS)

- Who is there? How has the community changed? How diverse?
- Not quantitative
- Relative abundance (% of total)
- Overall profile
- Usually taxonomic
- Large dataset

Classification	Number of Reads	% Total Reads	Description
Zoogloea	104,259	20.6	Floc forming obligate aerobes found in activated sludge
Flavobacterium	44,946	8.9	Degrades biopolymers such as chitin and cellulose. This genus is aerobic and is widely distributed in soil and water.
Pseudomonas	27,626	5.5	Genus of metabolically versatile, chemoorganotrophic aerobes. Generally, oxygen is the terminal electron acceptor, though nitrate reduction or complete denitrification may also be used under anaerobic conditions by many species. Overall, growth conditions vary greatly between species. Pseudomonas is ubiquitous in nature but may be less tolerant of acidic environments.
Thiobacillus	16,734	3.3	Genus of sulfur oxidizing aerobes or facultative anaerobes. Reduced sulfur compounds are oxidized to sulfate. Thiobacillus generally grow autotrophically but some species grow chemoorganotrophically or chemolithotrophically. All species are carbon dioxide fixers. Some species are facultative denitrifiers with T. denitrificans
Leucobacter	14,847	2.9	Genus of chemoorganotrophic strictly aerobic bacteria. Members of this genus are metabolically diverse and have been isolated from a variety of environments including soil, nematodes, air and activated sludge.
Mycoplana	14,319	2.8	Genus of aerobic bacteria that possess a strictly respiratory type of metabolism that produce acid oxidatively from sugars. Requiring biotin or thiamin for growth, Mycoplana utilize Casamino acids, ammonium salts, urea and peptone as both carbon and nitrogen sources. Growth is branching and filament forming.
Lewinella	14,060	2.8	Genus of sheathed aerobic chemoorganotrophs. Oxygen is used as the terminal electron acceptor. A variety of organic compounds are utilized as energy and carbon sources but specific type is species-dependent.
Shinella	10,511	2.1	Aerobic genus of non-spore forming bacteria. Shinella utilize sugars and sugar alcohols as energy and carbon sources.



Final Thoughts



- MBTs are routinely used to monitor biological processes



- More accurate quantification of key organisms



- Predictive power



- Less upsets



- Save money



- Better effluent quality

Questions?