May 12, 2020

Mr. Sun Liang, PhD, PE
Manager, Water Reuse Development
Metropolitan Water District of Southern California

Subject: NWRI Independent Science Advisory Panel Recommendations for Workshop 3

Dear Sun:

The National Water Research Institute (NWRI) is pleased to present this technical letter report on the findings and recommendations from Workshop No. 3 of the Independent Science Advisory Panel (Panel) for the Regional Recycled Water Program (RRWP), Advanced Purification Center Demonstration Project (Project). The full Panel met on April 9, 2020, via videoconference.

Ed Means of Means Consulting facilitated the meeting, with the following Panel members in attendance:

- Panel Chair: Charles Haas, PhD, BCEEM, Drexel University
- Joseph A. Cotruvo, PhD, BCES, Joseph Cotruvo and Associates
- Thomas E. Harder, PG, CHG, Thomas Harder and Co.
- Nancy Love, PhD, PE, BCEE, University of Michigan
- Adam Olivieri, DrPH, PE, EOA, Inc.
- Vernon Snoeyink, PhD, University of Illinois
- Paul K. Westerhoff, PhD, PE, BCEE, Arizona State University

Meeting Objectives

The goals for Workshop 3 were to present data that would enable the Panel to:

- Independently review the nitrification and denitrification optimization testing results for tertiary MBR performance.
- Independently assess the recommended changes of the current Tertiary MBR Testing and Monitoring Plan.

- Meet via videoconference and prepare a letter of support summarizing the Panel's technical review and recommendations regarding modification of Metropolitan's current testing and monitoring plan.

Questions to the Panel

The following questions were presented to the Panel, and are addressed in this technical letter report:

1. What is the Panel's evaluation of Metropolitan's recommendations for operating nitrification-only MBRs to meet targeted LRVs for the MBR process?
2. What is the Panel's evaluation of Metropolitan's recommendations for operating nitrification-only MBRs to meet basin plan objectives for the full process train?
3. What are the Panel's recommendations for proceeding with baseline testing?

Panel General Comments

The Metropolitan Water District of Southern California (Metropolitan) project team has extensively evaluated many unit processes, including the membrane bioreactor (MBR), for tertiary treatment. The team has obtained some useful results, but additional studies are necessary.

The Panel understands that the Regional Recycled Water Program is still in a study phase and no decision has been made regarding the optimal process configuration for full-scale advanced water treatment processes to treat either primary or secondary wastewater effluent from the Sanitation District of Los Angeles County's Joint Water Pollution Control Plant. Metropolitan has indicated that further fine-tuning and additional process evaluation will occur later in the program.

Metropolitan is proposing to proceed at this time with the nitrification-only operational mode, which deviates from the nitrification-denitrification (NDN) mode proposed in the current approved Testing and Monitoring Plan (TMP).

Treatment of non-nitrified secondary effluent in NdN mode at the demonstration facility has resulted in elevated nitrite levels in MBR filtrate which are anticipated to have adverse impacts on downstream reverse osmosis (RO) and ultraviolet/advanced oxidation process (UV/AOP) performance. While the Panel thinks that additional work to fine-tune the NdN process could adequately control nitrite, the Panel recommends that the development plan should be expanded to objectively consider and evaluate other plausible technologies (such as annamox) to manage nitrite as well as consideration of the other recommendations in the Panel memo.

One of the main goals of the approved TMP is to demonstrate pathogen removal through the MBR system. Metropolitan has noted that a pilot study in 2010-2012 to assess efficacy

of a nitrification-only mode MBR-RO-UV/AOP treatment train on effluent from the plant showed that treated water quality goals could be achieved based on the draft groundwater recharge regulations available during the time of the pilot study and that a nitrification-only operation mode is anticipated to provide conservative conditions to assess pathogen removal through the MBR process.

The Panel is prepared to review the results of the pathogen removal evaluation for the nitrification-only mode MBR-RO-UV/AOP treatment train to demonstrate that the process can reliably meet treated water quality goals once Metropolitan develops the data.

Metropolitan's ultimate choice of treatment train will greatly affect the long-term operations and maintenance costs for the region's recycled water supply. The goal should be to select the best technologies for the best long-term operational and economic results and to find the best available technology. The process of selecting the treatment train will require the project team to learn about and benefit from other developing technologies and to conduct some bench-scale and pilot testing.

Responses to Questions Provided by Metropolitan

1. What is the Panel's evaluation of Metropolitan's recommendations for operating nitrification-only MBRs to meet targeted LRVs for the MBR process?

Response - The Panel believes that insufficient information was presented during the workshop to draw firm conclusions about nitrification-only MBRs (there were only two slides on the nitrification-only MBR option). The Panel observes/recommends:

- The procedure and assumptions for calculating the nitrite reduction rate should be more clearly stated (see Slide 34). The nitrite reduction rate was calculated from raw (concentration vs. time) data via adjacent points. The data interpretation was not well developed or justified. Nitrite is accumulating, yet is shown as an uptake rate (still positive, along with nitrate, which decreased with time).
- Additional cost-benefit analysis should be conducted to better characterize the carbon cost for NdN versus the cost to use two-pass RO and potentially higher membrane replacement frequency should nitrate rejection rates decrease over the life of the RO membranes.
- Ancillary benefits for log reduction values (LRVs) may exist because of two-pass RO but were not identified.
- Additional industrial user identification and discharge characterization should be conducted to gain a better understanding of the potential organic nitrogen load entering and passing through the secondary treatment process at the wastewater treatment plants.

- Industrial users may be sources of nitrifying inhibitors, but this has not been evaluated; it will need to be evaluated if the technology moves toward nitrification-only MBRs.
- The Panel assumes that the LRV sampling plan is still consistent with the plan the project team previously provided to the Panel. The LRV plan and approach are appropriate. The Panel agrees that all LRVs should be adjusted based on matrix spike information, which we assume is being done for each sample.
- Does the project team anticipate a difference in the removal of protozoans under aerobic versus anaerobic conditions and, if yes, should the LRV study plan take this into account?
- Given the performance observed in this round of testing, it is premature to remove mainstream anammox from consideration without more information.
    - The Panel suggests considering mainstream anammox plus RO. For example, the Hampton Roads Sanitation District (HRSD) has developed, demonstrated and is moving forward with design of an AvN (ammonia-oxidizing bacteria, or AOB, versus nitrite-oxidizing bacteria, or NOB) mainstream anammox process.
    - AvN uses established sensor control strategies to select for AOB, repress NOB, and enhance anammox performance. Consequently, mainstream anammox should be objectively investigated as part of this stage of project development. It has progressed from a few years ago.
    - A visit to plants and/or other facilities with mainstream anammox experience (for example Hampton Roads or DC Water) is warranted to develop confidence in the status of the technology, a better understanding about the approach, and potential partnership with these leading utilities. They are very willing to share their experience with Metropolitan about application and scale-up of their technologies. These two utilities collaborated to create the utility-utility partnership concept that is now LIFT.
    - The annamox process warrants a deeper evaluation and a side-by-side cost comparison with the other options. The process might work even better in California's climate (warmer water temperatures).
- Recent research on nutrient removal is included in three attachments to this report:
    - Presentation by Haydee De Clippeleir at IWA Nutrient Removal conference in November 2018 (Attachment 3). This includes an explanation of Partial Nitrification Anammox (PNA) and Partial Denitrification Anammox (PdNA) pathways through the nitrogen cycle that form the basis of deammonification strategies (anammox treatment).

- Paper by Tri Le, a PhD student who conducted his work at DC Water (Attachment 4). The paper discusses the use of NO3-N residual as a control parameter.
- Poster by Priyanka Ali, who completed her masters at DCW last year, showing she could get a similar result with primary sludge fermentation vs. acetate as the carbon source (Attachment 5).

2. What is the Panel's evaluation of Metropolitan's recommendations for operating nitrification-only MBRs to meet basin plan objectives for the full process train?

Response - The Panel believes more detailed justification should be given for the recommendation that the tertiary MBR should be for nitrification only rather than NdN.

The Panel observes/recommends the following:

- Conduct an economic comparison (capital and O&M costs) between NdN plus RO, nitrification only and two-pass RO, and mainstream anammox plus RO.
- If all the nitrogen is converted to nitrate, the nitrification-only MBR would have ~50 mg NO3-N/L. Please show projections for nitrate in the effluent of the first- and second-pass RO. Please show staging of RO arrays.
- The replacement frequency for RO modules may be more frequent to achieve nitrate treatment targets. These costs should be considered.
- The ancillary benefits for two-pass RO, beyond nitrate removal, should be identified (for example, impacts on boron).
- Secondary impacts of two-stage RO were only briefly identified (for example, brine composition and impact on brine disposal, plus how this affects recent and planned brine toxicity testing).
- It is important to establish an ammonia goal after nitrification-only MBR and determine how the system will be operated to assure complete nitrification. The factors influencing complete nitrification should be understood as well as the risks if complete nitrification is not achieved.
    - Without nitrification for 100 percent of the day, the residual ammonia could make it difficult to dose chlorine accurately and to maintain constant chloramine residuals in the water flowing onto the RO membrane.
    - Another risk is producing off-specification water that does not meet the nitrate goal.
    - Critical Control Points should be defined for off-specification water.

- The Panel would like to receive copies of the WEFTEC 2019 paper (ref 6) and the other conference paper from 2018 (ref 5).
- The Panel is curious to see how the hypothesis about predation going down with an N-only system pans out and encourages the collection of quantitative data. Nitrifiers are quite vulnerable to predation—more so than heterotrophs, since ammonia-oxidizers often are detached during perturbations and predators are more successful with detached cells. The paper, *A comparative analysis of drinking water employing metagenomics*, is attached (Attachment 6) for reference.

3. What are the Panel's recommendations for proceeding with baseline testing?

Response - The Panel observes/recommends the following:

- While it is understandable why the team only operated for approximately one solids retention time (SRT) under the nine different NdN conditions evaluated, it is possible that with a 15-day SRT, the nitrifiers simply did not have ample time to acclimate. Typical minimums before starting a study is three SRTs. The Panel feels that NdN has not been truly ruled out yet.
- The current conclusion that denitrification is not possible at the proposed scale of operation may be premature and difficult to explain to regulators.
- The proposed fiber cutting/integrity test approach should still be valid for NdN-MBR or nitrification-only MBR.
    - The working assumption is that sufficient water can be filtered that will not result in an unreasonably sized pellet for examination.
    - Does the microbial research team believe there may be a problem with filtering enough water with a two- to three-order reduction of flow through the MBR after cutting fibers?
- Continuous in-line nitrogen sensors (all species) will be installed soon, and it is imperative they collect MBR influent data on nitrate, nitrite, and ammonia to understand how to design and operate the MBR. It is possible that diurnal changes or industrial inputs may be leading to large variations in nitrogen species concentrations.
- No data on MBR effluent dissolved organic carbon (DOC) were presented. How did DOC vary with the nine different NdN operational configurations? Is there anything to learn about the NDN process from the MBR effluent DOC data?
- Projections of nitrate (and nitrite) rejection by RO should be documented, based upon membrane life observed at other facilities.

  - It appears that 80 percent would be the minimum nitrate rejection that would be acceptable.
  - Will nitrate rejection be the likely controlling factor influencing RO membrane replacement frequency? If not, what factor is likely?
- It was not clear why two-pass RO was under consideration. Was it as a backstop in case nitrate or nitrite was not being sufficiently removed by NdN or other processes?
  - The extra expense of two-pass RO should not be necessary with proper choice of earlier steps in the treatment train.
  - There was no documented evidence presented that two-pass RO has been operated at scale for nitrate removal. It was applied for seawater desalination before the World Health Organization revised its boron guideline to 2.4 mg/L, which made it unnecessary.
  - Very detailed work has been done on tertiary MBR.
  - Nitrogen reduction is occurring but it appears to be sensitive to several conditions.
  - Indications of need for additional phosphate are not favorable.
  - Before additional work on tertiary MBR is undertaken, the upcoming secondary bench-scale work should be conducted as soon as possible, because it might provide different results, it might not require additional phosphate, and the carbon requirements might be less.
- Modifications of the primary/secondary treatment process may be an option for NdN.
- Concern about the flammability of methyl alcohol as a candidate carbon source seems to be misplaced.
  - Methanol has many commercial applications. It is flammable, but it is also highly water soluble.
  - The worldwide methanol production capacity is about 36 billion gallons, so it is readily available at relatively low cost because it is produced by hydrogenation of carbon monoxide. If the concern is with storage and transporting the volumes required, that is certainly manageable as has been demonstrated by its multitude of applications as solvent and feed stock.
  - Nitrite is readily converted to volatile nitrogen oxides or ammonia under appropriate conditions, if necessary.
  - It is reduced by sulfur dioxide to NO and N_2O.
  - It is reduced by hydrogen sulfide to ammonia.

  - Basin plan water quality objectives have been presented as a single value and the plant effluent data for comparison against the basin plan objectives have as well. Would it be more appropriate to characterize both values on a statistical basis, if possible?
  - What minimum nitrate removal is projected after RO membranes begin to age (80 percent nitrate rejection)?
  - What pressures would membranes operate under?
  - If all the nitrogen is converted to nitrate, the nitrification-only MBR would have ~50 mg NO_3-N/L. Please show projections for nitrate in effluent of first- and second-pass RO. Please show staging of RO arrays.
  - It was stated the nitrite goal after the nitrification-only MBR would be less than 0.2 mg NO_2-N/L. Is this correct?
  - How will you prepare or protect against upset events (nitrification is notoriously vulnerable to toxic upset)?
  - What are the known risks from upstream industries?
  - What will the utility do if there is a toxic load coming at them?
  - A high-level plan should at least be part of a nitrification-only recommendation.
  - Will DOC be different for nitrification-only MBR compared to NdN-MBR? If so, what impacts on RO fouling would be expected?
  - No data on MBR effluent DOC was presented.
  - How did this vary with the nine different NdN operational configurations?
  - Can we learn anything about the NdN process from the MBR effluent DOC data?
  - Projections of nitrate (and nitrite) rejection by RO should be documented, based upon membrane life observed at other facilities.
- Will nitrate rejection be the likely controlling factor influencing RO membrane replacement frequency? If not, what factor is likely?
- The Panel has included a reference regarding recent work on metagenomics for your information.

Conclusion

This concludes the Panel responses to questions presented during Workshop 3. Please contact me if you have any questions.

Sincerely,

Dr. Charles Haas

Panel Chair

Attachment 1 – About NWRI

Attachment 2 – Panel Member Biographies

Attachment 3 – Keynote Presentation by Haydée De Clippeleir, PhD

Attachment 4 – Tri Le et al., Nitrate residual as a key parameter to efficiently control partial denitrification coupling with anammox

Attachment 5 – Priyanka Ali poster on primary sludge fermentation

Attachment 6 – Kyle Brumfield et al., A comparative analysis of drinking water employing metagenomics

Attachment 1 • About NWRI

The National Water Research Institute is a 501c3 nonprofit organization and Joint Powers authority, founded in 1991 by a group of California water agencies in partnership with the Joan Irvine Smith and Athalie R. Clarke Foundation to promote the protection, maintenance, and restoration of water supplies and to protect public health and improve the environment. NWRI's member agencies include Inland Empire Utilities Agency, Irvine Ranch Water District, Los Angeles Department of Water and Power, Orange County Sanitation District, Orange County Water District, and West Basin Municipal Water District.

Disclaimer

This report was prepared by an Independent Expert Advisory Panel (Panel), which is administered by National Water Research Institute. Any opinions, findings, conclusions, or recommendations expressed in this report were prepared by the Panel. This report was published for informational purposes.

For more information, please contact

National Water Research Institute
18700 Ward Street
Fountain Valley, California 92708 USA
Phone: (714) 378-3278
www.nwri-usa.org
Kevin Hardy, Executive Director
Mary Collins, Communications Manager
Suzanne Sharkey, Water Resources Scientist and Project Manager

Publication Number: NWRI-2020-09

Attachment 2 • Panel Member Biographies

Chair: Charles N. Haas, PhD, BCEEM

Professor of Environmental Engineering and Head, Department of Civil, Architectural and Environmental Engineering, Drexel University

Dr. Charles Haas has more than 45 years of experience conducting research in water treatment, risk assessment, environmental modeling and statistics, microbiology, and environmental health. He has led the Department of Civil, Architectural, and Environmental Engineering at Drexel University since 1991, and previously served on the faculties of Rensselaer Polytechnic Institute and Illinois Institute of Technology. Haas holds a BS in Biology and an MS in Environmental Engineering from Illinois Institute of Technology, and a PhD in Environmental and Civil Engineering from University of Illinois.

Joseph A. Cotruvo, PhD, BCES

President, Joseph Cotruvo and Associates, LLC

Dr. Joe Cotruvo is president of Joseph Cotruvo & Associates, an environmental and public health consulting firm in Washington, DC, and a Research Professor in the Departments of Chemistry and Biochemistry, and Environmental Sciences, at the University of Toledo. Previously, he was director of the Drinking Water Standards Division of the EPA Office of Drinking Water. He received a BS in Chemistry from the University of Toledo and a PhD in Physical Organic Chemistry from the Ohio State University. He is board certified by the American Academy of Environmental Engineers and Scientists and recipient of the AAEES Science Award for 2019.

Thomas E. Harder, PG, CHG

Principal Hydrogeologist, Thomas Harder & Co.

Mr. Thomas Harder has more than 22 years of professional groundwater consulting experience. He has provided technical direction and management for large water resource projects in southern California, including the Chino Desalter Well Field Design and Construction, the West Coast Basin Barrier Project, and the Mojave Water Agency's Regional Recharge and Recovery Project. His expertise includes regional groundwater basin analysis, perennial (safe) yield, artificial recharge, groundwater management and models, contaminant hydrogeology, and wells. Harder holds a BS in Geology from California Polytechnic University, Pomona, and an MS in Geology with emphasis in Hydrogeology from California State University, Los Angeles. He is a registered geologist and hydrogeologist in California.

Nancy G. Love, PhD, PE, BCEE

Borchardt and Glysson Collegiate Professor, University of Michigan

Dr. Nancy Love is the Borchardt and Glysson Collegiate Professor in the Department of Civil and Environmental Engineering at the University of Michigan. There, she directs the Love Research Group, which works at the interface of water, infrastructure, and public health in both domestic and global settings. They focus on assessing and advancing public and environmental health using chemical, biological, and analytical approaches applied to water systems using both physical experiments and computational models. Dr. Love received her BS and MS at the University of Illinois, Urbana, and her PhD is from Clemson University. She has also been recognized for her scholarship and leadership with the WEF, the Water Research Foundation, and the National Science Foundation.

Adam Olivieri, DrPH, PE

Principal/Founder, EOA, Inc.

Dr. Adam Olivier has more than 35 years of experience in the technical and regulatory aspects of water recycling, groundwater contamination by hazardous materials, water quality and public health risk assessments, water quality planning, wastewater facility planning, urban runoff management, and on-site waste treatment systems. Dr. Olivieri is currently Vice President of EOA, Inc., in Oakland, California, where he manages a variety of projects, including serving as Santa Clara County Urban Runoff Program's Manager since 1998. He received a BS in Civil Engineering from University of Connecticut, an MS in Civil and Sanitary Engineering from University of Connecticut, and both an MPH and DrPH in Environmental Health Sciences from University of California, Berkeley.

Vernon Snoeyink, PhD

Professor Emeritus, Civil and Environmental Engineering, University of Illinois

Dr. Vernon Snoeyink's research has focused on drinking water quality control, including removal of organic and inorganic contaminants from water using adsorption systems, especially granular and powdered activated carbon systems coupled with membrane systems. His expertise includes mechanisms of formation and means to control water quality in distribution systems in response to reactions of iron, aluminum, and other inorganics. He has also been recognized for excellence in teaching and advising. He holds a BS in Civil Engineering, an MS in Sanitary Engineering, and PhD in Water Resources Engineering from University of Michigan.

Paul K. Westerhoff, PhD, PE, BCEE

Professor, Sustainable Engineering/Built Environment, Arizona State University

Dr. Paul Westerhoff's research focuses on emerging contaminants, water treatment processes, and water quality, including: occurrence, characterization, and oxidation of natural organic matter; removal of oxo-anions from drinking water; algal metabolites and algal biotechnology; wastewater reuse; and nanotechnology and sensors. Westerhoff holds a BS in Civil Engineering from Lehigh University, an MS in Civil and Environmental Engineering from University of Massachusetts, Amherst, and a PhD in Civil, Architectural, and Environmental Engineering from University of Colorado at Boulder. He is a Registered Professional Engineer in Arizona.

Attachment 3 • Keynote Presentation by Haydée De Clippeleir, PhD

NOTE: The attached document is for informational purposes only. It has not been reviewed or endorsed by the NWRI Independent Science Advisory Panel.

On the road towards mainstream short-cut nitrogen removal implementation: the story of potholes and detours

Haydée De Clippeleir, PhD

Research program manager, DC Water

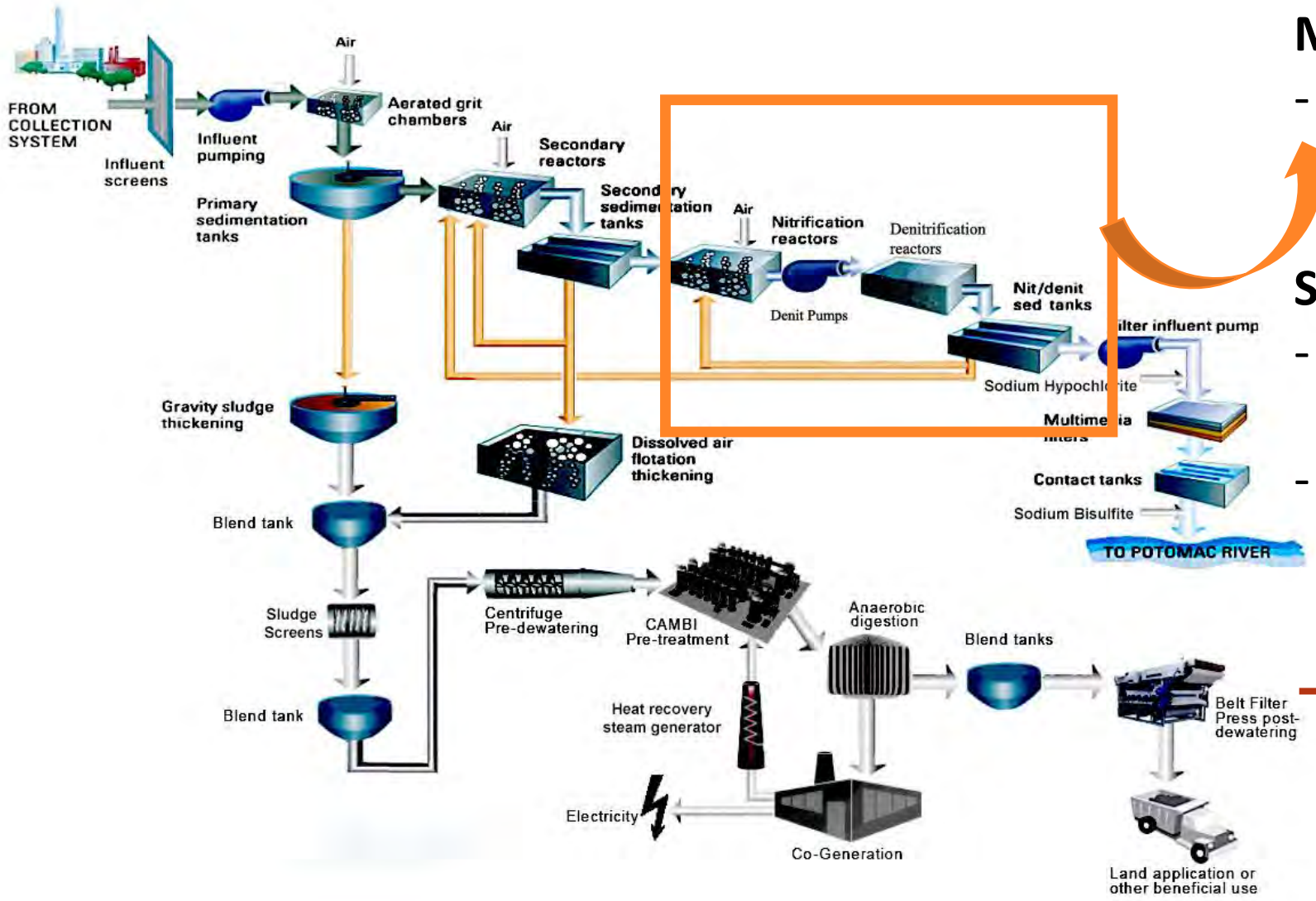
dc water is life®

IWA NRR conference, Brisbane, November 19th 2018

Overview Blue Plains

- 1400 MLD (384 mgd) average day capacity
- 153 acre footprint
- Serving DC, plus areas of MD and VA
  - Currently 2.2 million people
  - 1100 MLD (290 mgd)
- Discharges to Potomac River and Chesapeake Bay
- Stringent Nutrient Limits
  - TP - 0.18 mg/L
  - TN – 3.74 mg/L equivalent

Nutrient removal @ Blue Plains



Mainstream:

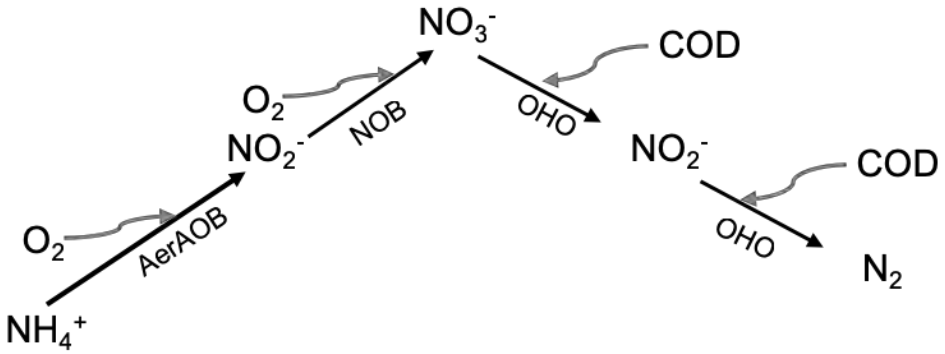
- Nitrification – denitrification with external carbon addition (MeOH)

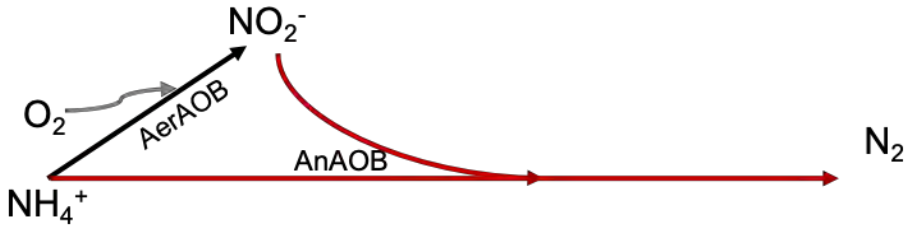
Sidestream:

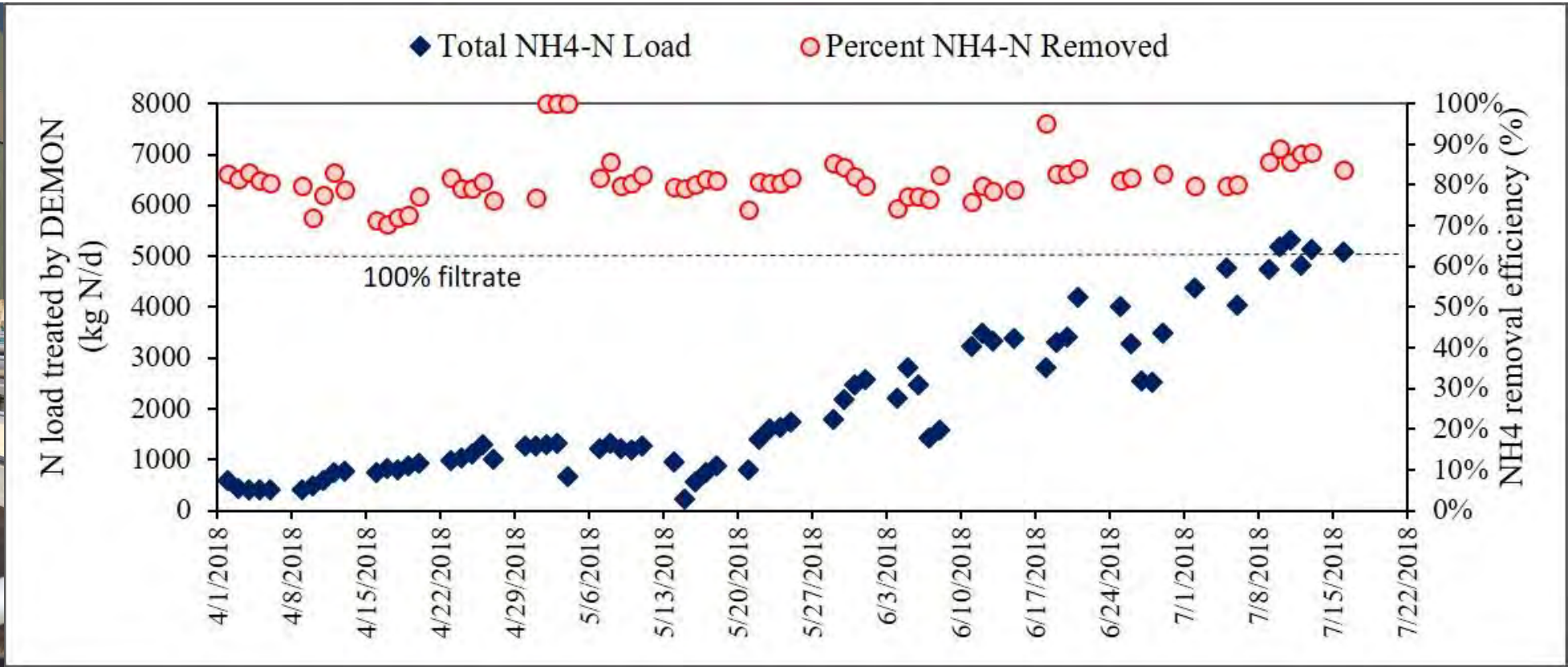
- DEMON process treating filtrate (THP-AD) (2018)
- Capacity: 10,000 kg N/d

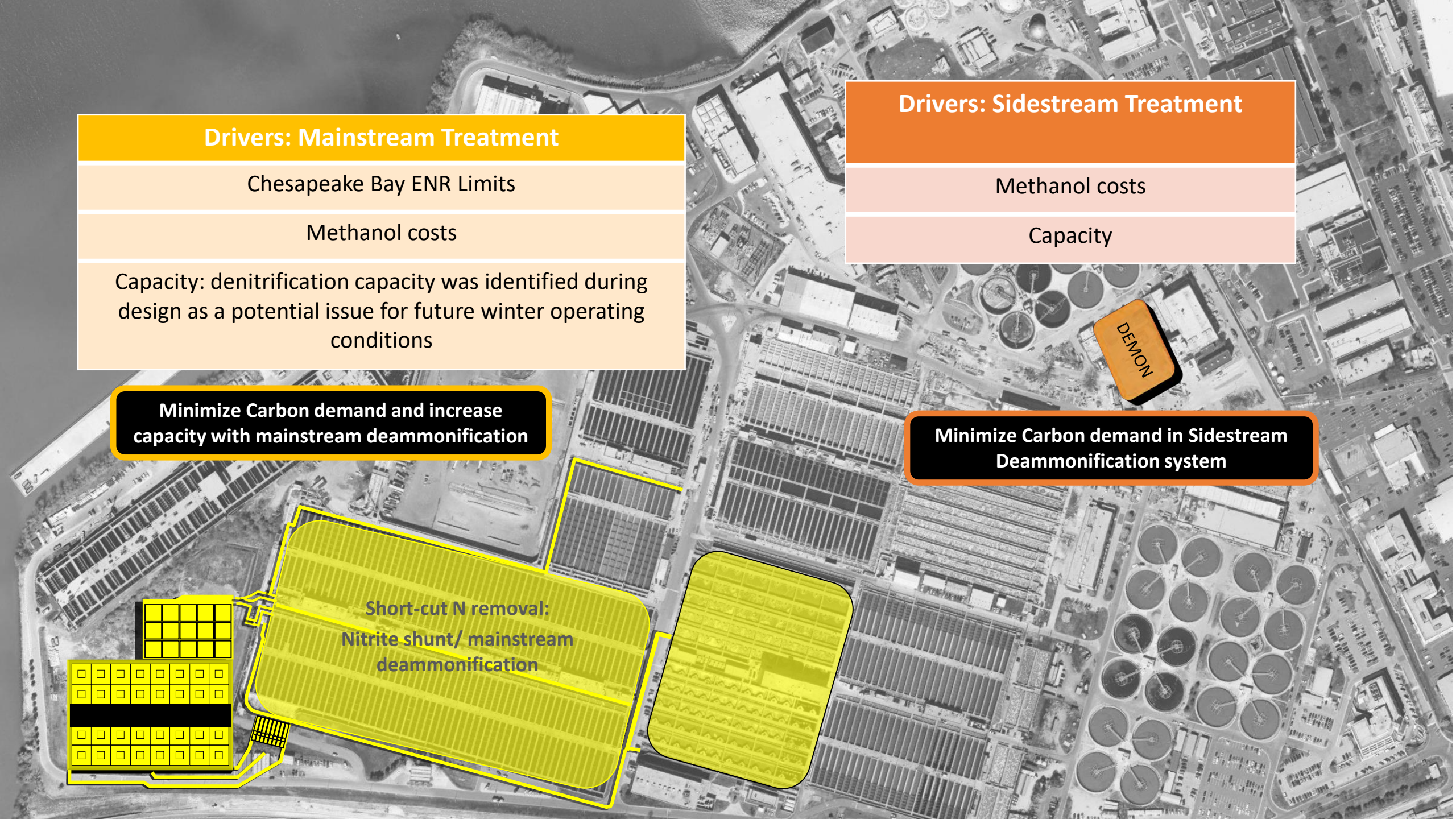
DEMON @ Blue Plains

Martinelli et al, NRR 2018, presentation 2.1

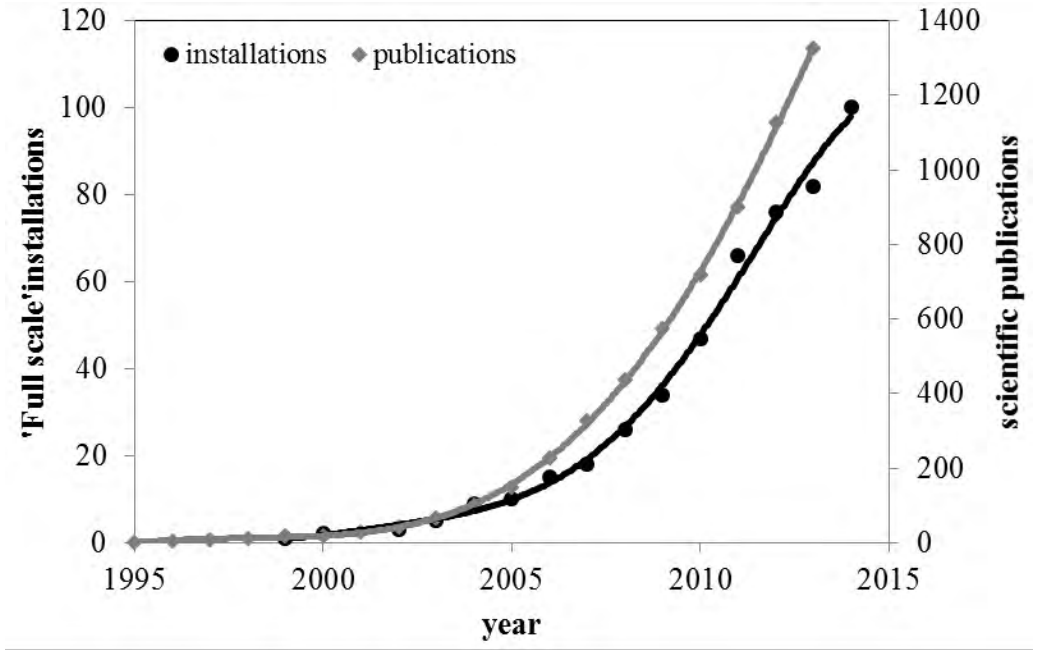




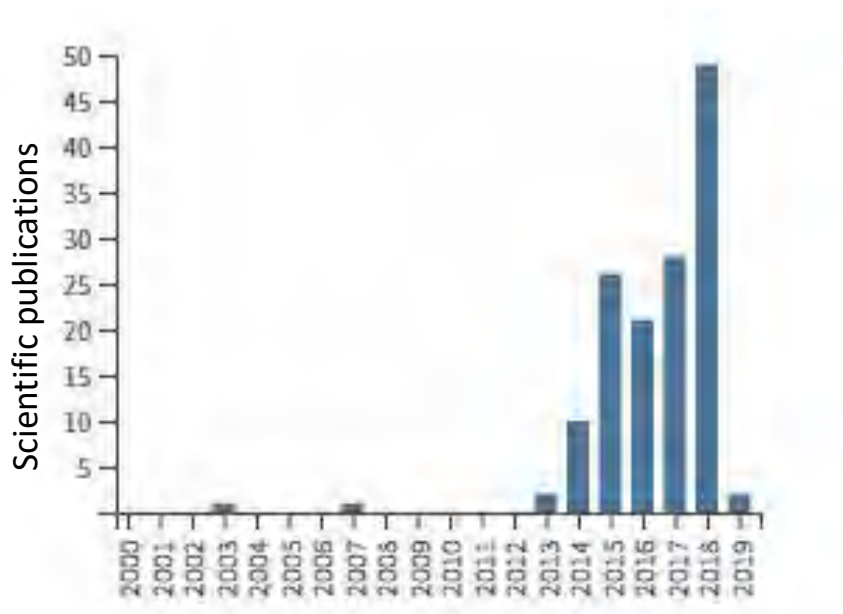



Drivers: Mainstream Treatment
Chesapeake Bay ENR Limits
Methanol costs
Capacity: denitrification capacity was identified during design as a potential issue for future winter operating conditions
Drivers: Sidestream Treatment
Methanol costs
Capacity
DEMON
Minimize Carbon demand and increase capacity with mainstream deammonification
Minimize Carbon demand in Sidestream Deammonification system
Short-cut N removal:
Nitrite shunt/ mainstream deammonification

Sidestream versus mainstream deammonification



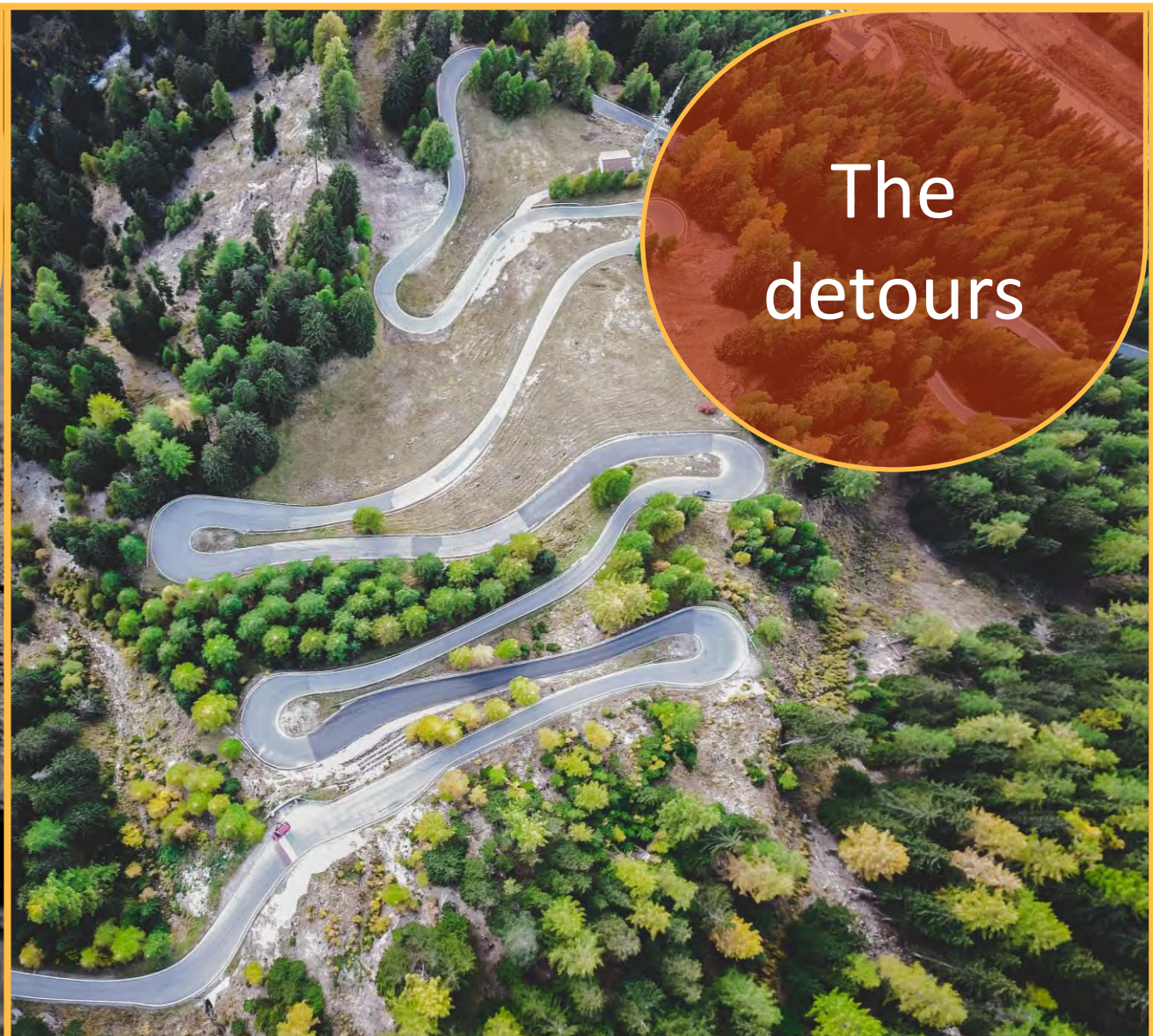
Lackner et al., 2014, WR



Except for AnAOB in Changi (PUB) and temporary trial in Strass, no true full-scale applications so far

What makes mainstream deammonification so much more challenging than sidestream deammonification?

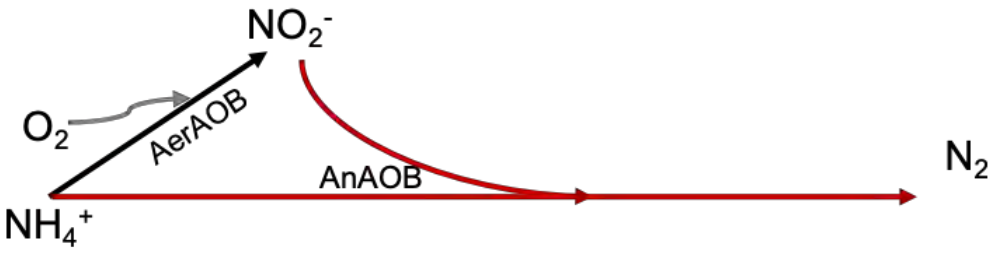
The road towards mainstream short-cut N removal



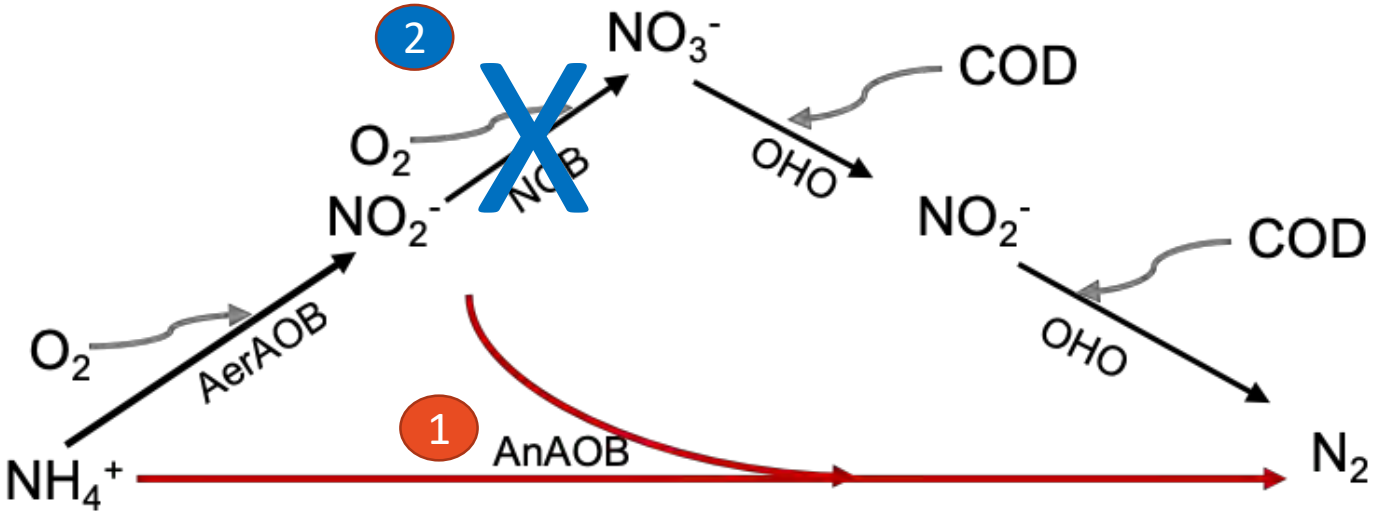

The potholes

mainstream short-cut N removal

The potholes



Mainstream deammonification



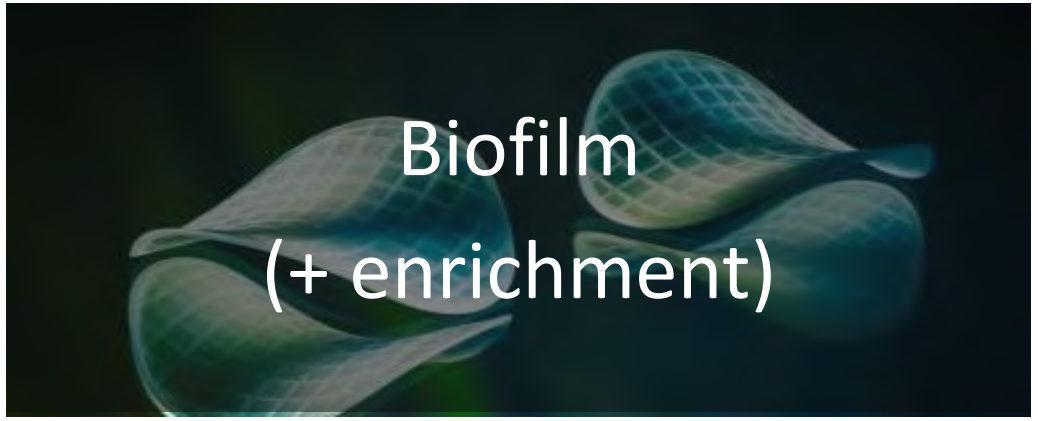
Mainstream short-cut N removal

Main challenges:

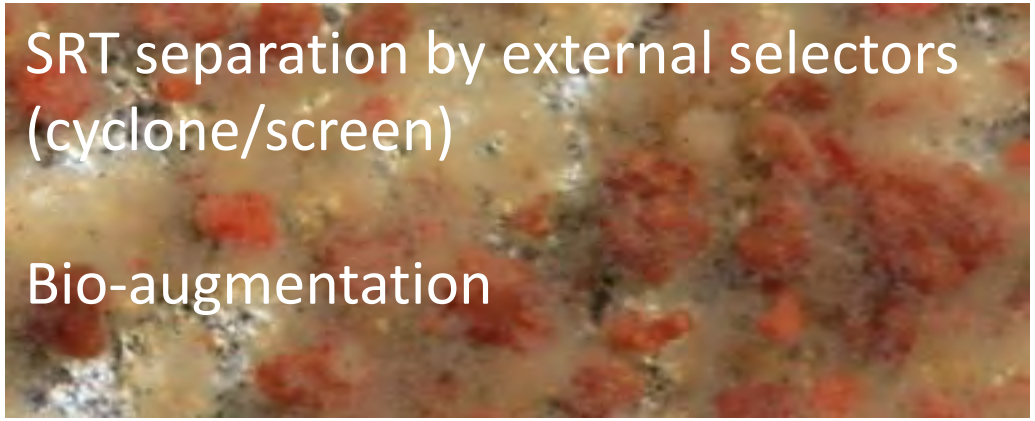
1. Sufficient retention of AnAOB while allowing for SRT pressure on other organisms
2. Nitrite availability for AnAOB through NOB out-selection

AnAOB retention

- Sufficient AnAOB SRT is needed (lower temperatures and limited substrate concentrations)
- Sidestream applications form the model for mainstream application

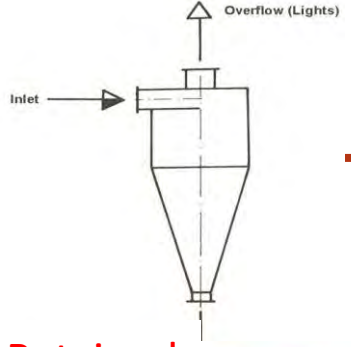


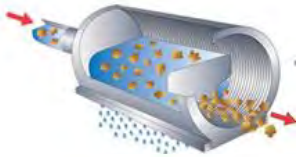
1 SRT



SRT separation

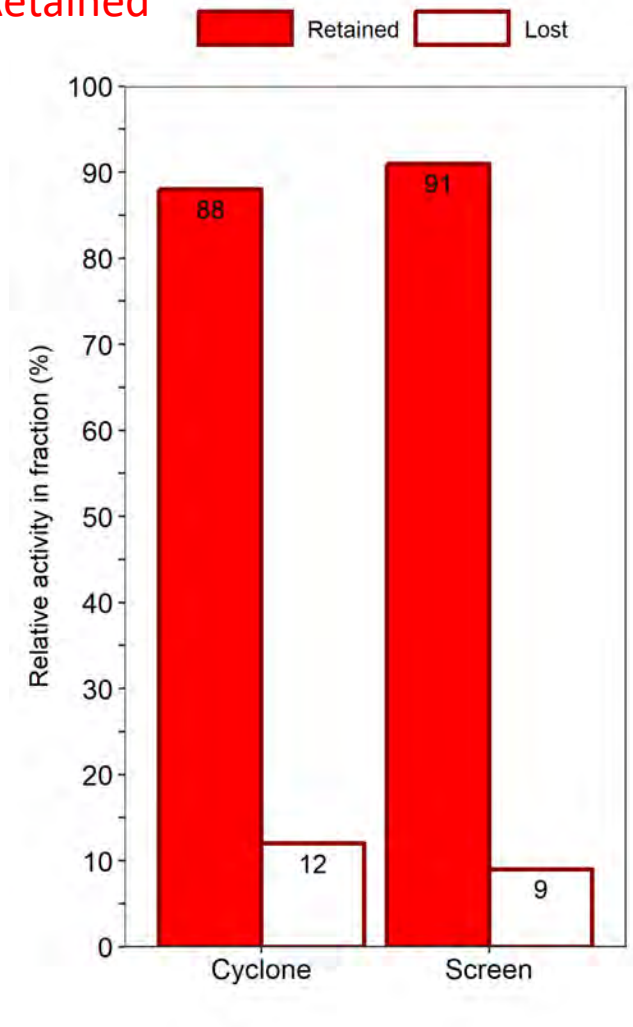
AnAOB retention with external selector

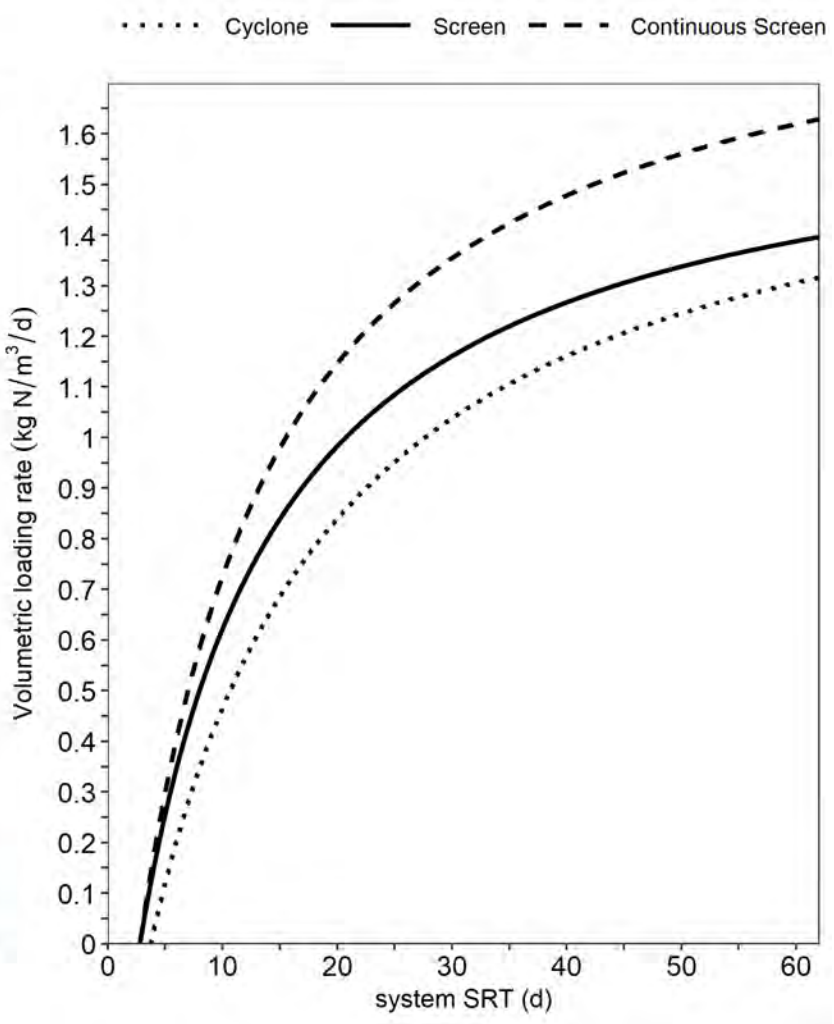




Retained

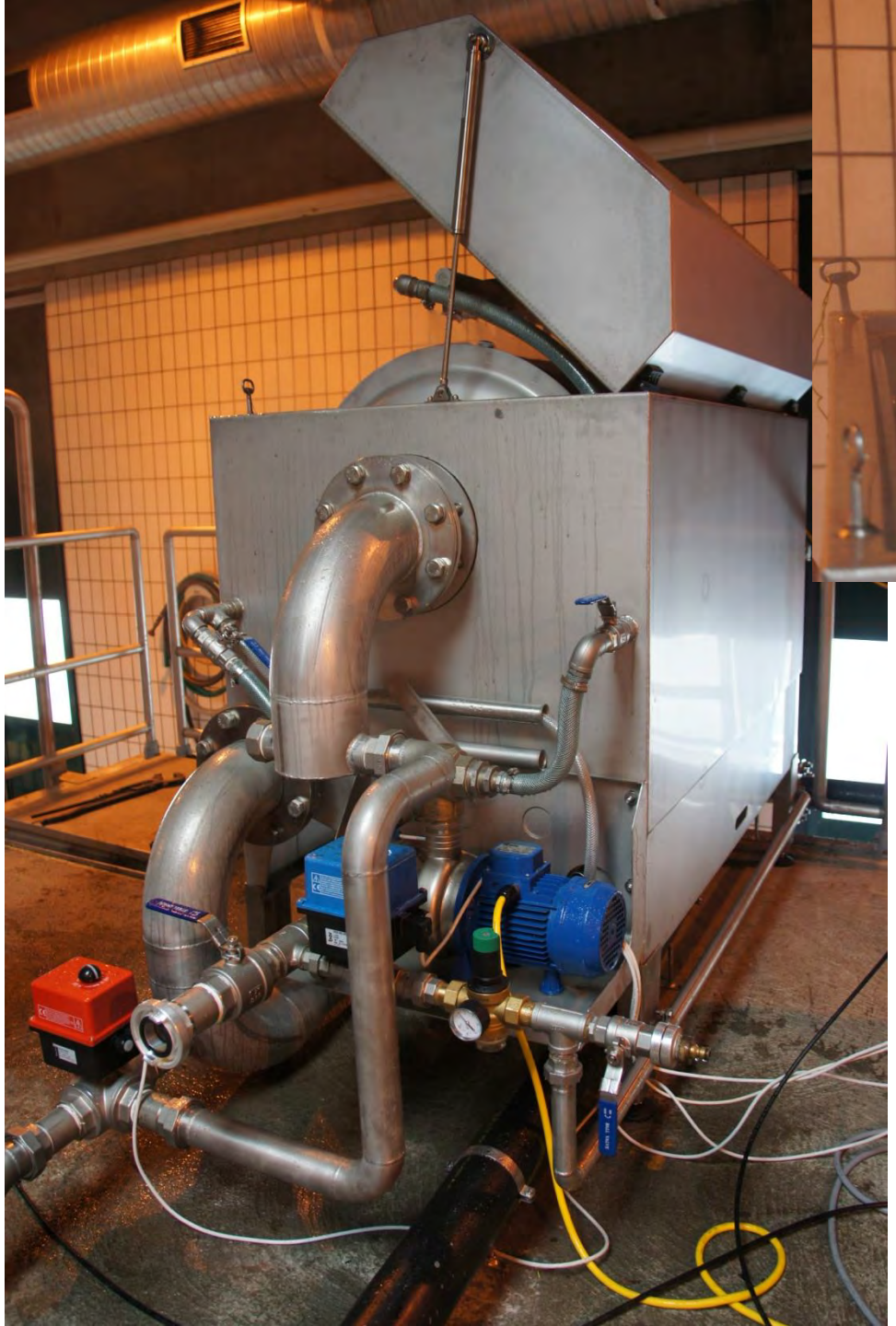
Retained





- External selector vital for anammox retention.
- Choice of selector can further improve capacity.
- Minor improvements can have big effects:
  - ↑ 3% retention = ↑ 12-29% capacity
- Under mainstream conditions (lower growth rates):
  - Cyclone: 42% activity retention
  - Screen: 72% activity retention

Van Winckel et al., *in preparation*



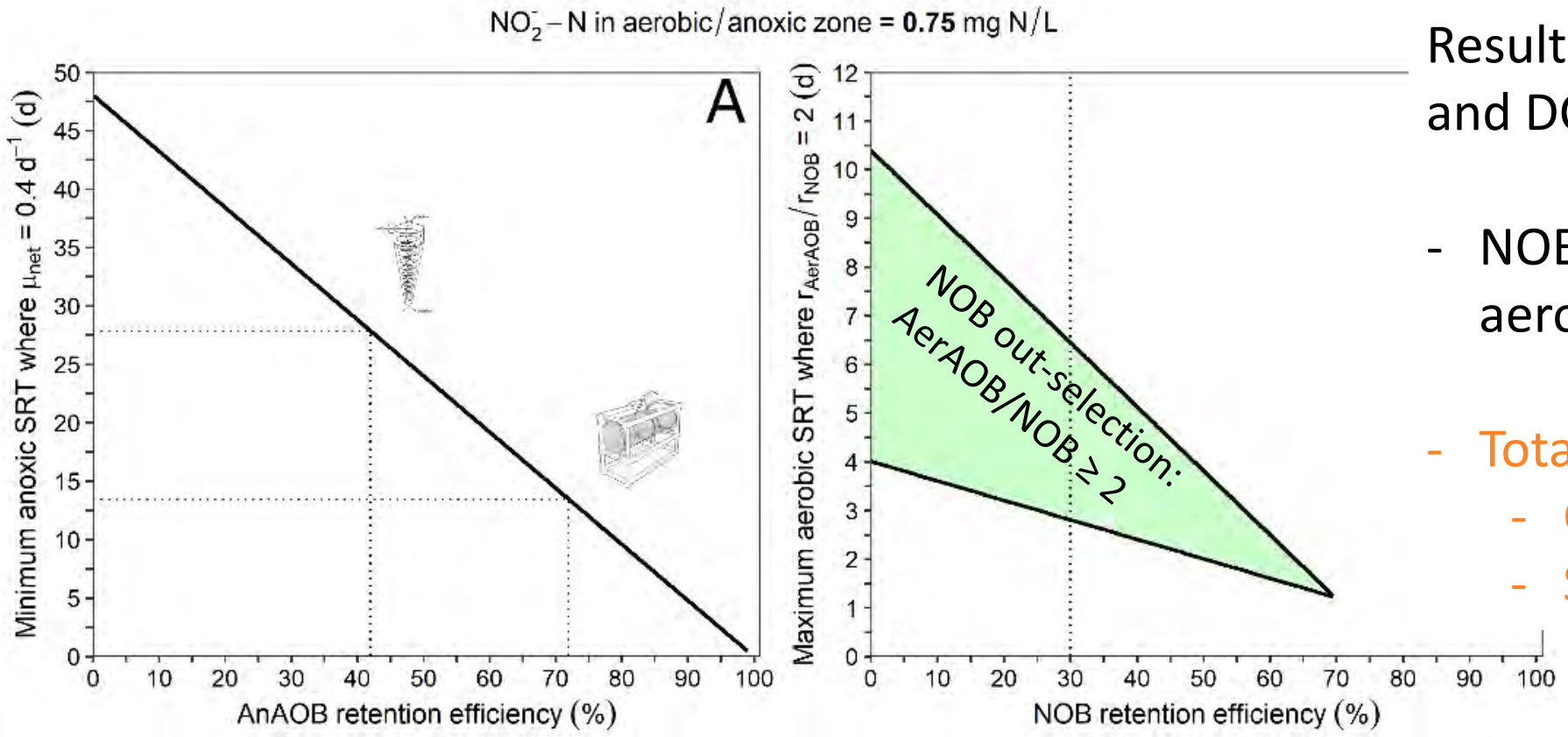
MLSS

filtrate

retained

AnAOB retention

1. The more efficient the AnAOB retention, the lower overall SRT needs to be
2. The better the SRT separation between NOB and AnAOB, the more chance of successful nitrite availability for AnAOB



Results from trials in Strass and DC Water:

- NOB retention ~ 30% : aerobic SRT: 3-6 days
- Total SRT requirements:
  - Cyclone: 31-34 days
  - Screen: 16-19 days

Van Winckel et al., *in preparation*

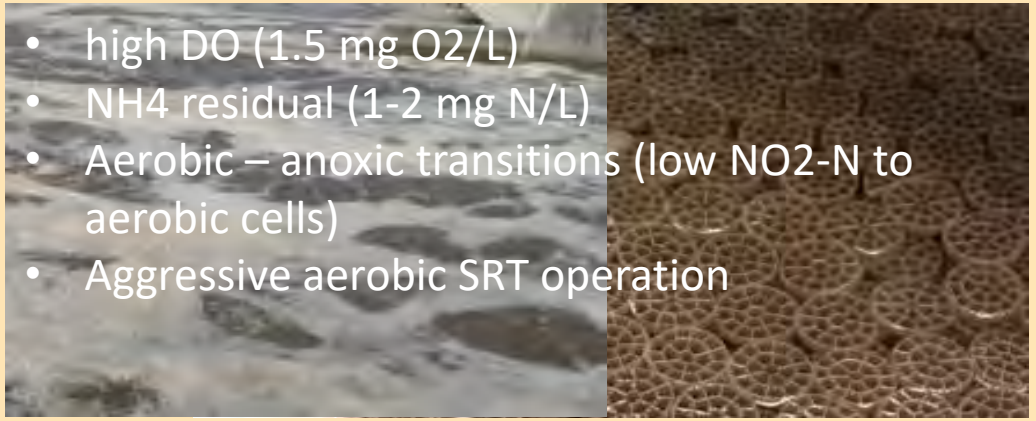
NOB out-selection

Ammonium residual

- Aerobic volume control
(DO ~ 1 mg O_2/L dependent on N load, DO:NH4)
- Ammonium residual
- Nitrite levels are low
- SRT of flocs = HRT = ~2.5h

- Low DO (0.2-0.8 mg O2/L)
- Ammonium residual
- Biofilm thickness control
- Centrate exposure

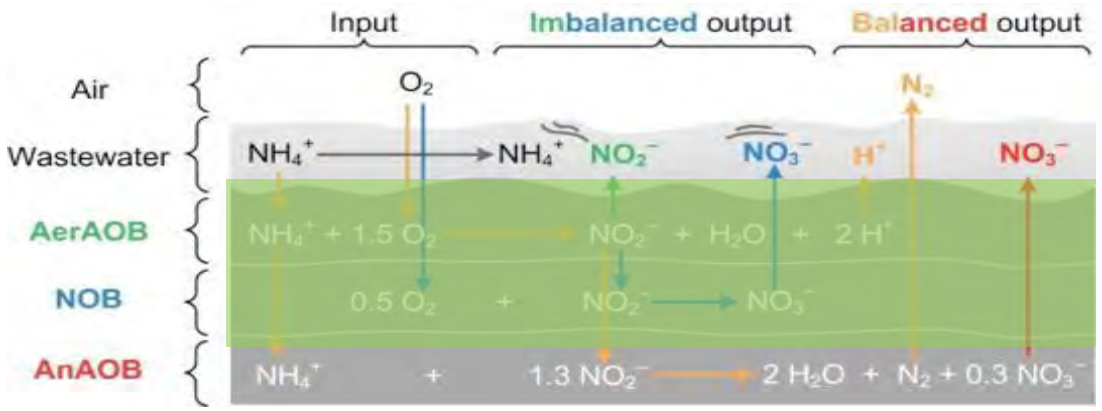
- high DO (1.5 mg O2/L)
- NH4 residual (1-2 mg N/L)
- Aerobic – anoxic transitions (low NO2-N to aerobic cells)
- Aggressive aerobic SRT operation



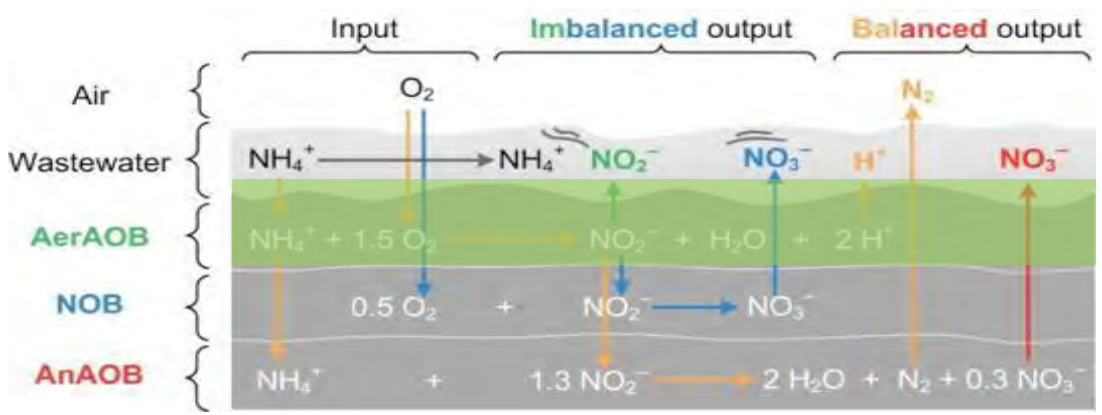
- high DO (1.5 mg O2/L)
- NH4 residual (1-2 mg N/L)
- Aerobic – anoxic transitions (low NO2-N to aerobic cells)
- Aggressive aerobic SRT operation

NOB out-selection

High DO

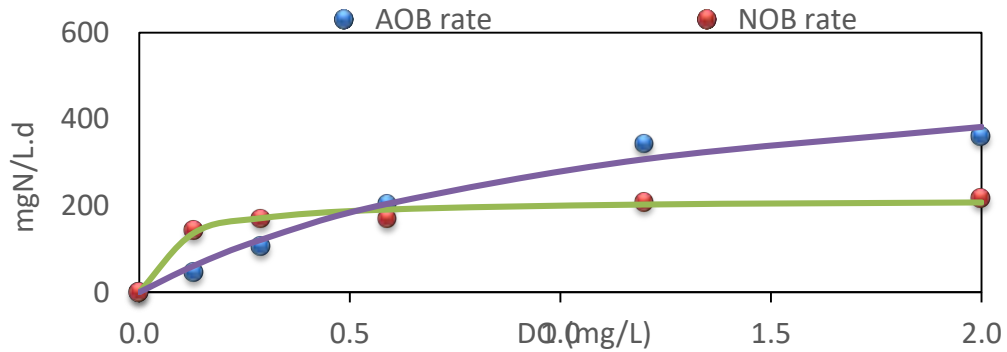


Low DO



SPACE

TIME



NOB out-selection

Summarized strategy:

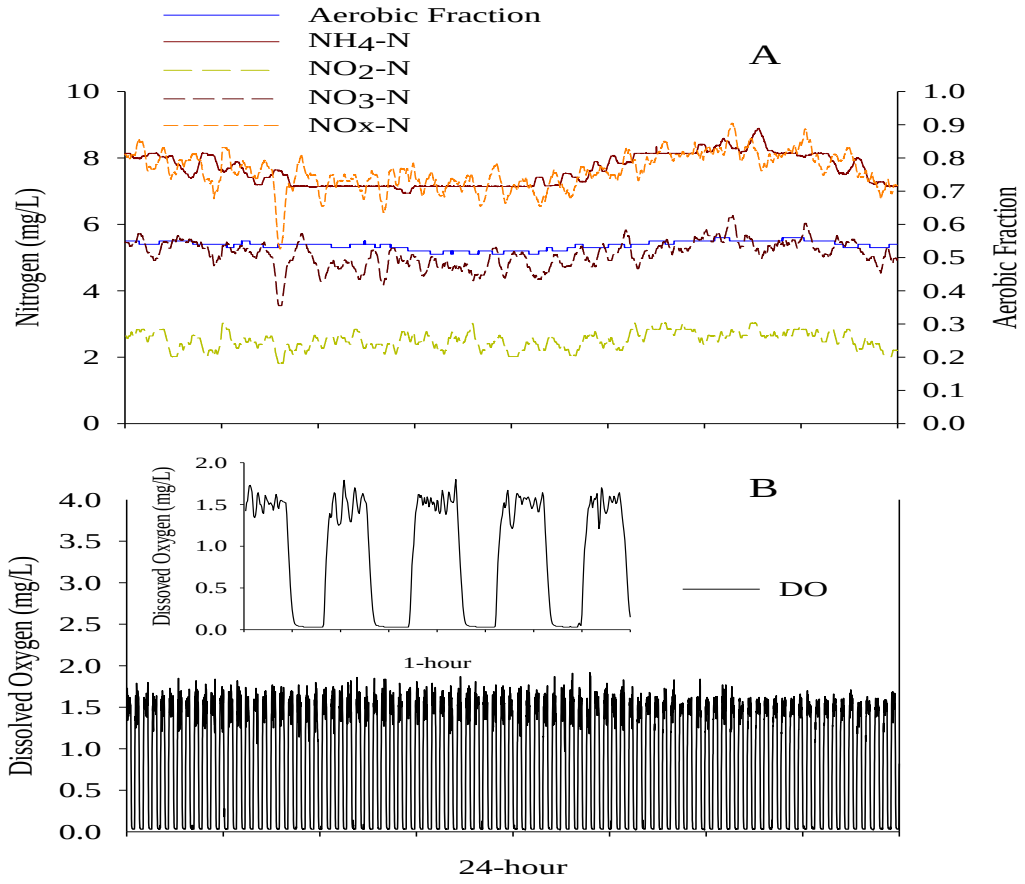
a) Ammonium residual
b) DO management to minimize aerobic SRT by either:
   - Control of aerobic volume present in the biofilm (DO penetration depth)
   - Control of aerobic time
c) Minimize nitrite availability for NOB:
   - Large anoxic biofilm volumes
   - Intermittent aeration
d) SRT control ↓ ↓ ↓

Action	AerAOB	NOB	AnAOB
A	⇧		⇧
B	⇧	⇩	
C		⇩	⇧
D		⇩	⇧

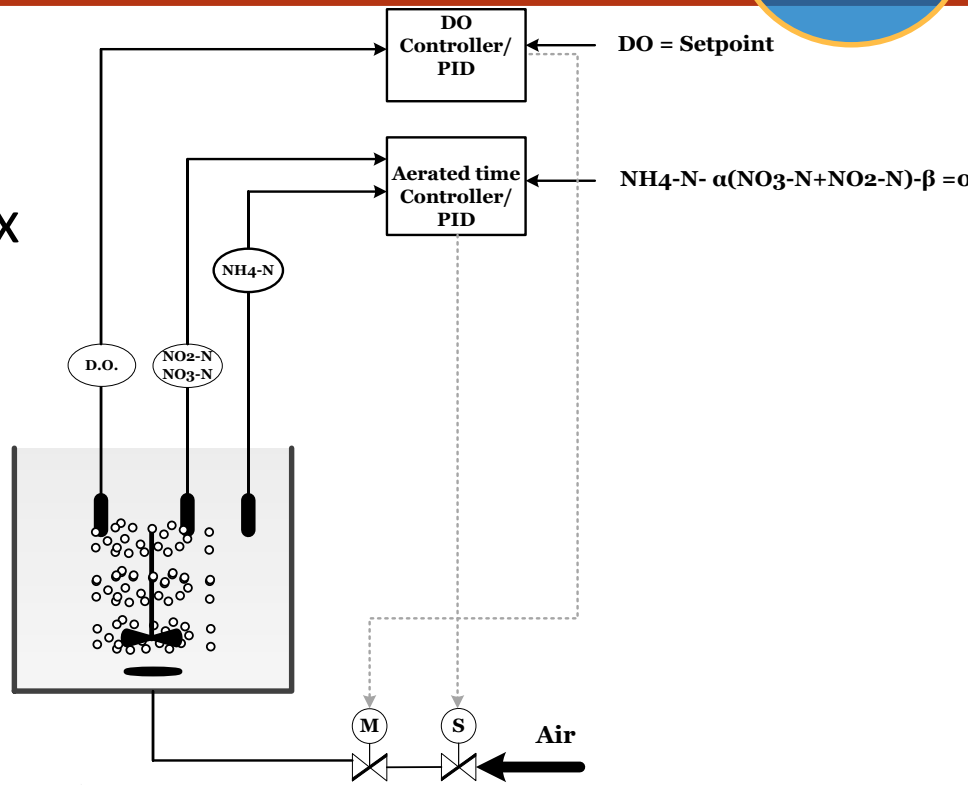
NOB out-selection through AvN Control

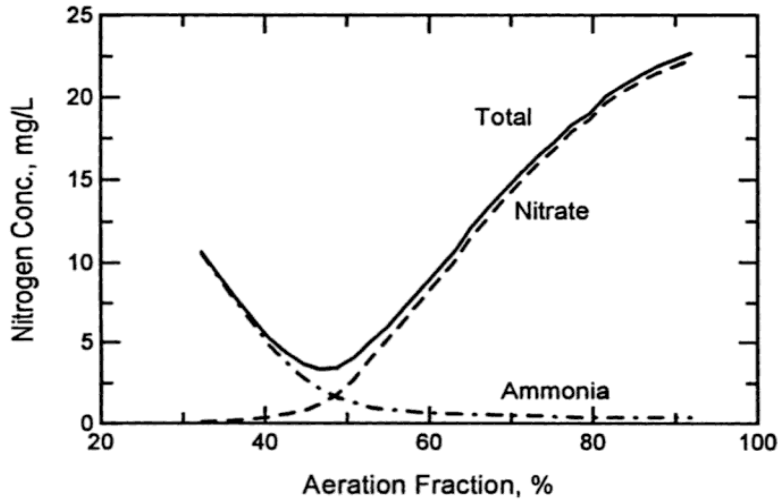
Main features:

- Includes all features for NOB out-selection
- Balances oxidation of ammonium with reduction of NOx



Regmi et al., 2015 Water Research



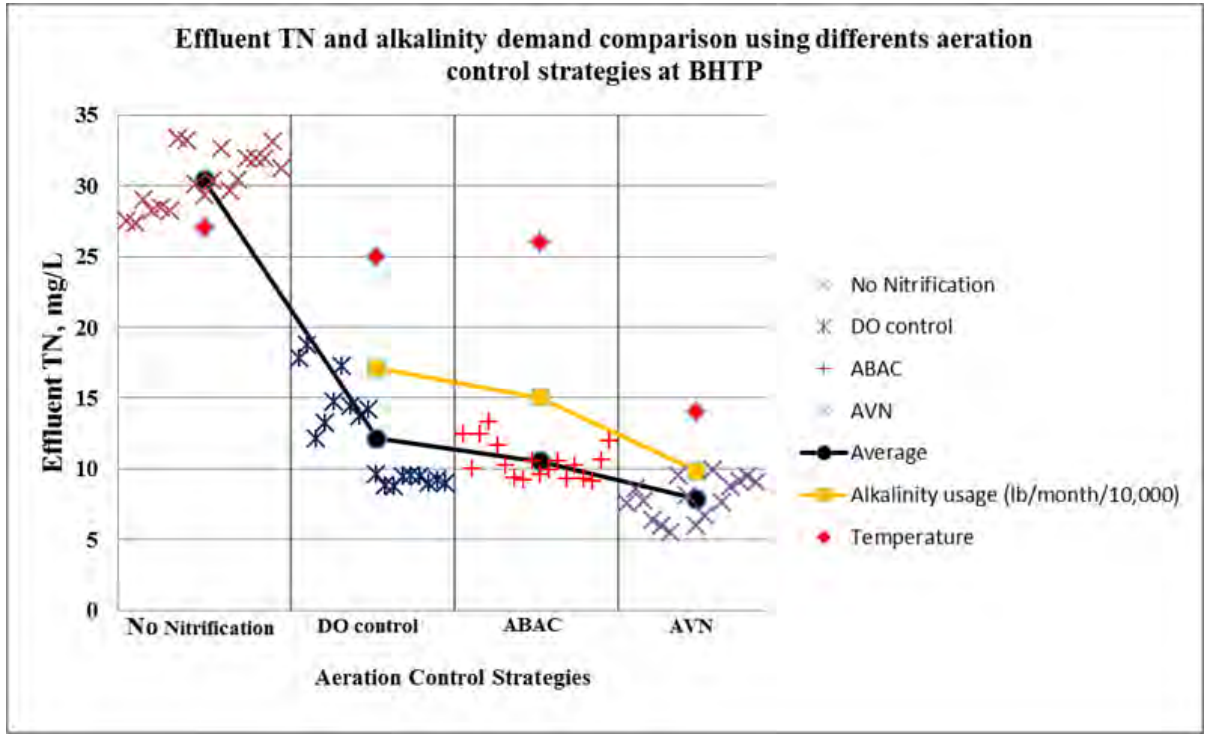


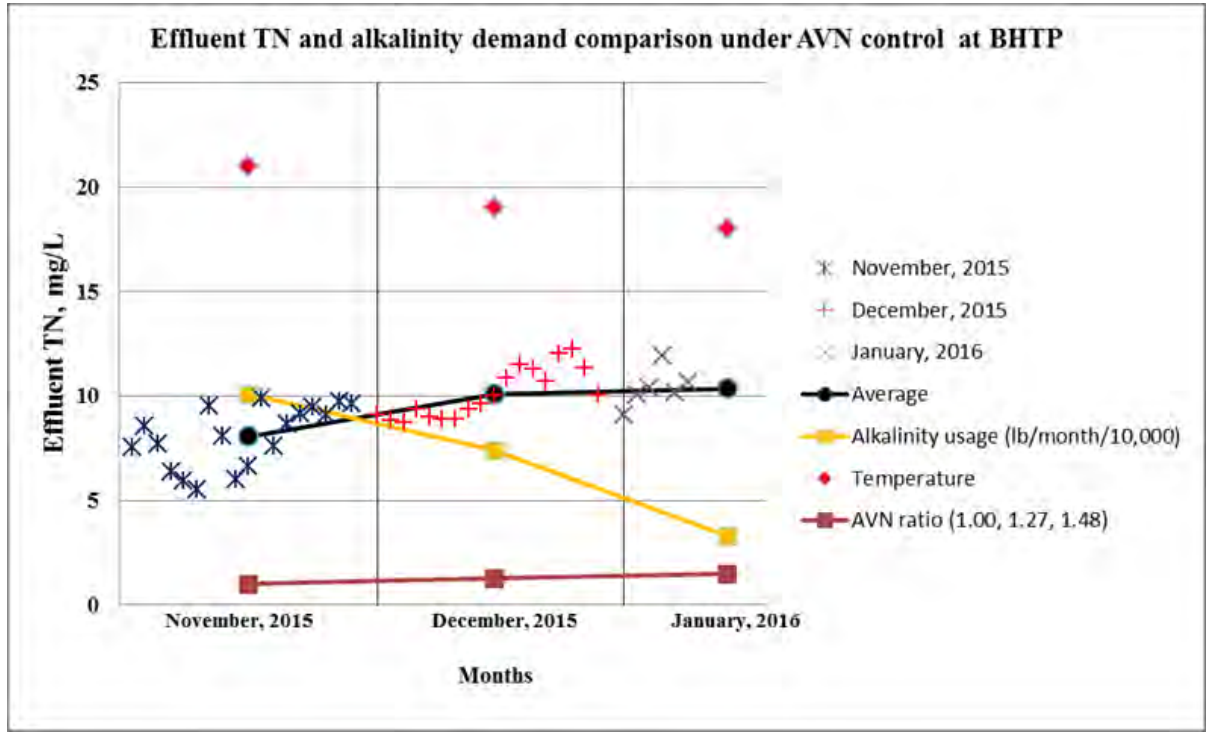
Batchelor, B (1983). Journal of Environmental Engineering

NOB out-selection through AvN Control

Main outcomes:

- Most efficient N removal given the available carbon
- Minimal aeration requirement for N removal
- Proper alkalinity management





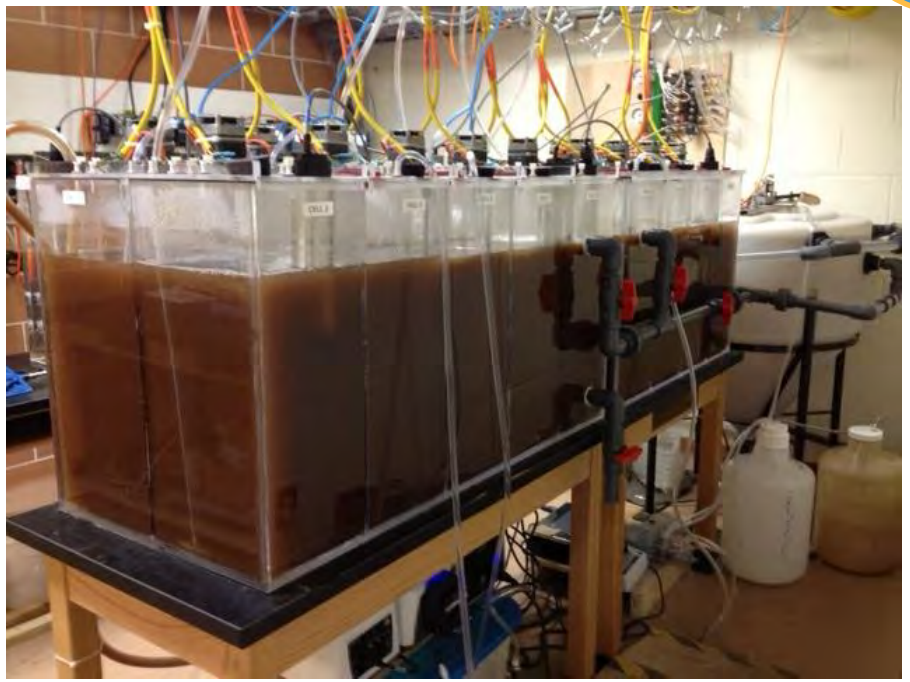
Bott et al. - Results testing @ Boat Harbor Treatment Plant (25 MGD), HRSD

Achievements for Blue Plains AWTP

Wastewater to be treated

- Secondary effluent (CEPT + HRAS)
- tCOD/N ratio: 1.4 ± 0.2
- no additional Carbon dosing

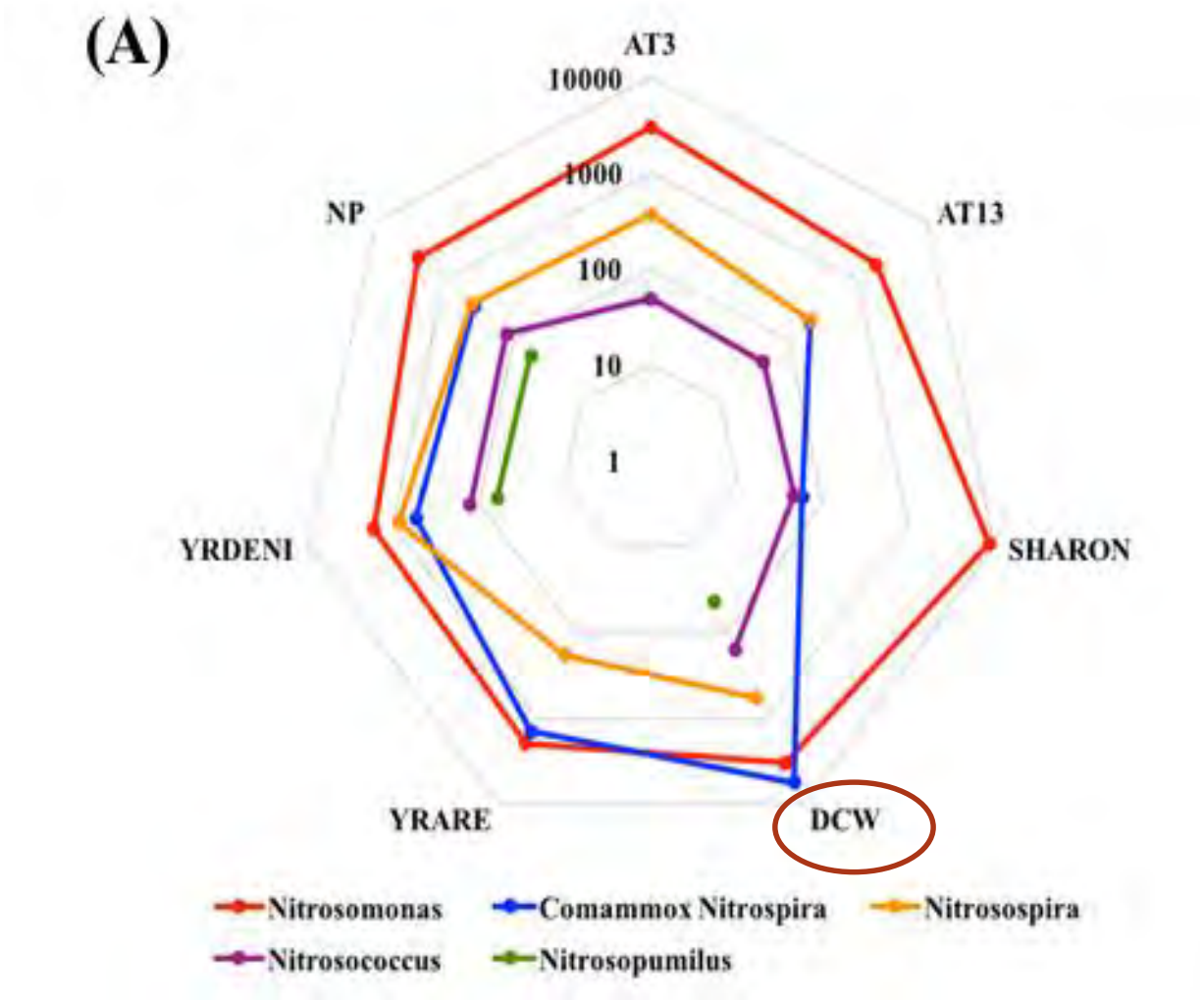
Mg N/L	IN	OUT	%
NH4 –N	21±4	1.5±1.7	93±7
NO2-N	2.7±2	0.7±0.5	-
NO3-N	1.1±0.8	5±1	Rel. 18±5
TIN	25±1.6	7±2	71±6
TSS	-	4±2	
MLSS	748 ±120		
Rv Ntot removal rate (mg N/L/d)			152±28

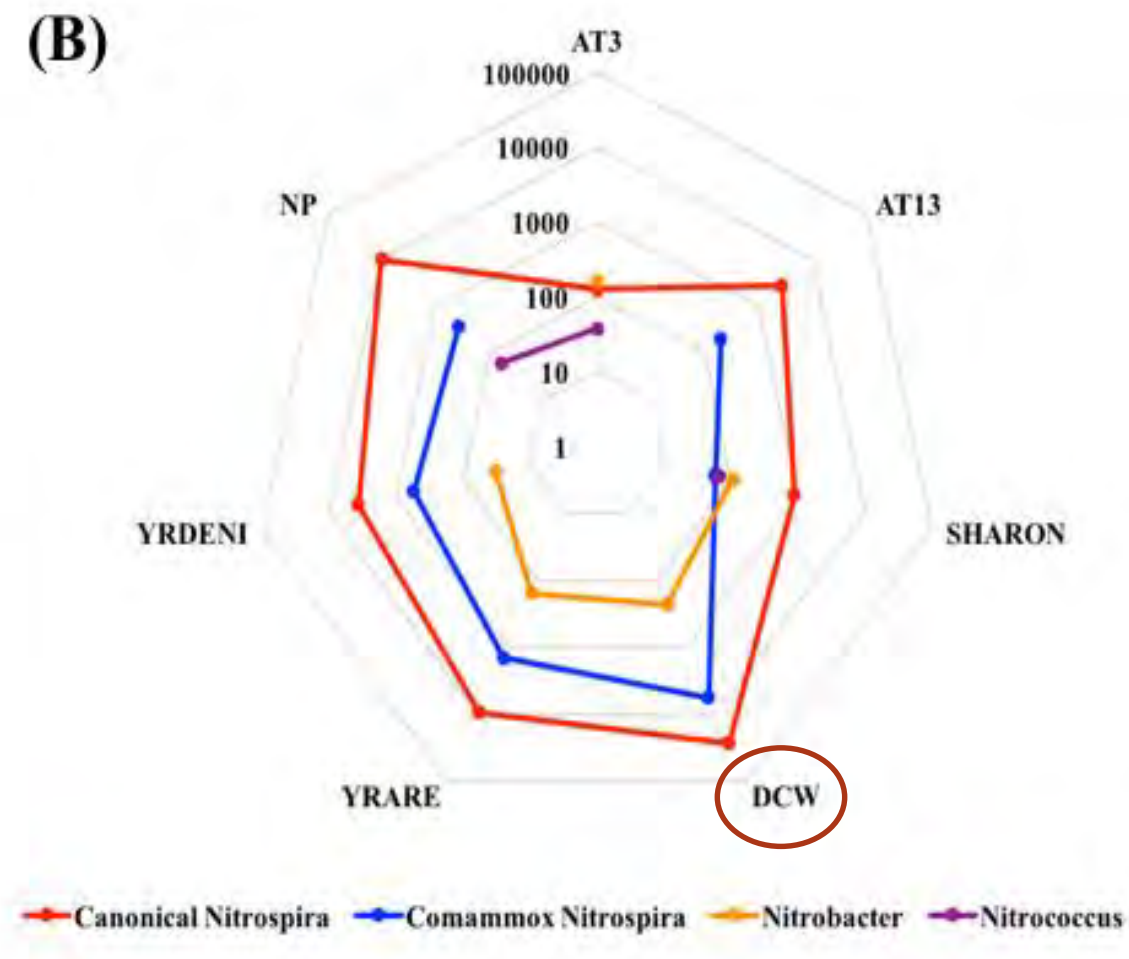


(Han et al., 2016, *Bioresource Technology*)

→ AerAOB/NOB ratio = 2

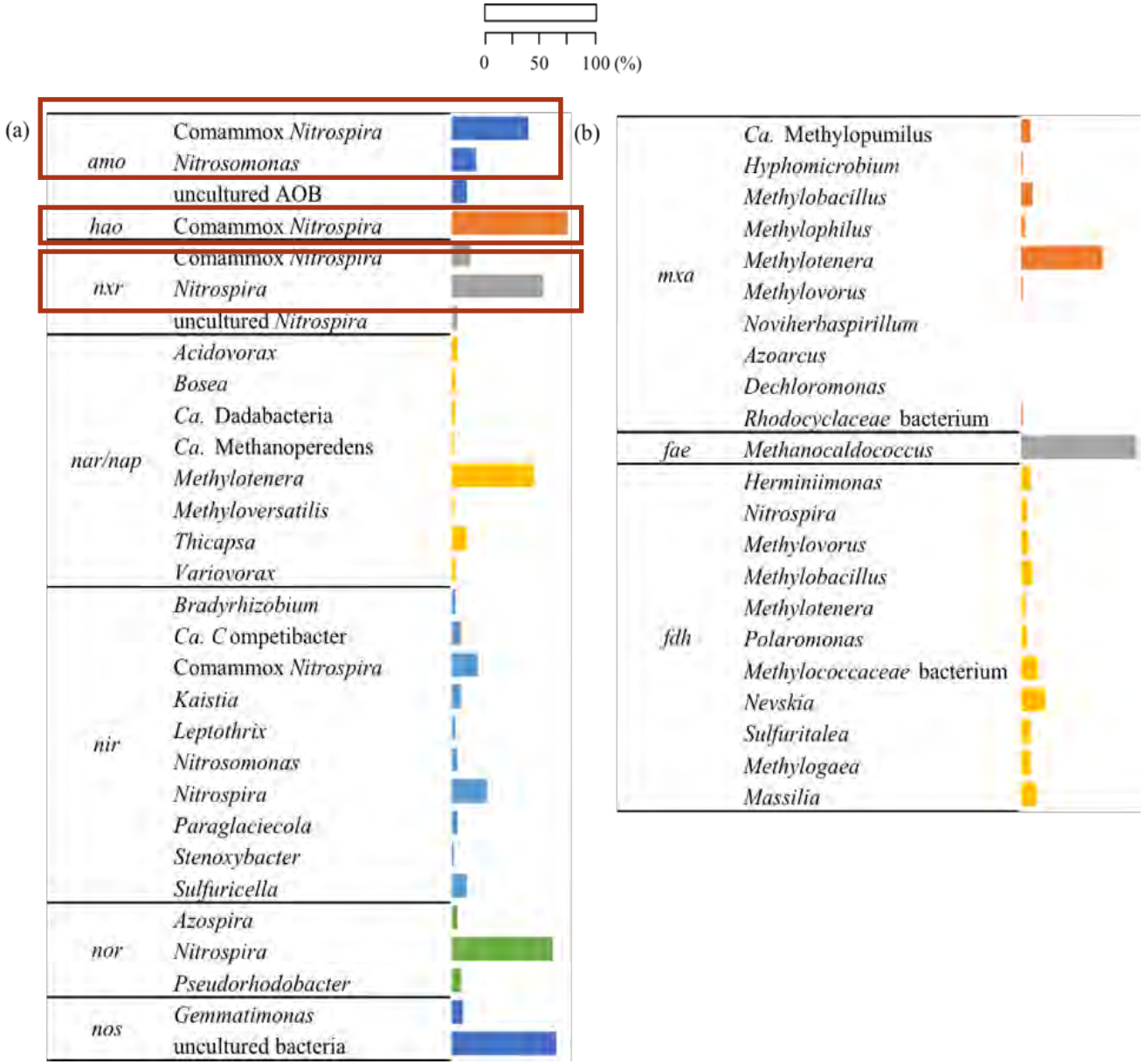
Comammox @ Blue Plains AWTP





(Park and Chandran, unpublished)

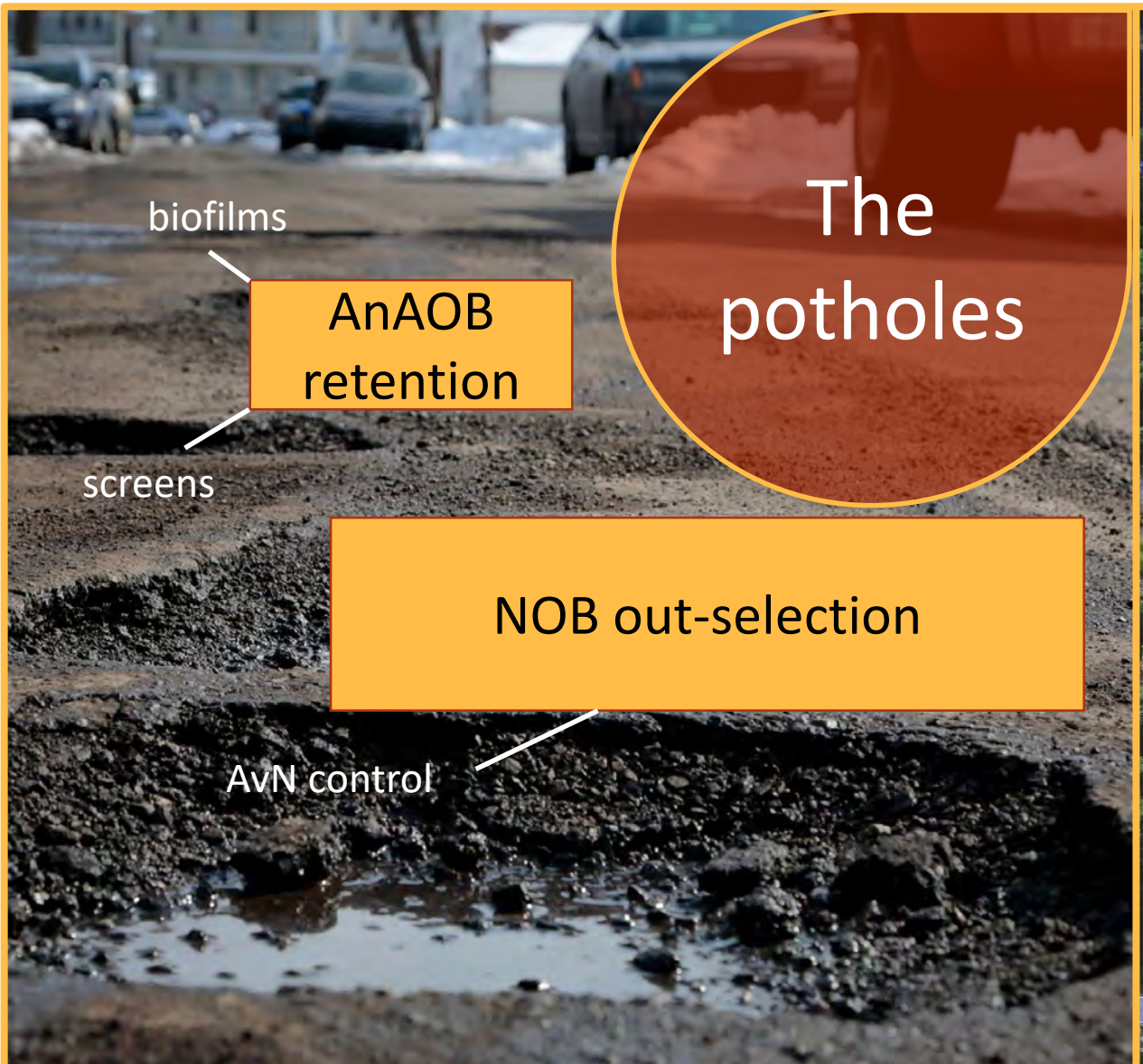
Comammox @ Blue Plains AWTP



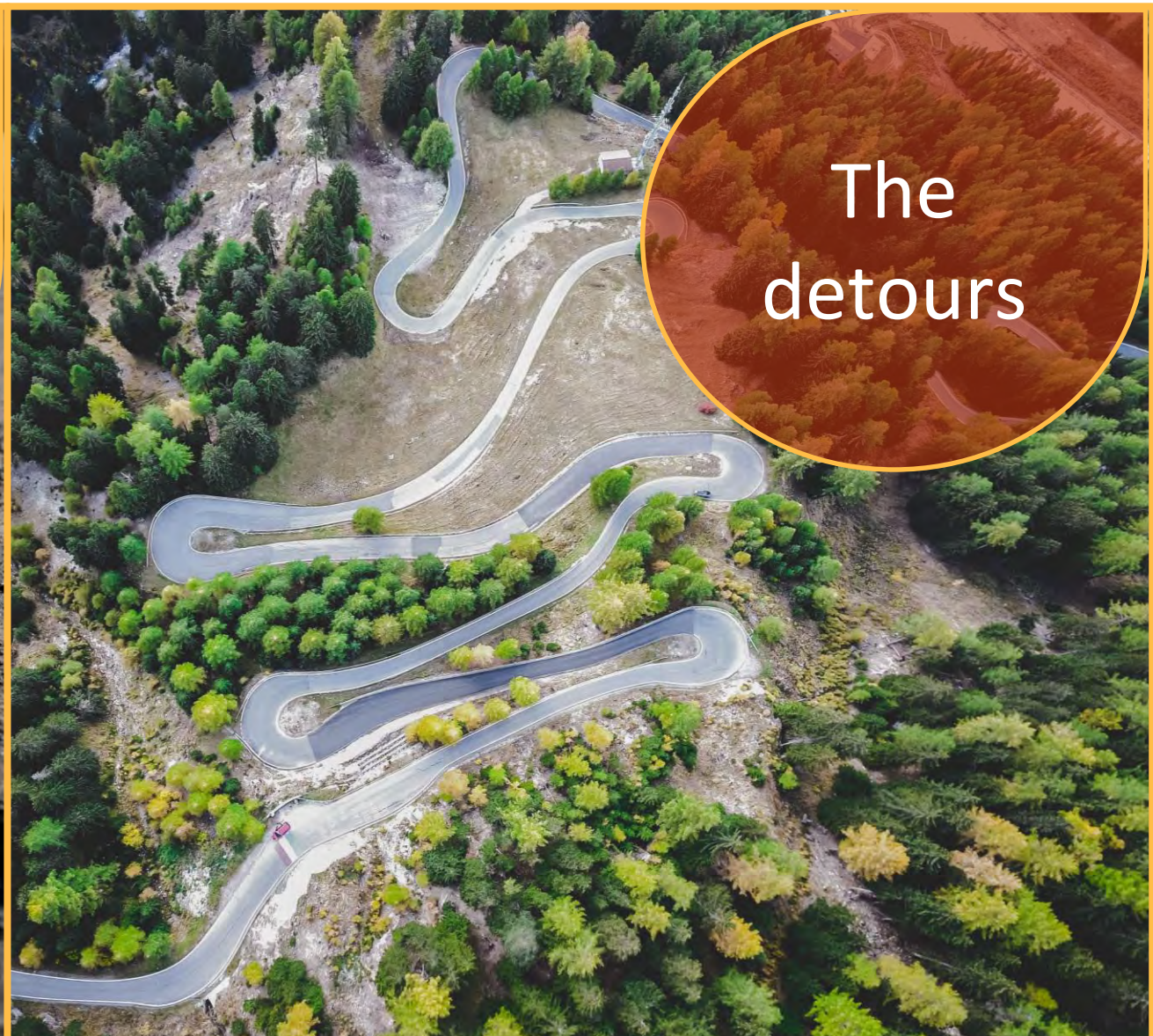
(Park and Chandran, unpublished)

amo	ammonium monooxygenase
hao	hydroxylamine oxidoreductase
nxr	nitrite oxidoreductase
nar	membrane-bound nitrate reductase
nap	periplasmic nitrate reductase
nir	nitrite reductase
nor	nitric oxide reductase
nos	nitrous oxide reductase
mxa	methanol dehydrogenase
fae	formaldehyde-activating enzyme
fdh	formate dehydrogenase

The road towards mainstream short-cut N removal



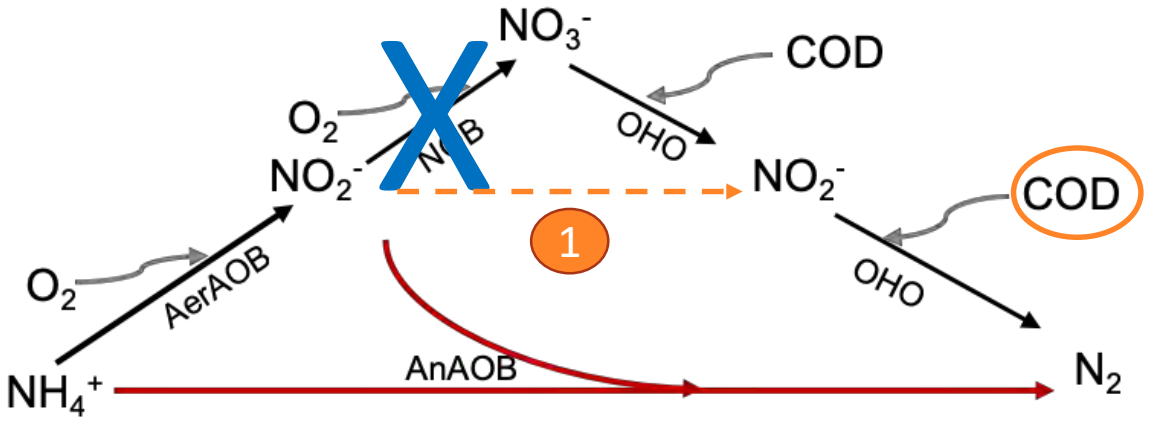
The potholes



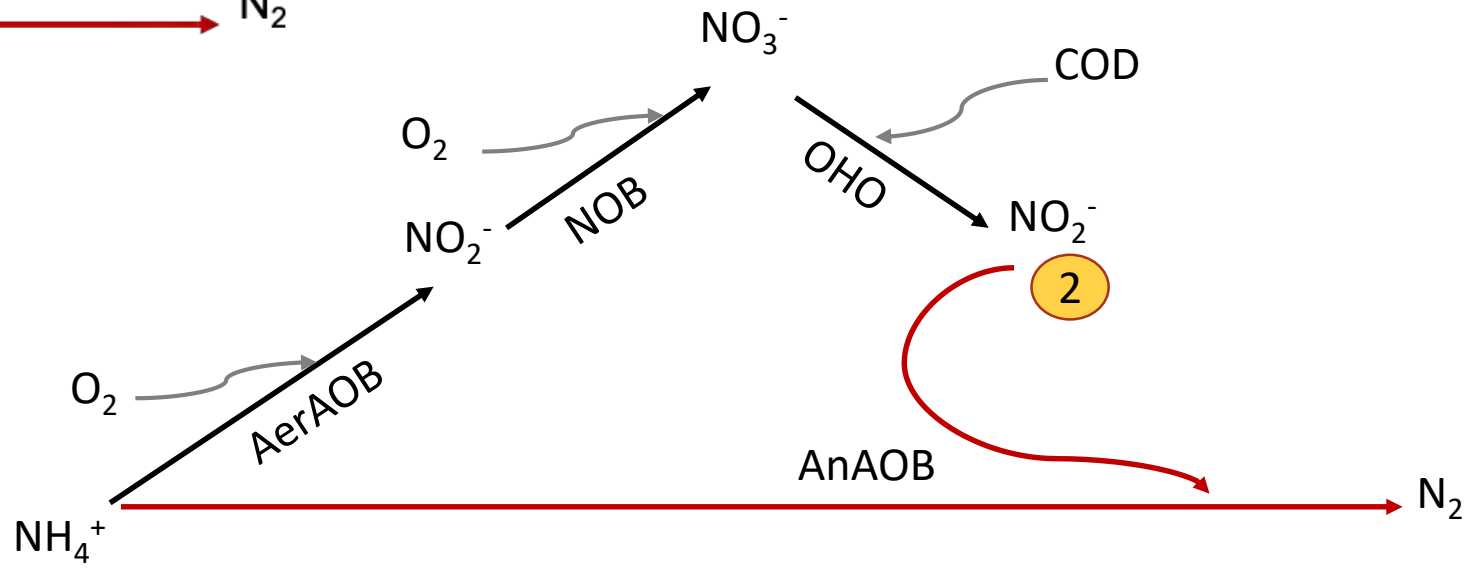
The detours


The detours

mainstream short-cut N removal



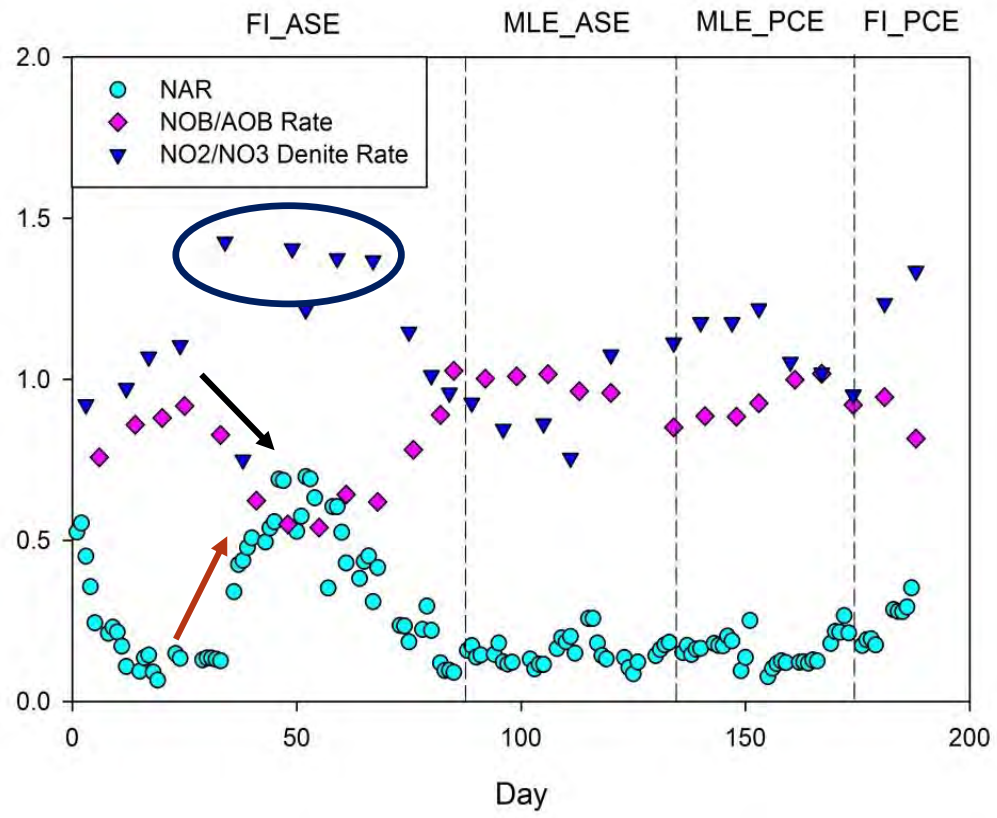
1. Use available COD for denitritation



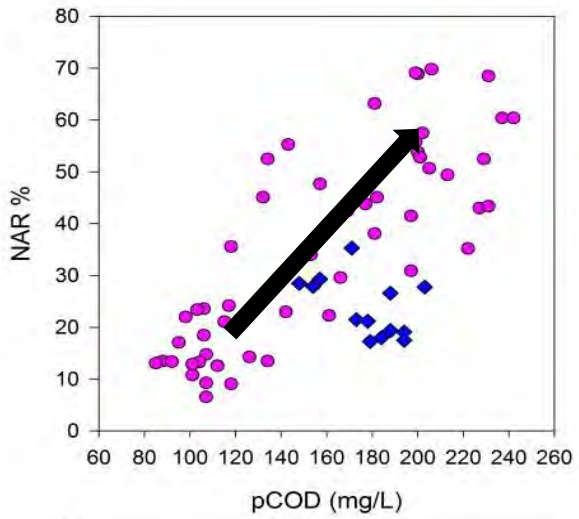
2. Generate nitrite availability through PdN rather than NOB out-selection

Use available COD for denitritation

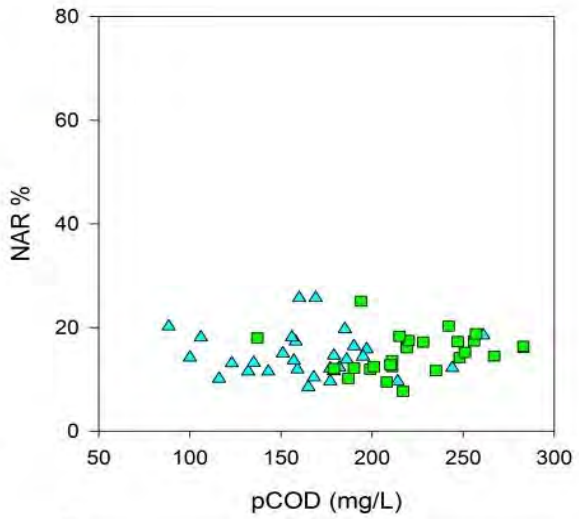
- Slowly biodegradable COD rather than total COD
- Aeration strategy

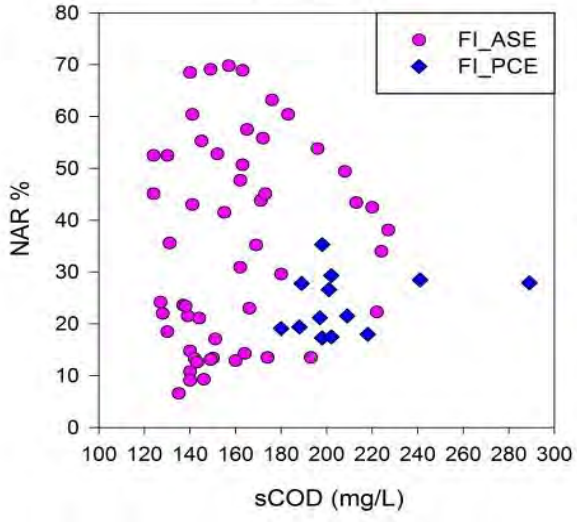


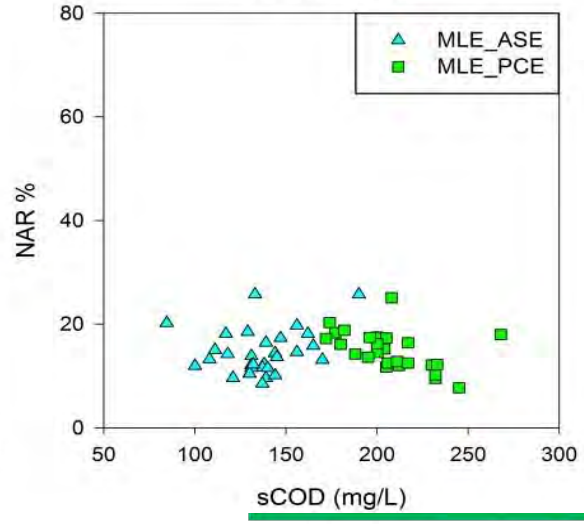
Intermittent aeration



MLE mode







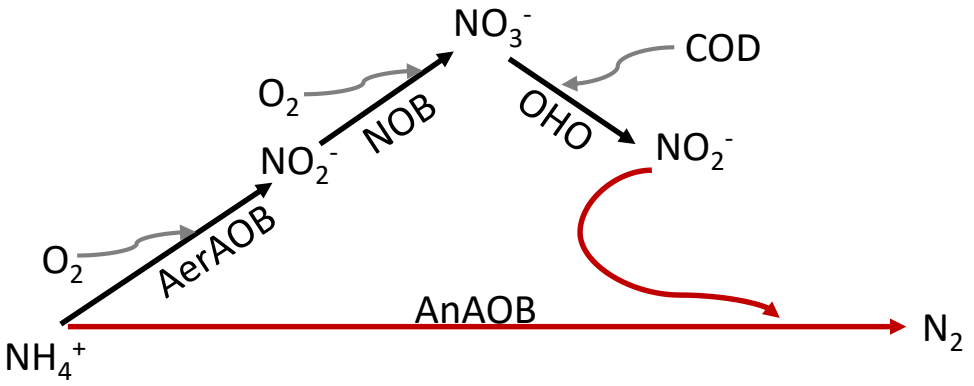
Klaus et al., *in preparation;* Kinyua et al., 2018 NRR, presentation 10.4

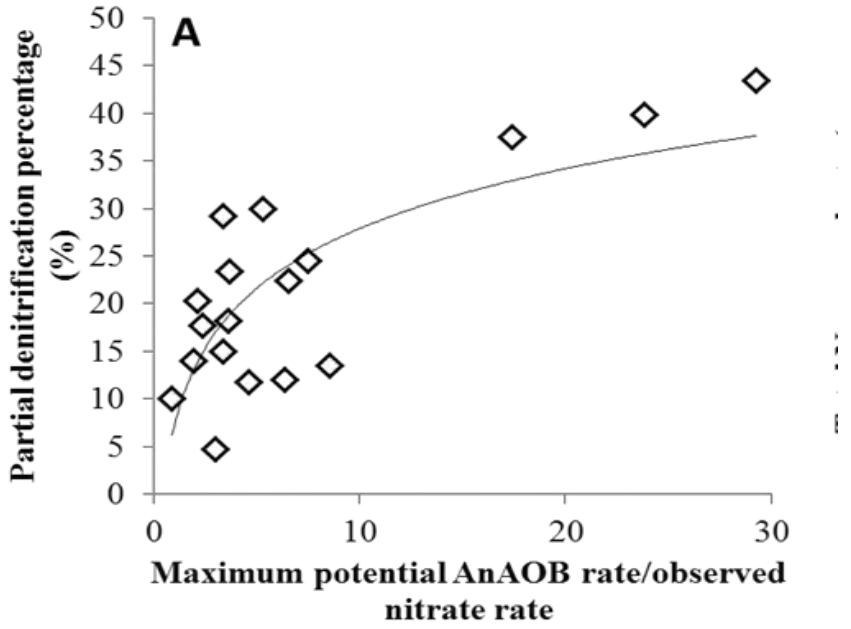
PdN route

- Nitrite accumulation has been frequently observed during denitrification

- PdN-AnAOB has been studied for sidestream applications:
  - DEAMOX (sulfide or acetate based)
  - PANDA
  - Recently, transfer of concept to mainstream

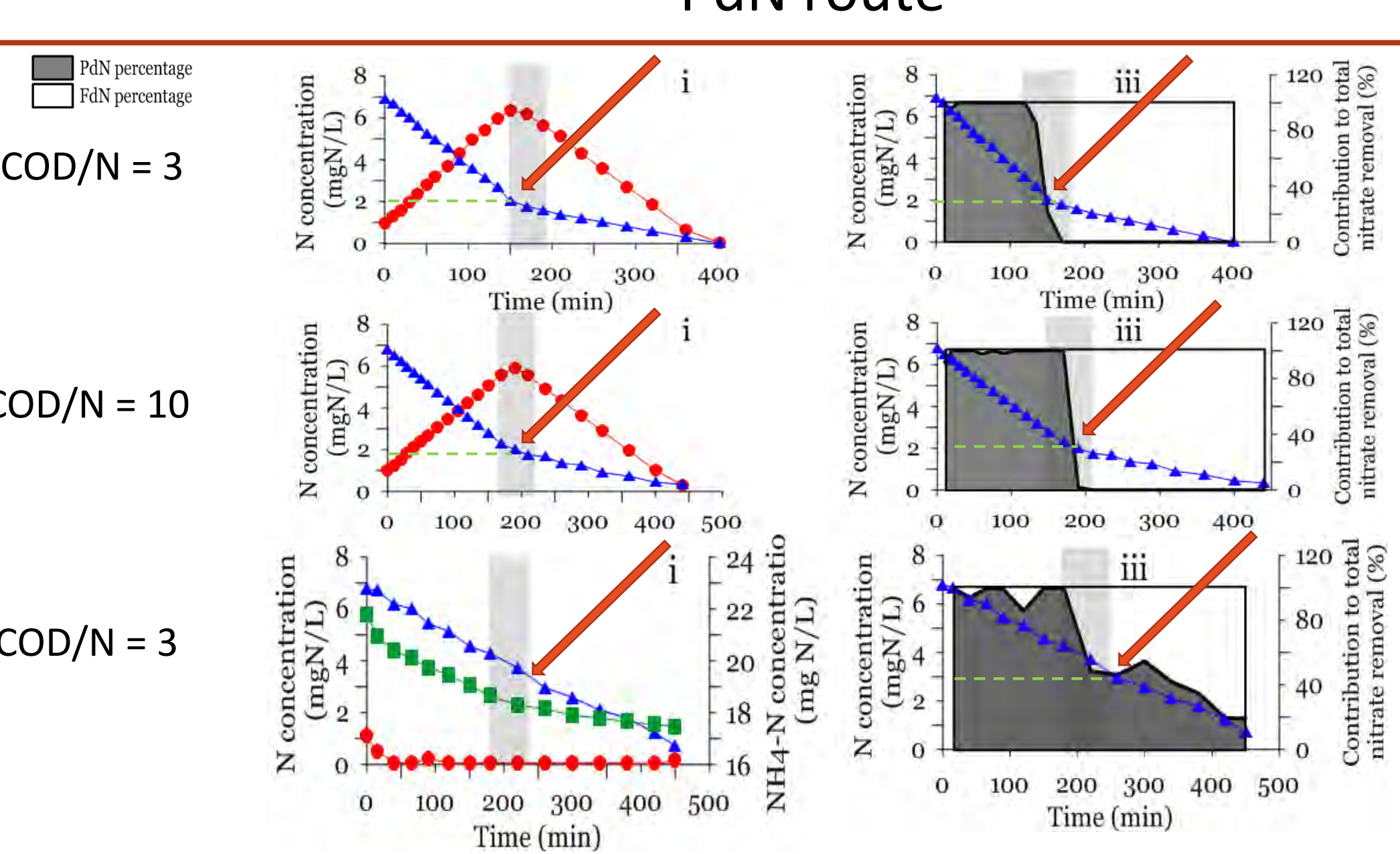
- PdN control based on literature depends on following:
  - COD/N ratio: 0.5- 8
  - SRT
  - Alkalinity, pH
  - Type of carbon
  - Competition for nitrite



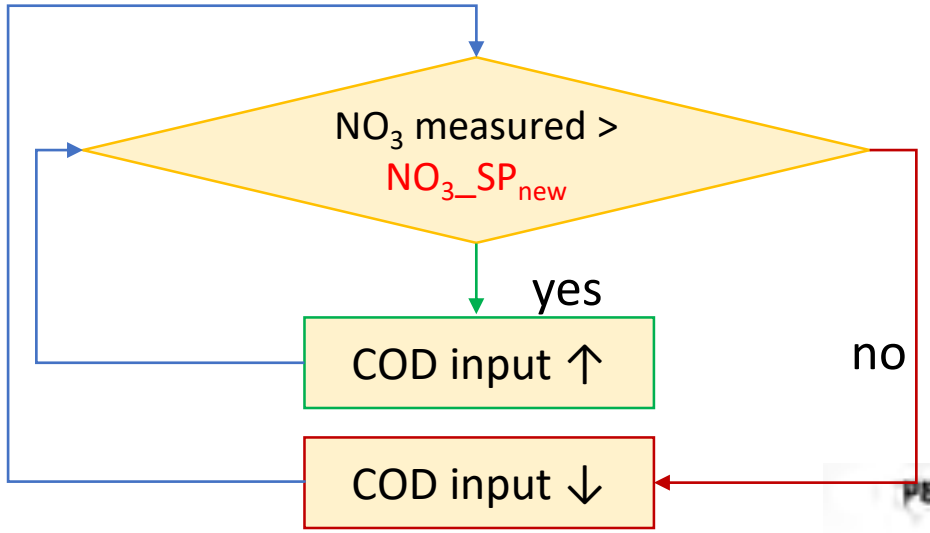


(*Sharp et al., Int. J. Sus. Dev. Plann. 2017 ;* Du et al., WR 2017; Let et al.,WER. 2019; Ma et al., ES&T, 2017; Baideme et al., 2017; Campolong et al., 2018; *Cao et al., 2016*)

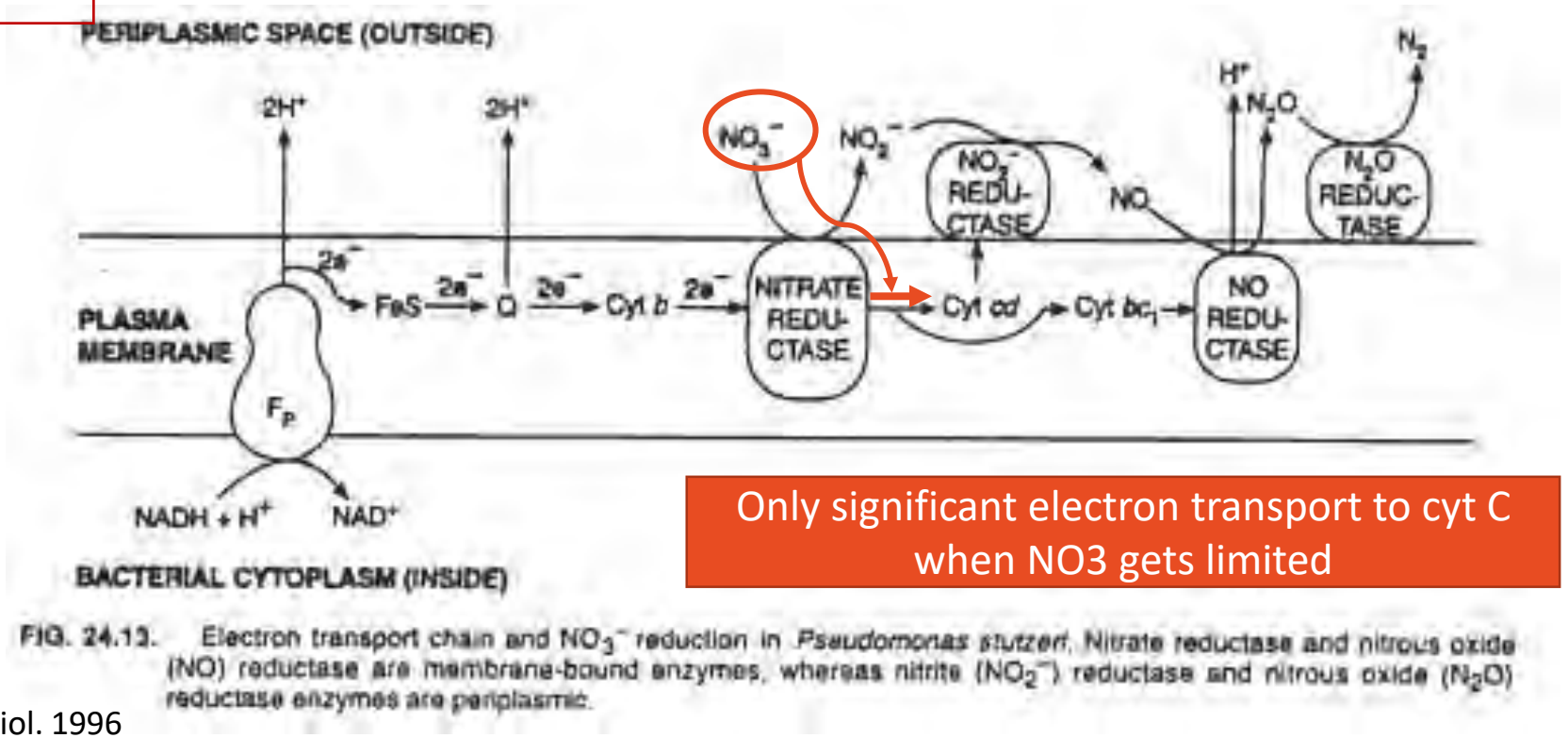
PdN route


NO2-N concentration
NO3-N concentration
NH4+-N concentration
PdN percentage
FdN percentage
COD/N = 3
COD/N = 10
COD/N = 3
without AnAOB
without AnAOB
with AnAOB
N concentration (mgN/L)
Time (min)
Contribution to total nitrate removal (%)
NH4-N concentratio (mg N/L)
i
iii

PdN control logic



- Keep Nitrate residual > 1.5-2 mg N/L
- Directly control COD dose rate, not necessarily COD/N
- Independent of AnAOB sink as long as VFA or glycerol are used

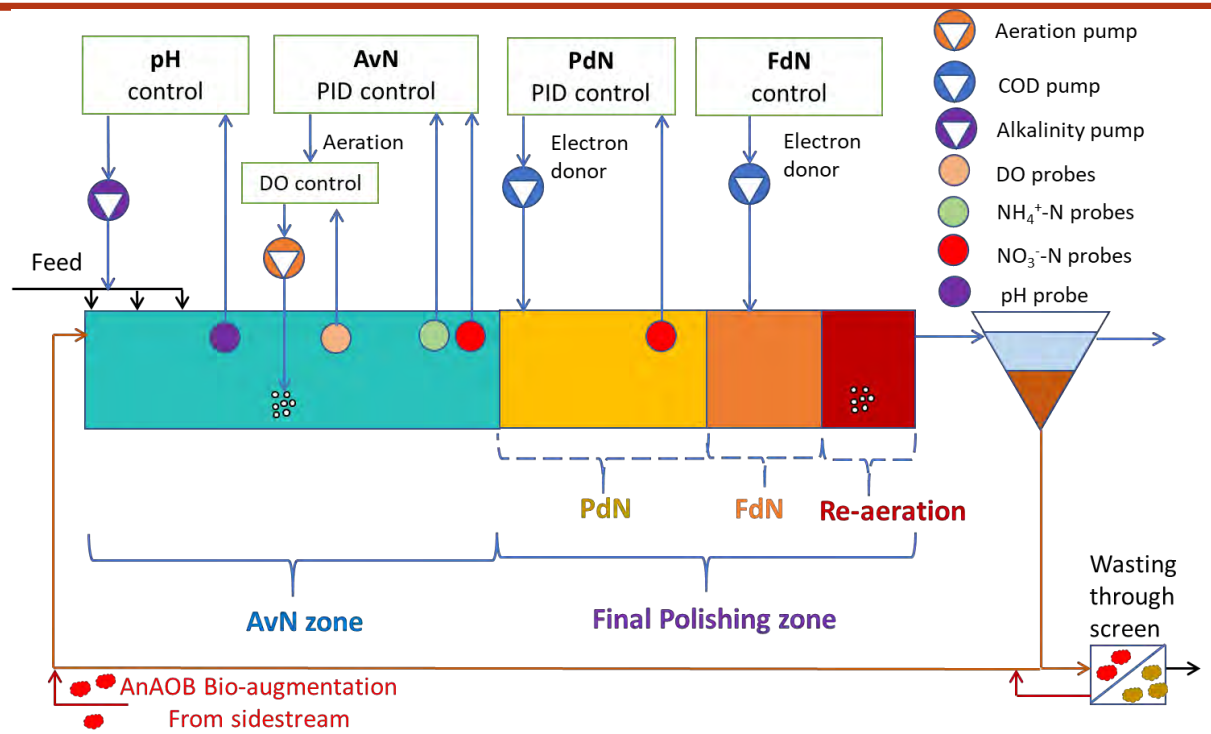


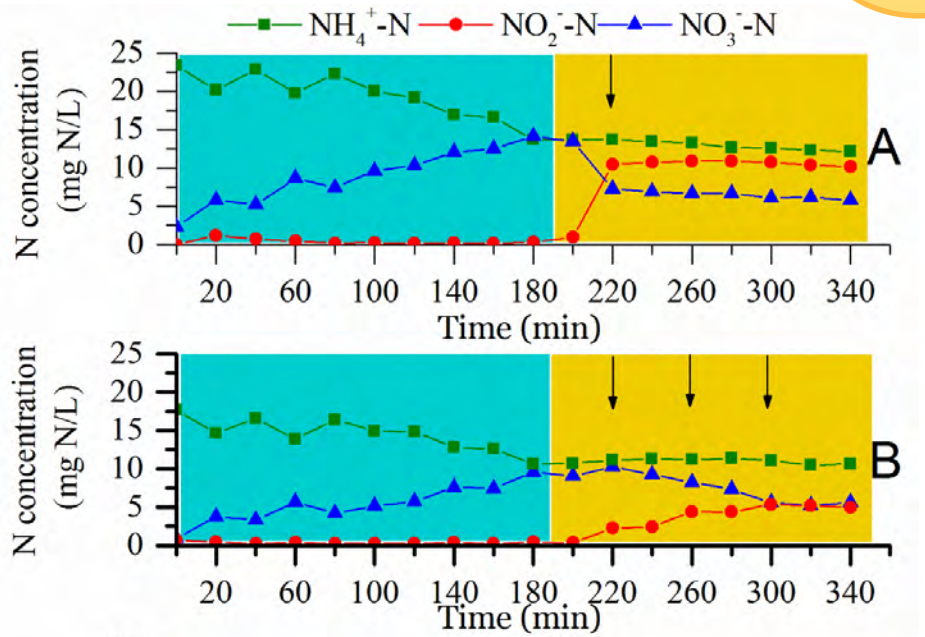
Only significant electron transport to cyt C when NO3 gets limited

FIG. 24.13. Electron transport chain and NO_3^- reduction in *Pseudomonas stutzeri*. Nitrate reductase and nitrous oxide (NO) reductase are membrane-bound enzymes, whereas nitrite (NO_2^-) reductase and nitrous oxide (N_2O) reductase enzymes are periplasmic.

Van Rijn et al., appl. Environ. microbiol. 1996

PdN-AnAOB polishing in same sludge

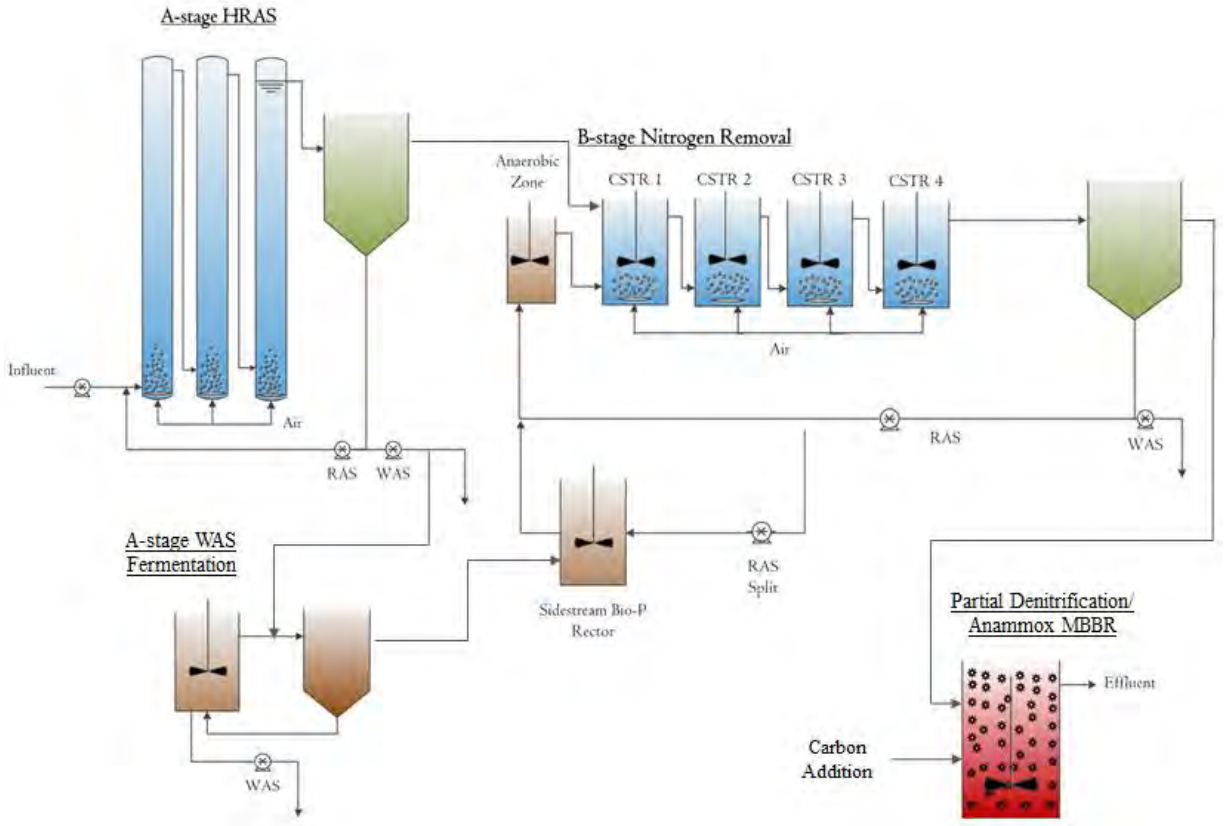


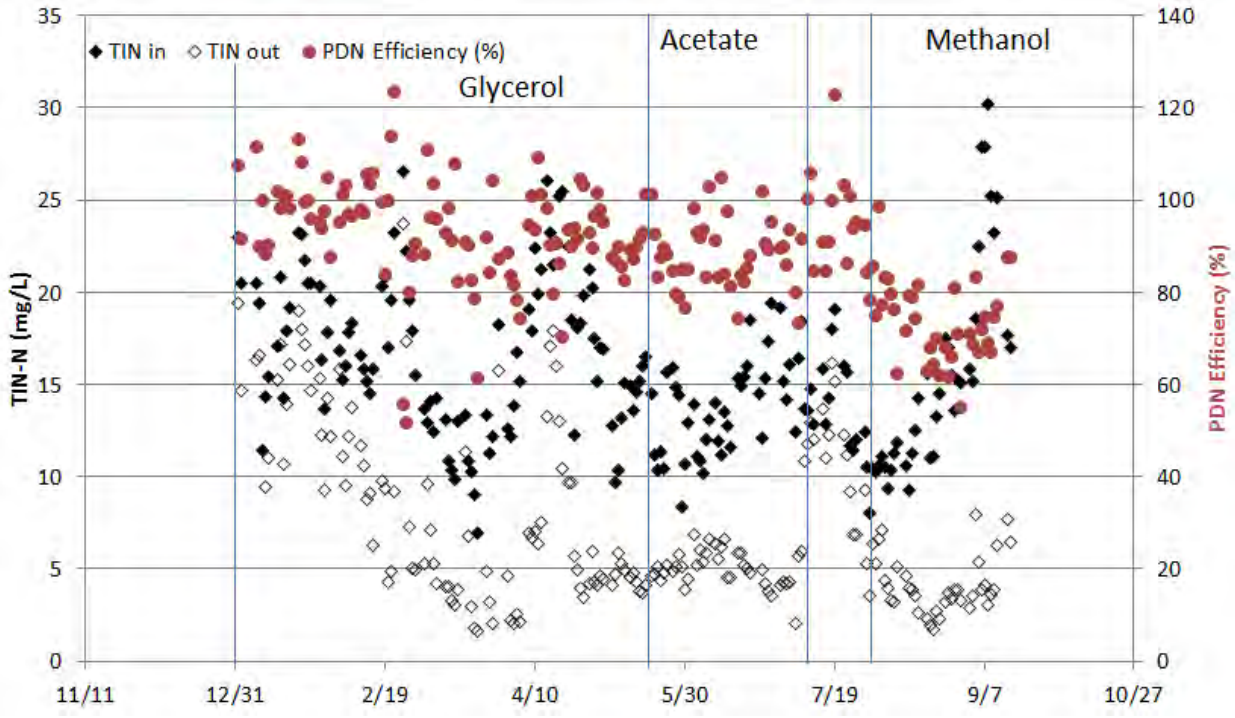


	AnAOB	PdN eff (%)	TIN effluent (mg N/L)	COD add/TIN removed (g/g)
A	no	88 ± 14	24 ± 4	-
B	no	97 ± 7	21 ± 7	-
C	no	88 ± 16	10 ± 3	4.7 ± 2.7
D	yes	80 ± 16	6 ± 4	2.2 ± 0.7

Le et al., *submitted;* Le et al., 2018 NRR, presentation 4.2

PdN-AnAOB polishing in separate sludge

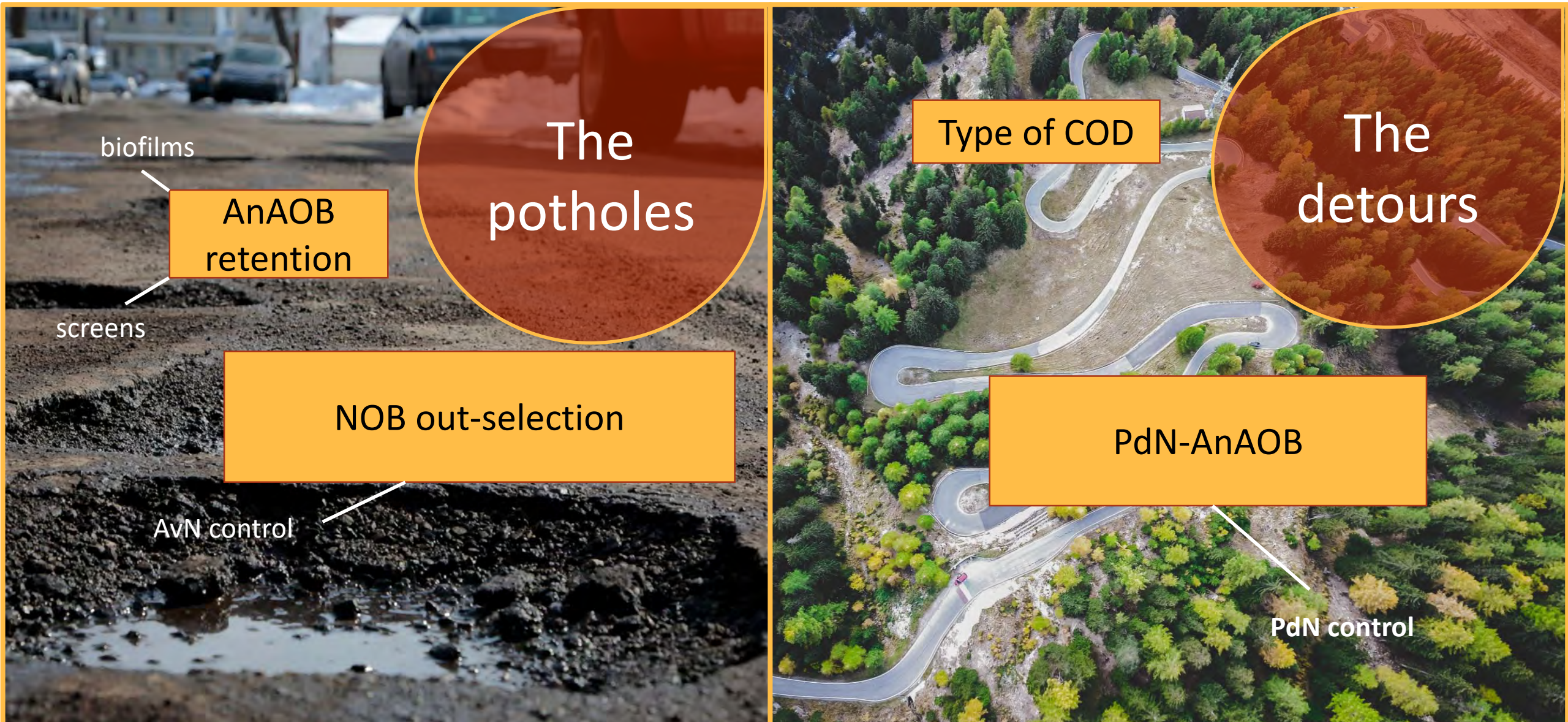




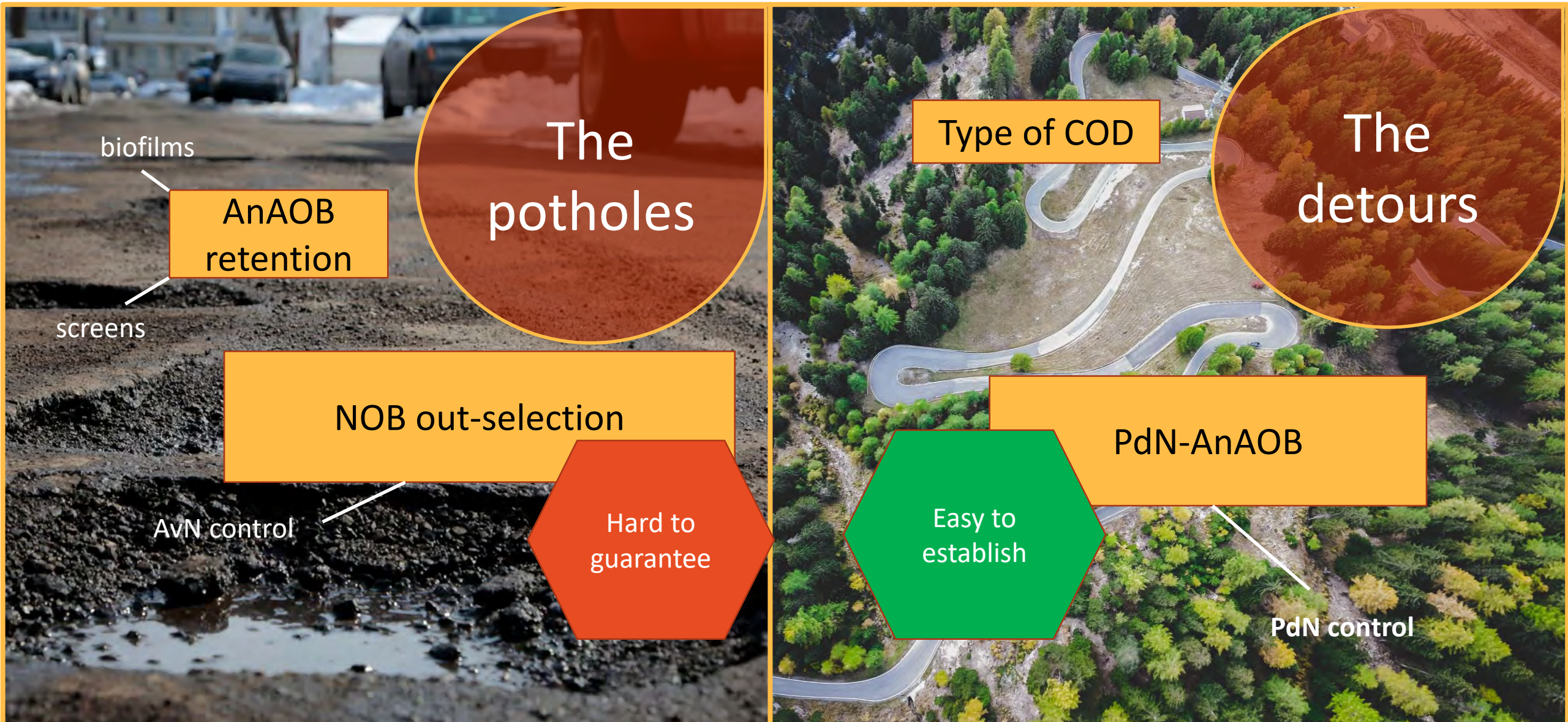
- PdN efficiency > 85% for glycerol and acetate
- PdN efficiency with methanol ~75% - due to presence of AnAOB sink?

Campolong et al., *2018;* Klaus et al., 2018 NRR, presentation 2.3

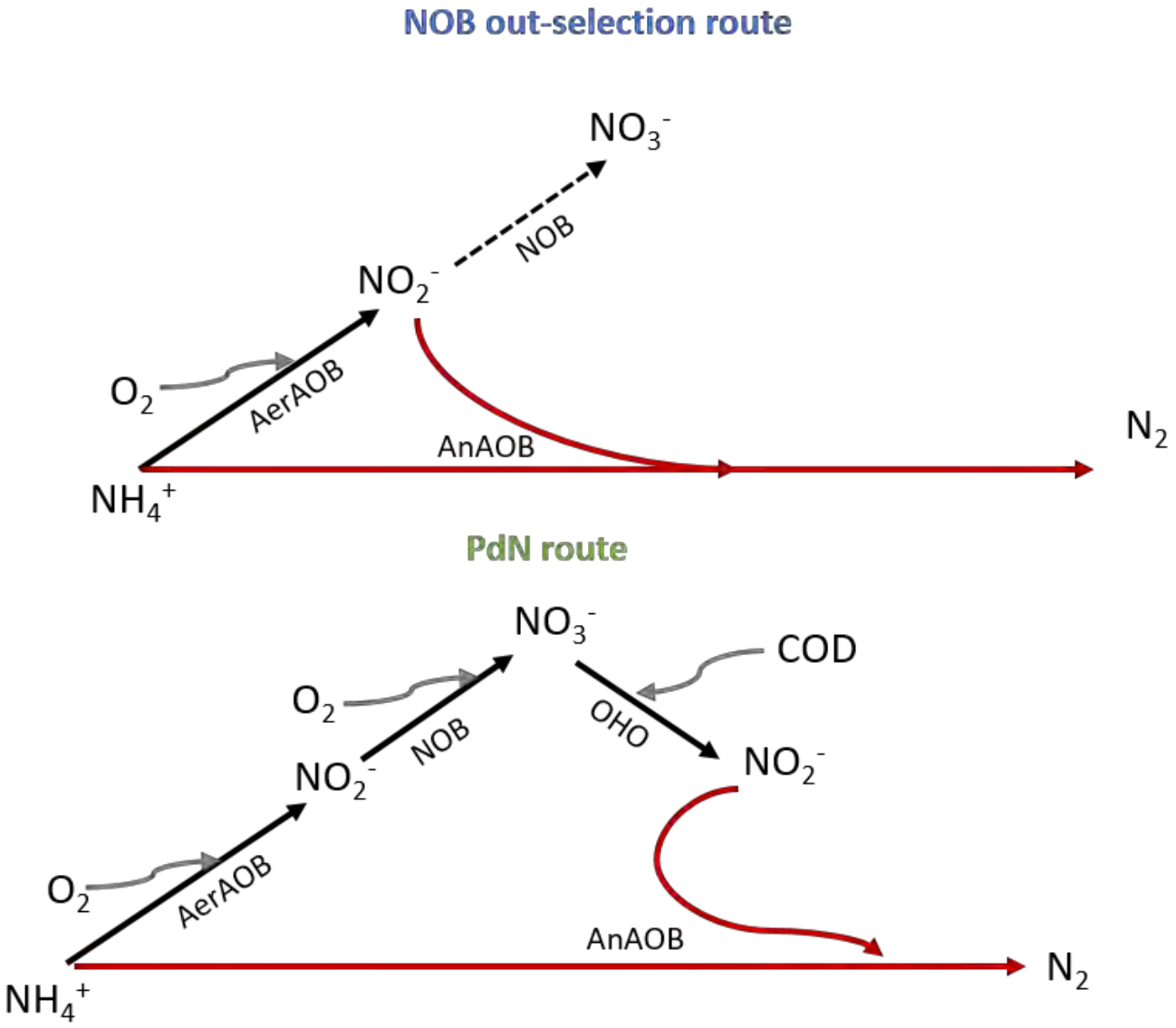
The road towards mainstream short-cut N removal



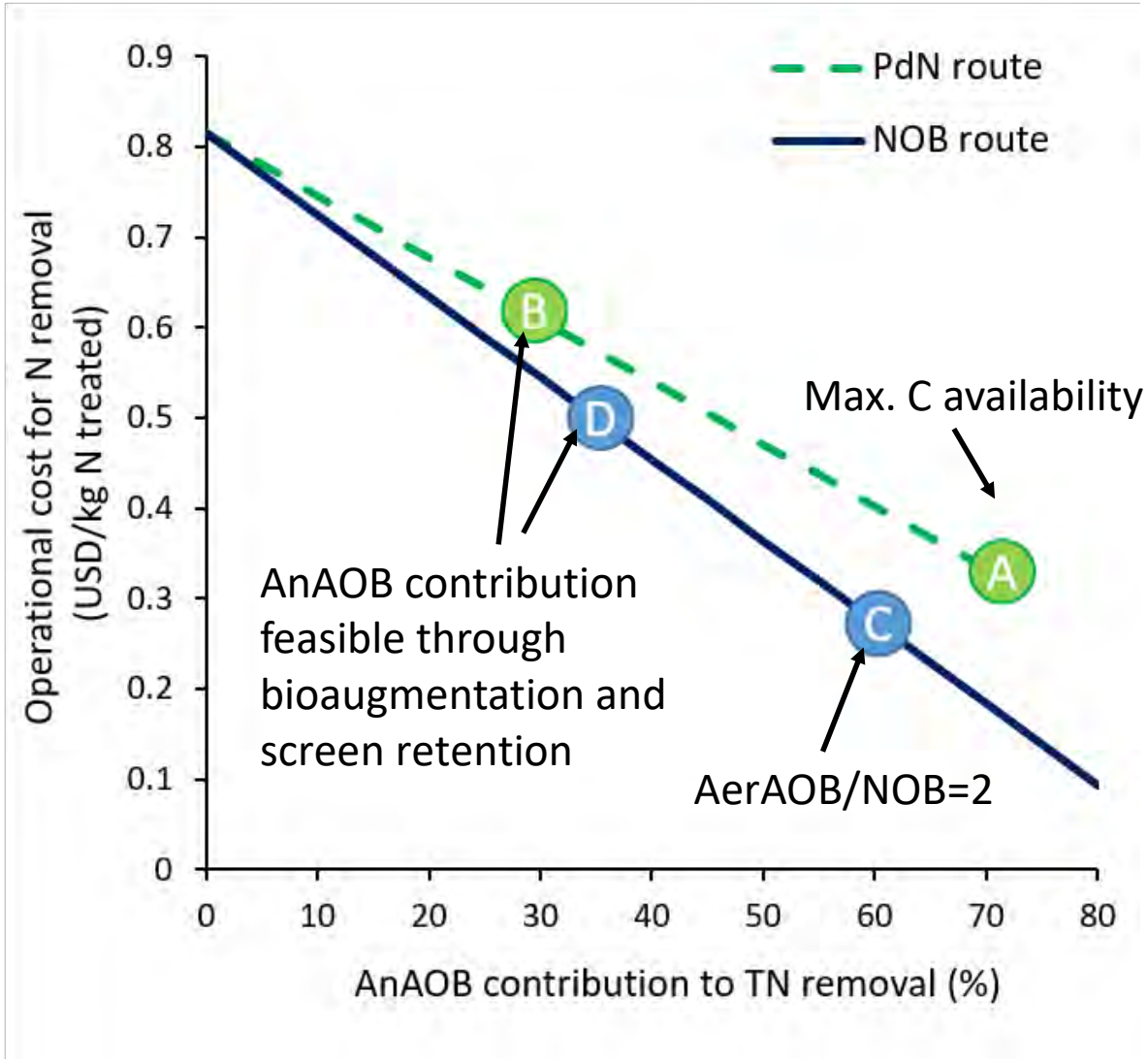
The road towards mainstream short-cut N removal



Direction for Blue Plains AWTP

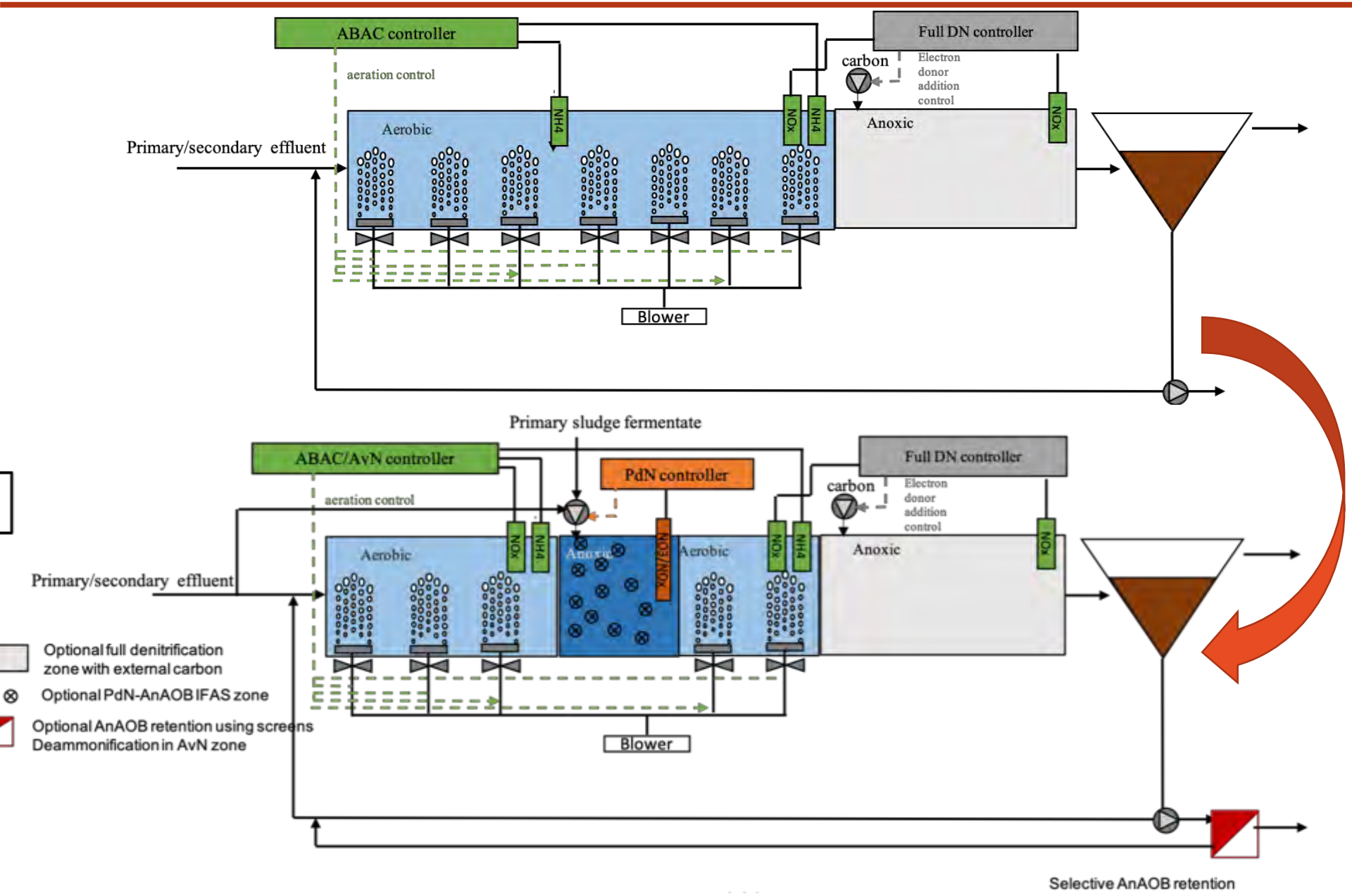


C source for PdN: primary sludge fermentate



Ali et al., 2018 NRR, presentation 10.3

Direction for Blue Plains AWTP



1. Guarantee effluent quality:
   - MeOH DN
   - Aerobic zone after PdN
2. PdN route for nitrite production
3. AnAOB retention
4. Aeration control (best chance for NOB out-selection

Thanks for the attention!

I love when the detour turns out the be a shortcut

(Katrina Mayer)

Haydée De Clippeleir, PhD
DC Water

Contact: Haydee.declippeleir@dcwater.com

dc water is life®

Attachment 4 • Tri Le et al., Nitrate residual as a key parameter to efficiently control partial denitrification coupling with anammox

NOTE: The attached document is for informational purposes only. It has not been reviewed or endorsed by the NWRI Independent Science Advisory Panel.

Water Environment Federation
the water quality people™

Nitrate residual as a key parameter to efficiently control partial denitrification coupling with anammox

Tri Le,[1,2] Bo Peng,[2,3] Chunyang Su,[2] Arash Massoudieh,[1] Alba Torrents,[3] Ahmed Al-Omari,[2] Sudhir Murthy,[2] Bernhard Wett,[4] Kartik Chandran,[5] Christine deBarbadillo,[2] Charles Bott,[6] Haydée De Clippeleir[2]

[1]Environmental Engineering, The Catholic University of America, Washington, District of Columbia

[2]DC Water and Sewer Authority, Washington, District of Columbia

[3]Department of Civil & Environmental Engineering, University of Maryland, College Park, Maryland

[4]ARAconsult, Innsbruck, Austria

[5]Department of Earth and Environmental Engineering, Columbia University, New York, New York

[6]Hampton Roads Sanitation District, Virginia Beach, Virginia

Received 5 February 2019; Revised 24 April 2019; Accepted 2 May 2019

District of Columbia Water and Sewer Authority

Correspondence to: Tri Le, Environmental Engineering, The Catholic University of America, Washington, DC.
Email: 64le@cua.edu

DOI: 10.1002/wer.1140

Abstract

Despite the increased research efforts, full-scale implementation of shortcut nitrogen removal strategies has been challenged by the lack of consistent nitrite-oxidizing bacteria out-selection. This paper proposes an alternative path using partial denitrification (PdN) selection coupled with anaerobic ammonium-oxidizing bacteria (AnAOB). A nitrate residual concentration (>2 mg N/L) was identified as the crucial factor for metabolic PdN selection using acetate as a carbon source, unlike the COD/N ratio which was often suggested. Therefore, a novel and simple acetate dosing control strategy based on maintaining a nitrate concentration was tested in the absence and presence of AnAOB, achieving PdN efficiencies above 80%. The metabolic-based PdN selection allowed for flexibility to move between PdN and full denitrification when required to meet effluent nitrate levels. Due to the independence of this strategy on species selection and management of nitrite competition, this novel approach will guarantee nitrite availability for AnAOB under mainstream conditions unlike shortcut nitrogen removal approaches based on NOB out-selection. Overall, a COD addition of only 2.2 g COD/g TIN removed was needed for the PdN-AnAOB concept showing its potential for significant savings in external carbon source needs to meet low TIN effluent concentrations making this concept a competitive alternative.

Practitioner points

- Nitrate residual is the key control parameter for partial denitrification selection.
- Metabolic selection allowed for flexibility of moving from partial to full denitrification.
- 2.2 g COD/g TIN removed was needed for partial denitrification-anammox process.

Key words

acetate; deammonification; mainstream; nitrate residual; partial denitrification

Introduction

Energy autarky can be reached by a combination of improved energy recovery and optimized nutrient removal. Energy recovery depends on the carbon pretreatment process chosen. Technologies such as chemically enhanced primary treatment (CEPT), high-rate activated sludge systems (HRAS) (Jimenez et al., 2015; Miller et al., 2017), and high-rate contact stabilization processes (Rahman et al., 2016) are known to capture 23%–85% of the incoming COD thus increasing the chance of reaching energy autarky. However, the latter technologies decrease the relative carbon feed into the nutrient removal stage (COD/N ratio < 2–3) and create a need for optimized nutrient removal through shortcut nitrogen removal or mainstream deammonification. Mainstream deammonification consists of two reactions in series: Firstly, half of the ammonium is aerobically converted to nitrite by ammonium-oxidizing bacteria (AerAOB), and secondly, the remaining ammonium and

produced nitrite are consumed by anaerobic ammonium-oxidizing bacteria (AnAOB) to form nitrogen gas anaerobically. A maximum operational cost saving of 60% in aeration demand and 100% in external carbon demand through application of mainstream deammonification can be theoretically estimated in comparison with conventional biological nitrogen removal (BNR). Deammonification has been globally applied for treatment of high strength ammonium streams as a more sustainable and cost-effective alternative for conventional nitrification–denitrification (Lackner et al., 2014). Despite the adoption for the sidestream's application (Lackner et al., 2014; Wett, 2007), no clear transition toward full-scale mainstream deammonification has been observed. The lower ammonium concentrations (<100 mg $NH_4^+ - N$ /L) and fluctuation in temperature and loading have caused challenges in nitrite-oxidizing bacteria (NOB) out-selection under mainstream conditions, and this has been recognized as the main challenge for ultimate full-scale adoption (Han, Clippeleir, et al., 2016a; Han, Vlaeminck, et al., 2016b; Lotti et al., 2015; Ma et al., 2016; Vlaeminck, Clippeleir, & Verstraete, 2012). In addition, maintaining an ammonium residual of 1–2 mg $NH_4^+ - N$/L for suspended sludge (Regmi et al., 2014) or up to 5 mg $NH_4^+ - N$/L for granular sludge (Poot, Hoekstra, Geleijnse, Loosdrecht, & Pérez, 2016) was needed to suppress NOB as well as provide kinetic advantage for AerAOB and AnAOB in mainstream conditions. This can be problematic when stringent nitrogen limits need to be met, especially given that effluent quality in reported systems has been mostly above 4 mg N/L (Cao, Loosdrecht, & Daigger, 2017; Gilbert et al., 2014; Han, Vlaeminck, et al., 2016b; Laureni et al., 2016; Lotti et al., 2015; Trojanowicz, Plaza, & Trela, 2016).

To accelerate mainstream deammonification's implementation in full scale, a development of a robust nitrogen polishing is required to remove both residual ammonium and nitrate and meet low effluent limits. Conventionally, full denitrification (FdN) with external organic carbon source addition has been widely practiced in wastewater treatment plants (WWTP) to reach the low effluent nitrogen limits (<5 mg TN/L; Mokhayeri et al., 2006). Methanol is the most commonly used external carbon source due to its cheap price (Katehis, 2007), reliable operation, low yield, and no nitrite accumulation (Dold et al., 2008). However, the usage of methanol also poses several drawbacks including slow growth rate and unstable performance under low temperature, and it is highly flammable (Mokhayeri et al., 2006). Therefore, many studies have been conducted to look for alternative organic carbon sources. Although ethanol, acetate, and glycerol were reported for having higher denitrification rates under low temperature, all of them had higher cost and some nitrite production which was not desired in conventional full denitrification (Bill, Bott, & Murthy, 2009; Mokhayeri et al., 2006). However, with a new era of mainstream shortcut nitrogen application, nitrite accumulation might be a benefit for coupling with the AnAOB process to remove residual ammonium anoxically.

Partial denitrification-anammox (PdN-AnAOB) is a novel, more economical alternative in terms of chemical cost savings, in which denitrifiers anoxically reduce residual nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$) that AnAOB can utilize to convert ammonium to nitrogen gas. Theoretically, PdN-AnAOB pathway can offer 50% saving in aeration and 80% saving in external carbon dosing compared with conventional BNR. In addition, such savings can be achieved without any need for NOB out-selection, unlike mainstream deammonification. Initially, PdN-AnAOB concept was proposed in sidestream treatment referred as DEnitrifying AMmonium OXidation (DEAMOX) which was designed to combine the AnAOB reaction with partial denitrification using sulfide (Kalyuzhnyi, Gladchenko, Mulder, & Versprille, 2006), acetate (Kalyuzhnyi, Gladchenko, Kang, Mulder, & Versprille, 2008), or glycerol (Sharp, Niemiec, Khunjar, Galst, & Deur, 2017) as an electron donor. Mainstream application of PdN-AnAOB has been getting more attention recently (Cao, Peng, Du, & Wang, 2016; Du et al., 2017; Ma, Qian, Yuan, Yuan, & Peng, 2017; Niemiec, Sharp, & Duer, 2018). While the AnAOB process is easily controlled whenever nitrite is available, PdN selection has become a critical step determining success of the PdN-AnAOB approach (Kumar & Lin, 2010). Nitrite accumulation as indicator of PdN has been reported in many studies when fast carbon sources such as acetate (Du et al., 2016; Ge et al., 2012; Gong et al., 2013; Le et al., 2018; Van Rijn, Tal, & Barak, 1996; Yang, Wang, & Zhou, 2012) or glycerol (Baideme et al., 2017; Bill et al., 2009; Park, Brotto, Loosdrecht, & Chandran, 2017; Sharp et al., 2017; Uprety, 2013) were used for denitrification. Furthermore, other research also showed that COD/N ratio, pH, DO level, and SRT could be important factors impacting PdN performance (Almeida, Júlio, Reis, & Carrondo, 1995; Beccari, Passino, Ramadori, & Tandoi, 1983; Glass & Silverstein, 1998; Obaja, MacÉ, & Mata-Alvarez, 2005). Niemiec et al. (2018) illustrated a glycerol driven partial denitritation/deammonification treating primary effluent with up to 85% of TIN removal, using a sophisticated combined control of COD/N ratio, pH, alkalinity, and solids retention time (SRT). Simpler control using influent COD/N ratio to achieve nitrite accumulation in PdN-AnAOB process was proposed in a variety of studies. Chen, Liu, Yang, Xue, and Wang (2009) and Pathak, Kazama, Saiki, and Sumino (2007) demonstrated a high AnAOB activity which was associated with PdN performance under low influent COD/N ratio of 0.6–0.7. Even with the higher COD/N ratio of 2.6 however, 73% of nitrate was mostly converted to nitrite (Baideme et al., 2017). In addition, Ge et al. (2012) reported that a higher nitrite accumulation rate was achieved when increasing COD/N ratio from 1 to 15 was added. As a result, a broad range of COD/N ratios for PdN selection has been reported and importance of COD/N ratio was inconclusive. In addition, as systems were run only based on PdN capability it was unclear whether one selected for a specialist community, losing capability for full denitrification (FdN) over time, or selected for a generalist community maintaining the flexibility to move toward FdN when needed. The latter would offer significant benefits for full-scale application as it would allow to meet effluent quality with PdN or FdN independent of the variability of the system. The latter however has not been studied or proven so far.

In this study, a simple control system was developed for selection of PdN on metabolic level, maintaining the capability

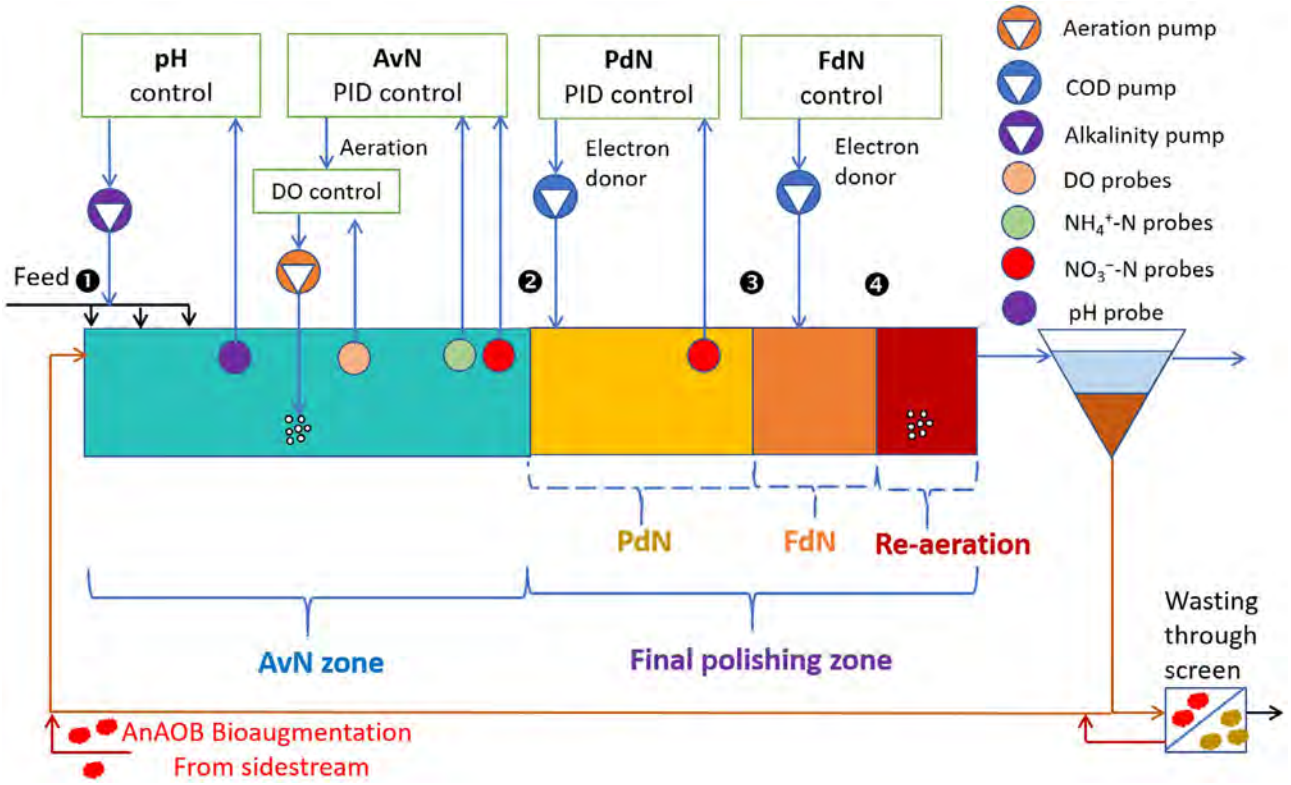


Figure 1. Mainstream shortcut nitrogen removal pilot consisting of AvN controlled zone in which equal ammonium and nitrate levels were targeted. In this zone, alkalinity was provided based on a pH target to compensate the CO_2 stripping. The second zone was a fully anoxic polishing zone in which the PdN and FdN strategies were tested and implemented. A final post-aeration zone was added in period IV. Overall zoning used in the different phases is presented in Figure 5.

of full denitrification using acetate as carbon source. The PdN selection strategy was focused on efficient production of nitrite even under the absence of AnAOB and thus the absence of competition for nitrite. This study shows a proof of principle of the PdN-AnAOB concept. Due to its flexibility in moving between PdN and FdN, guaranteeing effluent quality, we believe this will accelerate the implementation of shortcut nitrogen removal approaches in full-scale systems as it might offer a more reliable alternative for AnAOB incorporation into mainstream nutrient removal.

Mater ial and me tho ds

Pilot setup

Mainstream nitrogen removal pilot was located at the Blue Plains Advanced Wastewater Treatment Plant (AWTP) in Washington, DC (USA). The pilot schematic is shown in Figure 1. The overall volume of the pilot was 360 L; of which, 200 L was dedicated as Ammonium versus NO_x (AvN) controlled zone and 160 L was dedicated as polishing zone (PdN-AnAOB and FdN). Within the AvN zone, 50% of the volume was controlled by the AvN intermittent aeration controller (Regmi et al., 2014). Aeration-controlled zones were alternated with dedicated anoxic zones in which step feed was applied to increase potential for denitrification based on influent carbon. Aerobic SRT was calculated based on the aerobic time applied in the intermittent aeration zones in comparison with overall SRT (Han, Vlaeminck, et al., 2016b). Secondary effluent from the full-scale plant was fed into the pilot using step feed at three locations equivalent to 0, 40, and 60 min of reaction time. The overall total inorganic nitrogen (TIN) loading was 165 mg TIN L^{-1} day^{-1}, and total HRT was 340 min. Reactors were initially inoculated with nitrification/denitrification sludge from the full-scale biological nutrient removal step which was stably operating at a total SRT of 25 days and was acclimated to methanol dosing for denitrification. Bioaugmentation of AnAOB sludge, originating from a side-stream DEMON system in Strass (Austria), was done at day 300 (16 g wet sludge equivalent to a removal rate potential of 72 mg $NH_4^+ - N$ L^{-1} day^{-1}). COD dosing points were variable during the study and are indicated in Figure 5 in relation to the equivalent reaction time.

Control strategies and pilot operation

Online control consisted of an AvN PID aeration control (Regmi et al., 2014) which was implemented using online in situ ammonium (IQ SensorNet VaRION Plus, YSI), nitrate (IQ SensorNet VaRION Plus, YSI), and dissolved oxygen (DO) sensors (LDO Model 2, HACH) (Figure 1). The AvN controller optimized the aerobic duration within a 5-min cycle time to meet equal ammonium versus nitrate levels before the polishing zone and thus alpha values of 1 (Regmi et al.,

2014). During aeration, DO was maintained within the range of 1–1.5 mg DO/L. In addition, PdN control was achieved by either a feedforward control dosing acetate at fixed incoming COD/$NO_3^- - N$ ratio (period I) or by a PdN feedback PID control managing acetate dosing to meet a nitrate set point at the end of PdN zone (period II–IV). An additional COD flow proportional to the PdN-controlled COD addition was implemented during phase III and IV to achieve full denitrification. Manual wasting was conducted throughout the pilot performance to maintain overall sludge retention times (SRT) of about 20 days (Table 1). Aerobic SRT was determined automatically by the AvN controller, and thus, the intermittent aeration regime applied (Table 1). During phase IV, the wasted sludge was poured through a 125 μm sieve to retain AnAOB in the system (Han, Vlaeminck, et al., 2016b). Furthermore, a pH controller was used to sufficiently provide alkalinity for nutrient removal as the shallow pilot introduced increased CO_2 stripping.

Daily samples were taken from the feed, end of AvN zone, and the different polishing zones for overall performance assessment. In addition, a full concentration profile as presented in Figure 5 was taken on weekly basis.

Kinetic batch test

Batch tests were designed to investigate the mechanism of partial denitrification under anoxic condition. The anoxic batch tests were performed in 1 L reactor with nitrogen purging to maintain anoxic condition. Mixed liquor samples were collected from mainstream pilot. Sludge samples were washed using dechlorinated tap water and decanting steps to decrease residual substrate concentrations before starting the batch tests (Figure 3 during period I). Test D in Figure 3 was conducted using a mixture of the same sludge from the pilot (0.7 g VSS) during period I with addition of AnAOB sludge (0.5 g VSS) which originated from a sidestream DEMON system at WWTP Strass (Austria) (maximum activity rate of 6 mg $NH_4^+ - N$ (g VSS)$^{-1}$ hr^{-1}). The AnAOB sludge (stored at 4°C, under nitrate residual concentration) was first activated with ammonium and nitrite before conducting activity rates measurement. During the test, the temperature was controlled between 21 and 25°C, the pH was controlled in the range of 7–7.5 and samples were collected over a period of 4–8 hr for rate calculations. Acetate was spike at COD/$NO_3^- - N$ ratio of 3 or 10 to evaluate the impact of limited versus nonlimited COD supply. Samples for nitrogen speciation and COD were taken every 10 min for the first 90 min and every 30 min for the rest of the testing time until nitrate and nitrite levels dropped below 0.5 mg N/L.

Analytical procedures and calculations

All the nitrogen species ($NH_4^+ - N$, $NO_2^- - N$, $NO_3^- - N$), phosphorus ($PO_4^- - P$), and soluble COD were measured using HACH vials [HACH GmbH] and analyzed according to standard methods (American Public Health Association, 1999). Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods. In order to evaluate the process performance, partial denitrification (PdN) and full denitrification (FdN) contributions were calculated based on the equations below assuming that all nitrite converted will go to dinitrogen gas. AnAOB rates were calculated based on the ammonium removal rates and stoichiometry factor of 1.32 g $NO_2^- - N$/g $NH_4^+ - N$ removed.

$$\mathrm{PdN}\,(\%) = \frac{\text{AnAOB based nitrite removal rate } \left(\frac{\mathrm{mgNO_2^- - N}}{\mathrm{L.hr}}\right) + \text{Net } NO_2 \text{ accumulation } \left(\frac{\mathrm{mgNO_2^- - N}}{\mathrm{L.hr}}\right)}{\text{Nitrate removal rate } \left(\frac{\mathrm{mgNO_3^- - N}}{\mathrm{L.hr}}\right)}$$

$$\mathrm{FdN}\,(\%) = 100\% - \mathrm{PdN}$$

t Tests were done as a statistical tool to determine whether there were significant differences when comparing between different operational periods in Table 1.

Results

Pilot operation under COD/$NO_3^- - N$-based PdN control

In the first 89 days of operation, PdN selection strategy for the final polishing process was based on a feedforward control targeting acetate dosing at an incoming COD/$NO_3^- - N$ ratio of 3 (Table 1). Based on previous literature, this seemed a good target range to limit carbon for full denitrification (Du et al., 2017; Ma et al., 2017; Sharp et al., 2017). During the first 40 days, PdN selection and success were quite variables and efficiencies fluctuated between 5% and 100%. Although the stoichiometry of carbon addition was maintained well, ammonium and nitrate concentrations fluctuated significantly due to unstable AvN control. After optimizing the tuning of the AvN controller, a more stable ammonium (13 ± 2 mg $NH_4^+ - N$/L) and nitrate (12 ± 2 mg $NO_3^- - N$/L) concentration was produced from AvN zone to feed into the PdN zone resulting in an average nitrate concentration in the effluent of 5.6 ± 1.4 mg $NO_3^- - N$ /L. During this period (period I), PdN selection was stable and achieved efficiencies of 88 ± 14% (Figure 2, Table 1). Figure 5a shows the detailed concentration profile of the reactor showing the production of equal ammonium and nitrate concentration in the AvN zone and efficient conversion of nitrate to nitrite in the PdN zone. As no AnAOB was present, no additional ammonium removal took place in the PdN zone other than some ammonium removal for assimilation.

PdN kinetics

Although successful PdN selection was achieved under COD/$NO_3^- - N$ dosing of 3 (period I), during unstable nitrogen concentration but constant stoichiometries PdN selection

Table 1. Overview of the operational strategies applied during the different periods and performance of AvN zone, polishing zone, and overall pilot as a result of such strategy. Tags ❶, ❷, ❸, and ❹ identify the locations in Figure 1 where associated parameters in this table were measured

		I	II	III	IV
Operation day	day	40–89	90–165	166–219	220–254
COD control		COD/NO_3-N of 3-based	NO_3-N-based	NO_3-N-based	NO_3-N-based
AnAOB		No	No	No	1×
COD dosing		PdN	PdN	PdN + FdN	PdN + FdN
Overall					
TIN influent❶	mg N/L	29 ± 4	29 ± 3	29 ± 2	29 ± 3
COD/N influent	g/g	1.5 ± 0.5	1.9 ± 0.5	1.4 ± 0.3	1.2 ± 0.3
TIN effluent❹	mg N/L	22 ± 4	23 ± 5	10 ± 3	7 ± 2
Total SRT	day	20.5 ± 9.2	21.3 ± 9.2	19.1 ± 7.5	20.4 ± 3.4
Aerobic SRT	day	6.4 ± 3.1	4 ± 0.6	2.4 ± 0.6	1.1 ± 0.3
AvN					
TIN removal efficiency	% TIN in	16 ± 6	20 ± 7	28 ± 3	36 ± 4
NH_4 effluent❷	mg N/L	13 ± 2	12 ± 2	9 ± 1	8 ± 1
NO_3 effluent❷	mg N/L	12 ± 2	11 ± 1	10 ± 1	9 ± 1
NO_2 effluent❷	mg N/L	0.2 ± 0.1	0.5 ± 0.2	2.1 ± 1	1.7 ± 0.5
Final polishing					
TIN removal efficiency	% TIN in	6 ± 4	7 ± 6	38 ± 10	39 ± 8
NH_4 removal efficiency	% TIN in	5 ± 3	4 ± 4	4 ± 3	13 ± 3
Volumetric NH_4 removal	mg N/L	1.2 ± 1.3	0.5 ± 2.1	1.1 ± 1	6.5 ± 1.6
PdN NO_3 residual❸	mg N/L	5.6 ± 1.4	7.3 ± 1.9	6.6 ± 1	6.5 ± 0.8
PdN efficiency❸	%	88 ± 14	97 ± 7	88 ± 16	80 ± 16
Effluent NO_3 residual❹	mg N/L	5.6 ± 1.4	4.2 ± 1.3	1.1 ± 1.4	1.9 ± 1.1
FP COD added/TIN rem.	g/g	8.5 ± 6.5	13 ± 10	4.7 ± 2.7	2.2 ± 0.7

suffered (day 0–40). This created a need to better understand the PdN kinetics and investigate whether there was a better control strategy available other than COD/$NO_3^- - N$. For this, several batch experiments were performed using mixed liquor from the pilot system during period I. At both COD/$NO_3^- - N$ dosing of 3 (limited carbon, Figure 3a) as well as COD/$NO_3^- - N$ of 10 (nonlimiting carbon, Figure 3b), an efficient nitrite production and thus PdN rate was observed. In both cases 100% PdN was achieved during the first 150 min after which a sharp switch to full denitrification was observed (Figure 3aiii,biii). The switch from PdN to FdN happened both at 2 mg $NO_3^- - N$/L residual concentration (Figure 3a,b). Both tests indicated that although efficient PdN could be achieved, the sludge maintained the capability of FdN. To test whether the switch was reversible, an additional test under COD/$NO_3^- - N$ dosing of 3 was done where nitrate was added to the test after 75 min, increasing the nitrate levels from 0.86 to 15.95 mg $NO_3^- - N$/L (Figure 3c). Also in this case, a distinct switch from FdN to PdN was observed. These three tests all indicated that rather than COD/$NO_3^- - N$, nitrate levels determined the PdN kinetics.

To elucidate the potential impact of nitrite levels on the selection for PdN, AnAOB biomass was added to the pilot mixed liquor to provide nitrite consumption capabilities during the test. Figure 3d shows the batch experiment results. A good AnAOB stoichiometry (1.13 g $NO_3^- - N$/g $NH_4^+ - N$) between nitrate and ammonium consumption was observed during the first 200 min, indicating efficient PdN selection. When nitrate reached 3–3.74 mg $NO_3^- - N$/L, a significant drop in PdN efficiency was observed (100%–49%). In contrast to the tests without AnAOB, some PdN was maintained under lower nitrate concentrations as AnAOB were providing competition for nitrite. It has to be noted that during the tests, nitrate removal rates did not change significantly when changes from PdN to FdN were observed (Figure 3dii). Overall, a similar level of nitrate, at which decreased PdN selection was observed, was identified when no nitrite was present (Figure 3).

To evaluate the hypothesis of nitrate residual controlling PdN in larger scale, a short pilot scale run on day 70 was conducted to generate results under three different COD dosing scenarios each generating a different nitrate concentration profile in the polishing zone (200–360 min retention time) (Figure 4). COD/$NO_3^- - N$ ratio of 3 was applied for each dosing point, and the time of dosing is represented as dash lines in Figure 4. Each COD dosing scenario was performed for one system HRT (340 min). For all scenarios, efficient PdN and thus no TIN removal were achieved at the first two dosing points as nitrate levels remained above 2 mg N/L (Figure 4). For the second dosing point, a slightly lower PdN efficiency of 81% was observed reaching nitrate levels of 2.1 mg N/L (Figure 4b), while for all the first dosing location 100% PdN efficiency was observed (Figure 4). When COD dosing continued in the

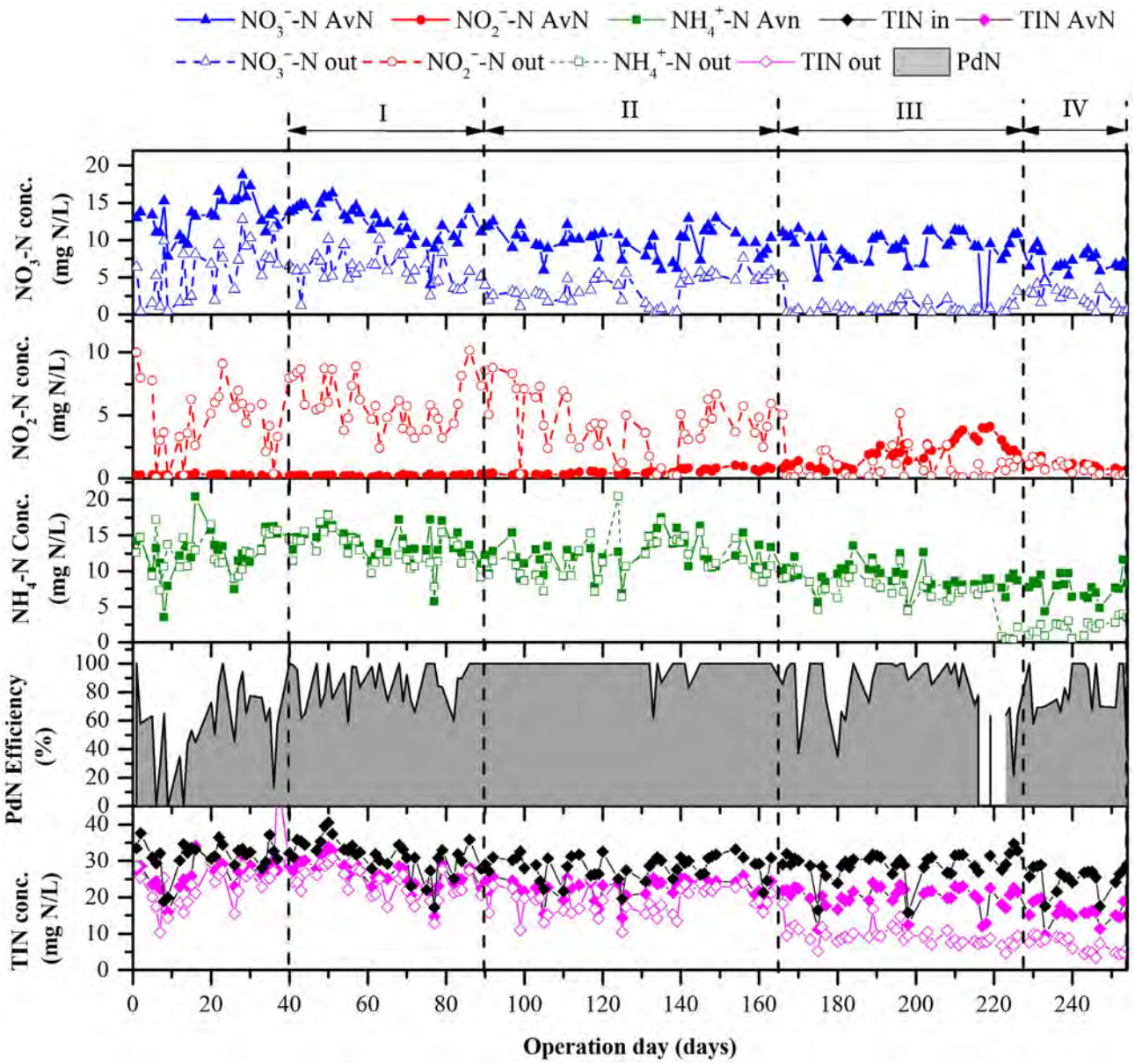


Figure 2. Mainstream polishing performance in terms of AvN effluent (influent to polishing zone) and final effluent nitrogen concentrations and PdN efficiency in four operational periods studied. Only in period IV, AnAOB biomass was added and ammonium removal in the polishing zone was demonstrated. Other phases focused on PdN control strategy (period I–II) and combining PdN with FdN (period III). Detailed description of strategies applied is presented in Table 1.

later zones (3 dosing points), nitrate concentration decreased to 0.1 mg $NO_3^- - N$/L and full denitrification kicked resulting in the TIN removal (Figure 4c). The results from Figure 4 confirmed that maintaining a nitrate residual concentration (around 2 mg $NO_3^- - N$/L) was essential for achieving PdN and that at lower nitrate concentration FdN was initiated.

Pilot operation under PdN control based on nitrate residual

After recognizing the role of nitrate residual, $COD/NO_3^- - N$ ratio-based COD dosing control was replaced with nitrate residual-based COD dosing control on day 89. This new control consisted of feedback PID loop which could dose a sufficient amount of COD to achieve a desired nitrate residual concentration in the PdN zone (highlighted in yellow, Figure 5). During period II, COD dosing was divided in three physical locations (equivalent to 220-, 260-, and 300-min reaction time) and the nitrate probe was located at the end of PdN zone (340 min). With a stable PdN control, nitrate residual in the PdN zone was maintained around 7.3 ± 1.9 mg $NO_3^- - N$/L, resulting in a high PdN efficiency of 97 ± 7% (Figure 2, period II; Table 1). A representative nitrogen profile during this period showed the three drops of nitrate concentration related to the points of COD dosing leading to three points of nitrite concentration

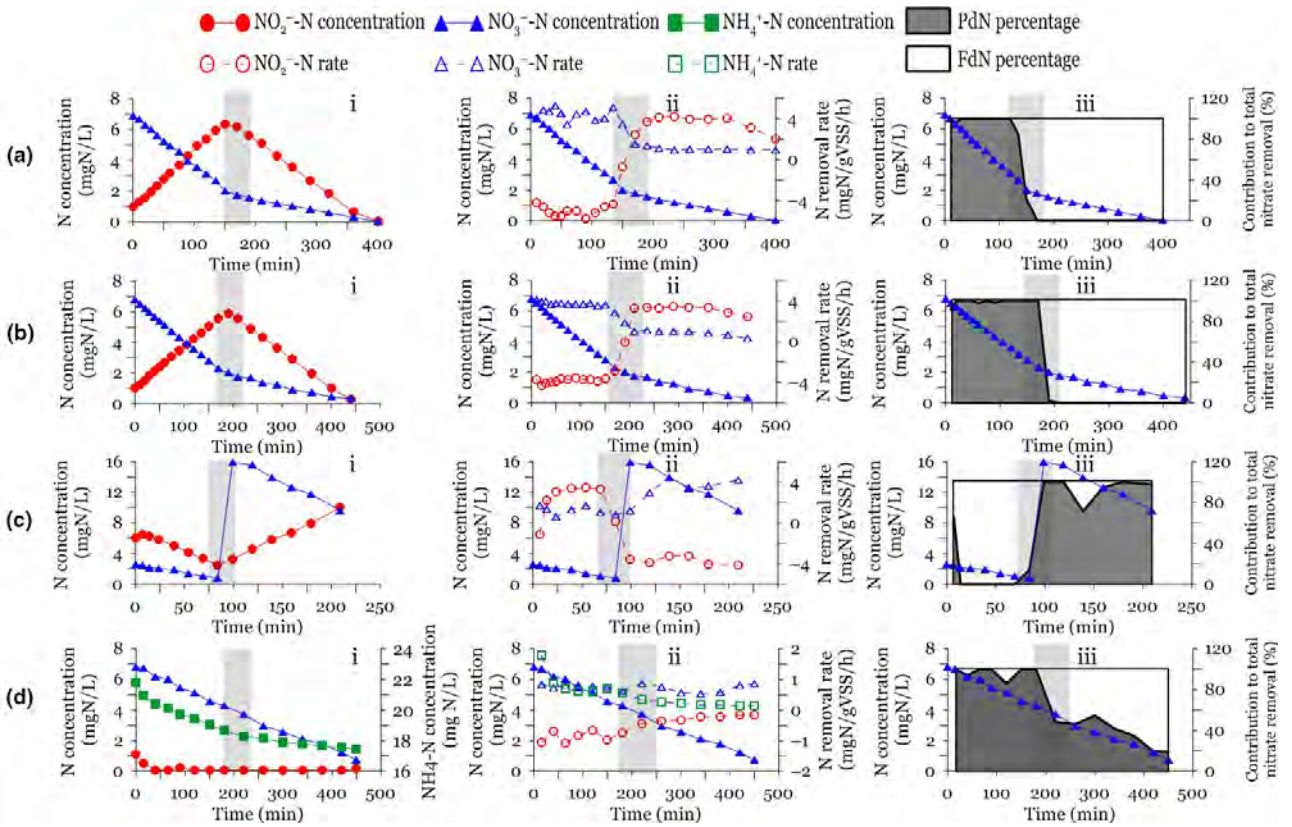


Figure 3. Nitrogen profiles of anoxic activity batch tests using sludge from the pilot during period I without the presence of AnAOB (a–c) and with addition of AnAOB (d). Tests were conducted under acetate addition namely COD/$N0_3^-$ – N of 3 for test a, c and d and COD/$N0_3^-$ – N of 10 for test b.

increases (Figure 5b). No significant TIN removal within the polishing zone was observed. COD stoichiometry measured over the polishing zone was variable during this period due to the low TIN removal which determined the denominator of the calculation (Table 1).

In the next period, an additional COD dosing point was added to allow for full denitrification (marked as FdN zone, highlighted in orange, Figure 5) and thus to allow nitrate concentration to drop below 2 mg NO_3^- – N/L after the PdN zone. During this phase, 88 ± 16% PdN efficiency was maintained at nitrate residual concentrations of 6.6 ± 1 mg N/L in the PdN zone (Figure 2, period III). Full denitrification was triggered in the FdN zone when nitrate residual dropped to 1.1 ± 1.4 mg N/L (Table 1). As a result, TIN removal efficiency significantly increased up to 38 ± 10% (5 times higher than period I and II; $p < 0.05$), yielding a final TIN effluent of 10 ± 3 mg N/L, of which 8 ± 1 mg NH_4^+ – N/L (Figure 2, Table 1 period III). The mass of COD added per mass of TIN removed stabilized to 4.7 ± 2.7, which was close to full denitrification stoichiometry (Table 1, period III). Figure 5c illustrates the simultaneous removal of nitrate and nitrite when full denitrification was triggered, leading to complete depletion of NOx in effluent.

To show the potential of PdN selection when coupled to AnAOB, AnAOB biomass (105 g VSS) was added to the system on day 222 (period IV). In addition, a post-aeration zone was installed to allow for some final ammonium oxidation and nitrogen gas release before the clarifiers (Figure 1). In the presence of AnAOB, 6.5 ± 1.6 mg NH_4^+ – N/L was removed over the polishing zone while PdN efficiency remained high (80 ± 16%) (Table 1, Figure 2). At similar TIN removal efficiencies ($p > 0.05$) compared with period III, during this phase a significant decrease in external COD demand ($p < 0.05$) was observed leading to COD stoichiometries of 2.2 ± 0.7 g COD added per TIN removed as a result of AnAOB activity. Figure 5d shows the simultaneous ammonium and nitrate removal over the PdN zone.

Discussion

A large variety of studies have quantified denitrification stoichiometries and yields for different carbon sources (Bill et al., 2009; Dold et al., 2008; Mokhayeri et al., 2009, 2006), and the latter information has been used for the operation and design of our denitrification systems. During those studies, nitrite accumulation has been observed frequently especially when carbon sources such as acetate or glycerol have been used. Although nitrite accumulation was well known, this has only recently been extended to the PdN-AnAOB concept for mainstream application. This study for the first time elucidated the

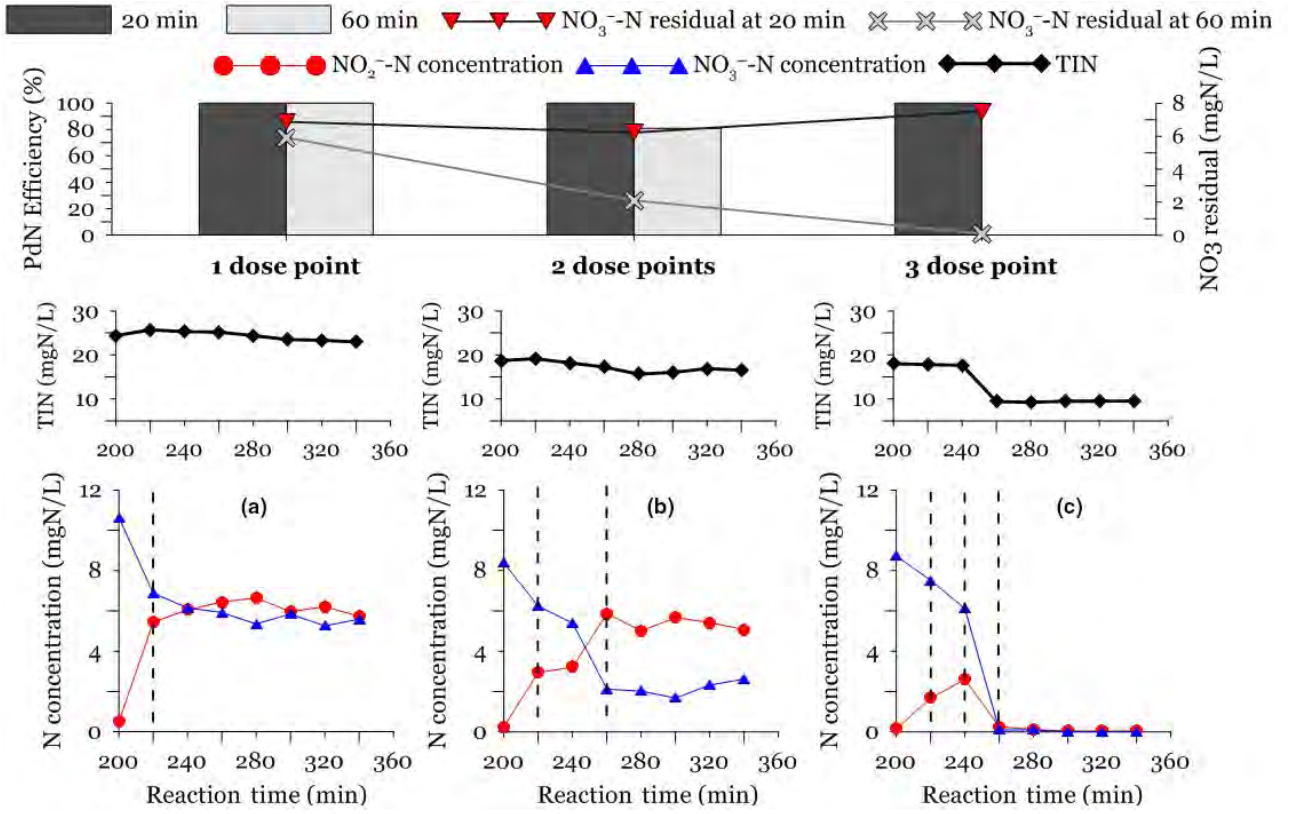


Figure 4. PdN efficiency and nitrogen profiles in the polishing zone of the pilot as a result of acetate dosing at COD/NO_3^- – N ratios of 3 at 220-min reaction time (a), at 220- and 260-min reaction time (b), and at 220-, 240-, and 260-min reaction time (c) resulting in different nitrate concentration profiles.

mechanism for PdN selection on metabolic level rather than species level and translated this is in an easy to apply control strategy.

PdN selection mechanism

To achieve PdN selection, the use of the right carbon source (glycerol or acetate but not methanol) is essential for potentially two main reasons. Acetate and glycerol have been reported to be converted to poly-3-hydroxybutyrate which can be stored intracellularly as efficient electron donors for denitrification (Carucci, Dionisi, Majone, Rolle, & Smurra, 2001; Moralejo-Gárate, Mar'atusalihat, Kleerebezem, & van Loosdrecht, 2011). Intracellular carbon storage may be a reason allowing for better PdN selection as a large pool of electrons is created within the cell. Nitrate reductase has a higher electron capacity than nitrite reductase as two electrons are transferred per mole nitrate reduced, while one electron is transferred per mole nitrite reduced. With the carbon storage, or electron storage, nitrate reductase could take up more electrons, thus creating sufficient rate differential between nitrate reduction and nitrite reduction thus leading to nitrite availability for AnAOB. Another perhaps better explanation for the more efficient partial denitrification selection when using acetate (or glycerol) compared with methanol is related to how and where electrons are donated. Nitrate reductase accepts electrons transferred through ubiquinone or cytochrome b in the upstream region of electron transfer chain whereas nitrite reductase accepts electrons from cytochrome c in a more downstream region (Van Rijn et al., 1996). Certain carbon sources donate electrons throughout the electron chain including cytochrome c while other sources donate preferentially in the upper regions (Almeida, Reis, & Carrondo, 1995; Liu, Mao, Bergaust, Bakken, & Frostegård, 2013). If electrons are donated in the upstream region (excluding cytochrome c), an increase of nitrite availability is expected. Indeed, acetate has been shown to donate electrons closer to nitrate reductase in the upstream region instead of nitrite reductase in the downstream region, leading to a faster nitrate reduction rate and thus more nitrite availability (Van Rijn et al., 1996). Similarly, glycerol donates the electrons to cytochrome b in the upstream region (Stewart, 1988). Methanol, however, donates its electrons close to cytochrome c and thus downstream region (Porte & Vignais, 1980).

The latter explanation correlated well with the observations that PdN performance was dependent on nitrate concentration, thus potentially indicating that electron transport from cytochrome b to cytochrome c only occurred after electron demand from cytochrome b decreased. This study therefore suggests that COD/NO_3^- – N is not the direct control mechanism unlike other studies suggested (Du et al., 2017; Ma et al., 2017; Sharp et al., 2017), rather operation under a minimum

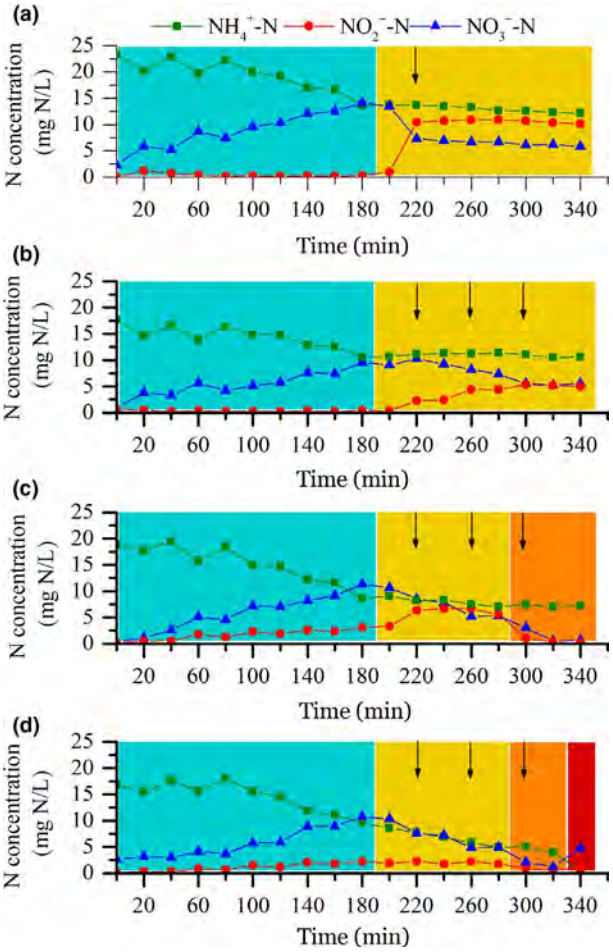


Figure 5. Overall nitrogen concentration profiles throughout the pilot including AvN and polishing zones expressed by equivalent reaction time for the different operational periods studies (Table 1). (a) period I day 54, (b) period II day 97, (c) period III day 200, and (d) period IV day 224. Background color indicates the zones presented in Figure 1. COD dosing points are represented as black arrows.

nitrate concentration was. Similar nitrate and nitrite profiles were established under limiting ($COD/NO_3^- - N$ of 3) and non-limiting carbon dosing ($COD/NO_3^- - N$ of 10; Figure 3a,b) confirming this hypothesis. Although free nitrous acid inhibition of denitrifiers has been reported at levels of 0.2 mg HNO_2-N/L (Ma et al., 2010), a combination of PdN with AnAOB (Figure 3d) confirmed that the nitrite concentration, and thus free nitrous acid concentrations, did not play any role in the PdN selection in our study.

Reviewing literature from perspective of nitrate residual, the achievement of PdN selection with nitrate residual can be spotted in a variety of studies. The initial accumulation of nitrite followed by nitrite reduction, when nitrate was depleted, was observed in both glycerol studies (Bill et al., 2009; Uprety, 2013) and acetate studies (Mokhayeri et al., 2006). In a case study by Gong et al. (2013), nitrogen profile under acetate feast-famine condition with different $COD/NO_3^- - N$ ratio varied from 1.4 to 3.5 and initial nitrate concentration varied from 16.8 to 79.3 mg $NO_3^- - N$/L, all the results shared the similar observation that nitrite accumulated when nitrate was above 2 mg N/L. Nitrate residual concentration as key factor to control the switch from PdN to FdN can also be extracted from studies combining PdN and AnAOB in suspended sludge (Du et al., 2017) and biofilms (Ma et al., 2017). Nonetheless, all of the above studies shared the same COD dosing approach which was based on low $COD/NO_3^- - N$ (2–3) to control PdN and had nitrate levels in the effluent of 2–3 mg N/L (Du et al., 2017; Gong et al., 2013; Ma et al., 2017). Based on our study, we suggest that their success was potentially based on the maintenance of a nitrate residual concentration (as controlled by COD dosing strategy) rather than directly a result of the $COD/NO_3^- - N$ stoichiometry dosed.

Our study demonstrated long-term operation at a high and stable PdN efficiency (>80%) using nitrate-based COD dosing control in the PdN zone. The PdN control was therefore purely based on the controlled addition of the right type of COD to maintain a nitrate residual concentration (>2 mg N/L) in the bulk. This finding significantly simplifies operational control and implementation of the PdN-AnAOB concept.

Application of PdN-AnAOB within shortcut N removal scheme

The simple PdN selection approach developed in this study offers new perspectives for full-scale mainstream AnAOB implementation. As PdN was fully controlled by nitrate residual, no management of nitrite competition was required to achieve good PdN efficiencies (period I–III, Figure 2). Therefore, nitrite availability should be easily achievable independent of AnAOB activity or inventory, giving an ideal mainstream condition for AnAOB enrichment and growth. This would allow for some of the proposed bioaugmentation approaches (Al-Omari et al., 2015) to be implemented with a higher success rate as AnAOB survival will be less dependent on NOB out-selection. In addition, the flexibility to switch back to full denitrification when needed will offer operational flexibility to optimize the system and more easily guarantee effluent quality. Based on this study, AvN control only removed 16%–20% TIN without any NOB out-selection in period I and II, using available carbon in the influent (Table 1). With more aggressive aerobic SRT control to wash out NOB in period III, nitrite started to accumulate around 2 mg $NO_2^- - N$/L in the end of AvN zone (Table 1). The efficient NOB out-selection during this period enhanced nitrite sink in anoxic cells of AvN zone, thus increased TIN removal efficiency up to 28% (Table 1). The same SRT control strategy was carried out in period IV which helped to provide nitrite for deammonification process during AvN zone. As the result, TIN removal efficiency in AvN zone further increased to 36% when AnAOB was present (Table 1). With the AnAOB biomass added however, only 6.5 of the 8 mg NH_+^4–N/L could be removed in the polishing zone, leading to increased TIN effluent numbers. Optimization of upfront aeration and SRT control strategy with downstream PdN and FdN contributions will be needed to better balance aerobic ammonium oxidation with anoxic ammonium oxidation. Especially with a suspended

AnAOB approach, it is anticipated that AnAOB rates will change seasonally and will depend on retention efficiency (, in preparation), PdN efficiency, and loading conditions.

The requirement of using acetate to perform denitrification may yield a higher operational cost due to its higher observed yield for full denitrification (8.15 USD/kg $NO_3^- - N$ removed) compared with methanol (1.14 USD/kg $NO_3^- - N$ removed) (Mokhayeri et al., 2009). Furthermore, as acetate can be stored intracellularly, one might need to manage carbon dosing strategy well to avoid overdosing in the full denitrification zone. Within this study, full denitrification yields were lower than expected (4.7 ± 2.7 g COD added per TIN removed vs. 8.4 g COD/g TIN removed using a yield of 0.66 g COD/g COD as reported by Mokhayeri et al. (2009)). Also, for PdN coupled with AnAOB yields, reflected nitrate reduction yields (2.2 g COD added per $NO_3^- - N$ removed observed in our study vs. 3.35 g COD added per $NO_3^- - N$ removed expected based on Mokhayeri et al. (2009)) and thus no indications of overdosing were present. Overall, a yield of 2.2 ± 0.7 g COD/TIN removed was achieved during period IV, showing the potential for more than 50% COD cost savings using the PdN-AnAOB concept. Longer term testing under more variable conditions will have to confirm the lower acetate yield when operating under PdN conditions compared with the FdN yields reported before. The latter confirmation will be crucial to assess economic viability.

Conclusion

In conclusion, this study sets the stage for PdN-AnAOB application under mainstream conditions through identification of the nitrate residual concentration as the key feature for PdN selection and translation of this knowledge in a simple online control strategy. The metabolic-based PdN selection allowed for flexibility to move between PdN and FdN when required to meet effluent nitrate levels. Due to the independence of the PdN selection strategy on species selection and management of nitrite competition, this novel approach will guarantee nitrite availability for AnAOB under mainstream conditions unlike shortcut nitrogen removal approaches based on NOB out-selection. Although the PdN-AnAOB concept requires more resources than mainstream deammonification based on NOB out-selection, the reliability and simplicity of this concept might help with accelerating full-scale integration of AnAOB-based systems for mainstream nutrient removal. Future research will have to show long-term performance of PdN coupled to AnAOB to achieve low TIN effluent levels under variable conditions.

Acknowledgment

This work was supported by District of Columbia Water and Sewer Authority (DC Water), Washington, DC. The authors gratefully thank Norman Dockett for technical support and everyone in the DC Water research laboratory for all assistance offered.

References

Almeida, J. S., Júlio, S. M., Reis, M. A. M., & Carrondo, M. J. T. (1995). Nitrite inhibition of denitrification by *Pseudomonas fluorescens*. *Biotechnology and Bioengineering*, *46*(3), 194–201. https://doi.org/10.1002/bit.260460303

Almeida, J. S., Reis, M. A. M., & Carrondo, M. J. T. (1995). Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens*. *Biotechnology and Bioengineering*, *46*(5), 476–484. https://doi.org/10.1002/bit.260460512

Al-Omari, A., Wett, B., Nopens, I., De Clippeleir, H., Han, M., Regmi, P., … Murthy, S. (2015). Model-based evaluation of mechanisms and benefits of mainstream shortcut nitrogen removal processes. *Water Science and Technology*, *71*(6), 840–847. https://doi.org/10.2166/wst.2015.022

American Public Health Association, A. W. W. A., Water Environment Federation. (1999). In Association, A. P. (Ed.), *Standard Methods for Examination of Water and Wastewater*. US.

Baideme, M., Long, C., Plante, L., Starke, J., Butkus, M., & Chandra, K. (2017). Optimization of partial denitrification to maximize nitrite production using glycerol as an external carbon source. In *WEFTEC 2017*. Chicago, USA.

Beccari, M., Passino, R., Ramadori, R., & Tandoi, V. (1983). Kinetics of dissimilatory nitrate and nitrite reduction in suspended growth culture. *Journal (Water Pollution Control Federation)*, 58–64.

Bill, K. A., Bott, C. B., & Murthy, S. N. (2009). Evaluation of alternative electron donors for denitrifying moving bed biofilm reactors (MBBRs). *Water Science and Technology*, *60*(10), 2647–2657. https://doi.org/10.2166/wst.2009.622

Cao, S., Peng, Y., Du, R., & Wang, S. (2016). Feasibility of enhancing the DEnitrifying AMmonium OXidation (DEAMOX) process for nitrogen removal by seeding partial denitrification sludge. *Chemosphere*, *148*, 403–407. https://doi.org/10.1016/j.chemosphere.2015.09.062

Cao, Y., van Loosdrecht, M. C. M., & Daigger, G. T. (2017). Mainstream partial nitritation–anammox in municipal wastewater treatment: Status, bottlenecks, and further studies. *Applied Microbiology and Biotechnology*, *101*(4), 1365–1383. https://doi.org/10.1007/s00253-016-8058-7

Carucci, A., Dionisi, D., Majone, M., Rolle, E., & Smurra, P. (2001). Aerobic storage by activated sludge on real wastewater. *Water Research*, *35*(16), 3833–3844. https://doi.org/10.1016/S0043-1354(01)00108-7

Chen, H., Liu, S., Yang, F., Xue, Y., & Wang, T. (2009). The development of simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) process in a single reactor for nitrogen removal. *Bioresource Technology*, *100*(4), 1548–1554. https://doi.org/10.1016/j.biortech.2008.09.003

Dold, P., Takács, I., Mokhayeri, Y., Nichols, A., Hinojosa, J., Riffat, R., … Murthy, S. (2008). Denitrification with carbon addition—kinetic considerations. *Water Environment Research*, *80*(5), 417–427. https://doi.org/10.2175/106143007X221085

Du, R., Cao, S., Li, B., Niu, M., Wang, S., & Peng, Y. (2017). Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters. *Water Research*, *108*, 46–56. https://doi.org/10.1016/j.watres.2016.10.051

Du, R., Peng, Y., Cao, S., Li, B., Wang, S., & Niu, M. (2016). Mechanisms and microbial structure of partial denitrification with high nitrite accumulation. *Applied Microbiology and Biotechnology*, *100*(4), 2011–2021. https://doi.org/10.1007/s00253-015-7052-9

Ge, S., Peng, Y., Wang, S., Lu, C., Cao, X., & Zhu, Y. (2012). Nitrite accumulation under constant temperature in anoxic denitrification process: The effects of carbon sources and COD/NO_3-N. *Bioresource Technology*, *114*, 137–143. https://doi.org/10.1016/j.biortech.2012.03.016

Gilbert, E. M., Agrawal, S., Karst, S. M., Horn, H., Nielsen, P. H., & Lackner, S. (2014). Low temperature partial nitritation/anammox in a moving bed biofilm reactor treating low strength wastewater. *Environmental Science & Technology*, *48*(15), 8784–8792. https://doi.org/10.1021/es501649m

Glass, C., & Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research*, *32*(3), 831–839. https://doi.org/10.1016/S0043-1354(97)00260-1

Gong, L., Huo, M., Yang, Q., Li, J., Ma, B., Zhu, R., … Peng, Y. (2013). Performance of heterotrophic partial denitrification under feast-famine condition of electron donor: A case study using acetate as external carbon source. *Bioresource Technology*, *133*, 263–269. https://doi.org/10.1016/j.biortech.2012.12.108

Han, M., De Clippeleir, H., Al-Omari, A., Wett, B., Vlaeminck, S. E., Bott, C., & Murthy, S. (2016a). Impact of carbon to nitrogen ratio and aeration regime on mainstream deammonification. *Water Science and Technology*, *74*(2), 375–384. https://doi.org/10.2166/wst.2016.202

Han, M., Vlaeminck, S. E., Al-Omari, A., Wett, B., Bott, C., Murthy, S., & De Clippeleir, H. (2016b). Uncoupling the solids retention times of flocs and granules in mainstream deammonification: A screen as effective out-selection tool for nitrite oxidizing bacteria. *Bioresource Technology*, *221*, 195–204. https://doi.org/10.1016/j.biortech.2016.08.115

Jimenez, J., Miller, M., Bott, C., Murthy, S., De Clippeleir, H., & Wett, B. (2015). High-rate activated sludge system for carbon management – Evaluation of crucial process mechanisms and design parameters. *Water Research*, *87*, 476–482. https://doi.org/10.1016/j.watres.2015.07.032

Kalyuzhnyi, S. V., Gladchenko, M. A., Kang, H., Mulder, A., & Versprille, A. (2008). Development and optimisation of VFA driven DEAMOX process for treatment of strong nitrogenous anaerobic effluents. *Water Science and Technology*, *57*(3), 323. https://doi.org/10.2166/wst.2008.044

Kalyuzhnyi, S. V., Gladchenko, M., Mulder, A., & Versprille, B. (2006). DEAMOX-New biological nitrogen removal process based on anaerobic ammonia oxidation coupled

to sulphide-driven conversion of nitrate into nitrite. *Water Research*, *40*(19), 3637–3645. https://doi.org/10.1016/j.watres.2006.06.010

Katehis, D. (2007). *General information on alternative supplemental carbon sources*. Washington, DC: Water Environment Research Foundation.

Kumar, M., & Lin, J. G. (2010). Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal-Strategies and issues. *Journal of Hazardous Materials*, *178*(13), 1–9. https://doi.org/10.1016/j.jhazmat.2010.01.077

Lackner, S., Gilbert, E. M., Vlaeminck, S. E., Joss, A., Horn, H., & van Loosdrecht, M. C. M. (2014). Full-scale partial nitritation/anammox experiences – an application survey. *Water Research*, *55*, 292–303. https://doi.org/10.1016/j.watres.2014.02.032

Laureni, M., Falås, P., Robin, O., Wick, A., Weissbrodt, D. G., Nielsen, J. L., … Joss, A. (2016). Mainstream partial nitritation and anammox: Long-term process stability and effluent quality at low temperatures. *Water Research*, *101*, 628–639. https://doi.org/10.1016/j.watres.2016.05.005

Le, T., Peng, B., Su, C., Massoudieh, A., Torrents, A., Al-Omari, A., … De Clippeleir, H. (2018). Impact of carbon source and COD/N on the concurrent operation of partial denitrification and anammox. *Water Environment Research*, *91*(3), 185–197. https://doi.org/10.1002/wer.1016

Liu, B., Mao, Y., Bergaust, L., Bakken, L. R., & Frostegård, Å. (2013). Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. *Environmental Microbiology*, *15*(10), 2816–2828. https://doi.org/10.1111/1462-2920.12142

Lotti, T., Kleerebezem, R., Hu, Z., Kartal, B., de Kreuk, M. K., van Erp Taalman Kip, C., … van Loosdrecht, M. (2015). Pilot-scale evaluation of anammox-based mainstream nitrogen removal from municipal wastewater. *Environmental Technology (United Kingdom)*, *36*(9), 1167–1177. https://doi.org/10.1080/09593330.2014.982722

Ma, B., Qian, W., Yuan, C., Yuan, Z., & Peng, Y. (2017). Achieving mainstream nitrogen removal through coupling anammox with denitratation. *Environmental Science and Technology*, *51*(15), 8405–8413. https://doi.org/10.1021/acs.est.7b01866

Ma, B., Wang, S., Cao, S., Miao, Y., Jia, F., Du, R., & Peng, Y. (2016). Biological nitrogen removal from sewage via anammox: Recent advances. *Bioresource Technology*, *200*, 981–990. https://doi.org/10.1016/j.biortech.2015.10.074

Ma, J., Yang, Q., Wang, S., Wang, L., Takigawa, A., & Peng, Y. (2010). Effect of free nitrous acid as inhibitors on nitrate reduction by a biological nutrient removal sludge. *Journal of Hazardous Materials*, *175*(1–3), 518–523. https://doi.org/10.1016/j.jhazmat.2009.10.036

Miller, M. W., Elliott, M., DeArmond, J., Kinyua, M., Wett, B., Murthy, S., & Bott, C. B. (2017). Controlling the COD removal of an A-stage pilot study with instrumentation and automatic process control. *Water Science and Technology*, *75*(11), 2669–2679. https://doi.org/10.2166/wst.2017.153

Mokhayeri, Y., Nichols, A., Murthy, S., Riffat, R., Dold, P., & Takacs, I. (2006). Examining the influence of substrates and temperature on maximum specific growth rate of denitrifiers. *Water Science and Technology*, *54*(8), 155–162. https://doi.org/10.2166/wst.2006.854

Mokhayeri, Y., Riffat, R., Murthy, S., Bailey, W., Takacs, I., & Bott, C. (2009). Balancing yield, kinetics and cost for three external carbon sources used for suspended growth post-denitrification. *Water Science and Technology*, *60*(10), 2485–2491. https://doi.org/10.2166/wst.2009.623

Moralejo-Gárate, H., Mar'atusalihat, E., Kleerebezem, R., & van Loosdrecht, M. C. M. (2011). Microbial community engineering for biopolymer production from glycerol. *Applied Microbiology and Biotechnology*, *92*(3), 631–639. https://doi.org/10.1007/s00253-011-3359-3

Niemiec, A., Sharp, R., & Duer, A. (2018). Testing of novel denitratation/deammonification process for mainstream total nitrogen removal. *Proceedings of the Water Environment Federation*, *2018*(5), 157–163.

Obaja, D., MacÉ, S., & Mata-Alvarez, J. (2005). Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. *Bioresource Technology*, *96*(1), 7–14. https://doi.org/10.1016/j.biortech.2004.03.002

Park, H., Brotto, A. C., van Loosdrecht, M. C. M., & Chandran, K. (2017). Discovery and metagenomic analysis of an anammox bacterial enrichment related to Candidatus "Brocadia caroliniensis" in a full-scale glycerol-fed nitritation-denitritation separate centrate treatment process. *Water Research*, *111*, 265–273. https://doi.org/10.1016/j.watres.2017.01.011

Pathak, B. K., Kazama, F., Saiki, Y., & Sumino, T. (2007). Presence and activity of anammox and denitrification process in low ammonium-fed bioreactors. *Bioresource Technology*, *98*(11), 2201–2206. https://doi.org/10.1016/j.biortech.2006.08.014

Poot, V., Hoekstra, M., Geleijnse, M. A. A., van Loosdrecht, M. C. M., & Pérez, J. (2016). Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge. *Water Research*, *106*, 518–530. https://doi.org/10.1016/j.watres.2016.10.028

Porte, F., & Vignais, P. M. (1980). Electron transport chain and energy transduction in Paracoccus denitrificans under autotrophic growth conditions. *Archives of Microbiology*, *127*(1), 1–10. https://doi.org/10.1007/BF00414348

Rahman, A., Meerburg, F. A., Ravadagundhi, S., Wett, B., Jimenez, J., Bott, C., … De Clippeleir, H. (2016). Bioflocculation management through high-rate contact-stabilization: A promising technology to recover organic carbon from low-strength wastewater. *Water Research*, *104*, 485–496. https://doi.org/10.1016/j.watres.2016.08.047

Regmi, P., Miller, M. W., Holgate, B., Bunce, R., Park, H., Chandran, K., … Bott, C. B. (2014). Control of aeration, aerobic SRT and COD input for mainstream nitritation/denitritation. *Water Research*, *57*, 162–171. https://doi.org/10.1016/j.watres.2014.03.035

Sharp, R., Niemiec, A., Khunjar, W., Galst, S., & Deur, A. (2017). Development of a novel deammonification process for cost effective separate centrate and main plant nitrogen removal. *International Journal of Sustainable Development and Planning*, *12*(1), 11–21. https://doi.org/10.2495/SDP-V12-N1-11-21

Stewart, V. (1988). Nitrate respiration in relation to facultative metabolism in enterobacteria. *Microbiological Reviews*, *52*(2), 190. https://doi.org/10.1126/science.1215643

Trojanowicz, K., Plaza, E., & Trela, J. (2016). Pilot scale studies on nitritation-anammox process for mainstream wastewater at low temperature. *Water Science and Technology*, *73*(4), 761–768. https://doi.org/10.2166/wst.2015.551

Uprety, K. (2013). *Evaluation of glycerol and waste alcohol as supplemental carbon sources for denitrification*. Blacksburg, VA: Virginia Tech.

Van Rijn, J., Tal, Y., & Barak, Y. (1996). Influence of volatile fatty acids on nitrite accumulation by a *Pseudomonas stutzeri* strain isolated from a denitrifying fluidized bed reactor. *Applied and Environmental Microbiology*, *62*(7), 2615–2620.

Van Winckel, T., Vlaeminck, S. E., Al-Omari, A., Bachmann, B., Sturm, B., Wett, B., … De Clippeleir, H. (2019). Balancing kinetic and physical selection mechanisms to achieve efficient deammonification. Manuscript submitted for publication.

Vlaeminck, S. E., De Clippeleir, H., & Verstraete, W. (2012). Microbial resource management of one-stage partial nitritation/anammox. *Microbial Biotechnology*, *5*(3), 433–448. https://doi.org/10.1111/j.1751-7915.2012.00341.x

Wett, B. (2007). Development and implementation of a robust deammonification process. *Water Science and Technology*, *56*(7), 81–88. https://doi.org/10.2166/wst.2007.611

Yang, X., Wang, S., & Zhou, L. (2012). Effect of carbon source, C/N ratio, nitrate and dissolved oxygen concentration on nitrite and ammonium production from denitrification process by *Pseudomonas stutzeri* D6. *Bioresource Technology*, *104*, 65–72. https://doi.org/10.1016/j.biortech.2011.10.026

Attachment 5 • Priyanka Ali poster on primary sludge fermentation

NOTE: The attached document is for informational purposes only. It has not been reviewed or endorsed by the NWRI Independent Science Advisory Panel.

THE GEORGE WASHINGTON UNIVERSITY
WASHINGTON, DC

Primary Sludge Fermentation: Sustainable and Economical Process of Supplementing Carbon for Short-Cut Nitrogen Removal

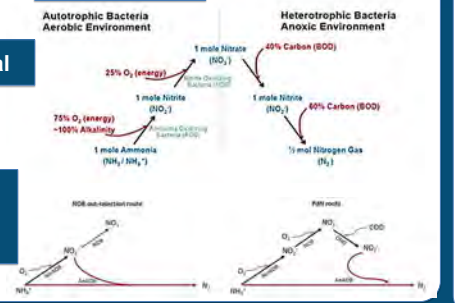
Priyanka Ali, Rumana Riffat and Haydee De Clippeleir

Introduction

Conventional

VS

Short-Cut Nitrogen Removal

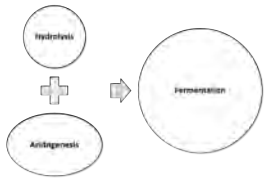


Blue Plains Advanced Wastewater Treatment Plant

- ❑1400 MLD plant
- ❑Stringent limits:
  - TN < 3.8 mg TN/L
  - TP < 0.18 mg P/L
- ❑Conventional BNR system
- ❑Costs per year:
  - **$8M** for methanol dosing
  - $0.6M on alkalinity
  - $1.5M for aeration

Fermentation

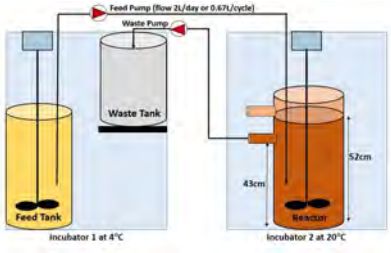
Primary Sludge
↓
Particulate COD
↓
Soluble COD
↓
Volatile Fatty Acid



Objectives

1. Find the SRT needed to reach required soluble COD yields as well as to limit the concurrent nutrient release during fermentation
2. Explore the viability of primary sludge fermentate as the recycled carbon source for selecting PdN in order to reduce chemical costs
3. Analyze the operational cost savings when incorporating primary sludge fermentation in Blue Plains Advanced wastewater treatment plant

Materials and Methods

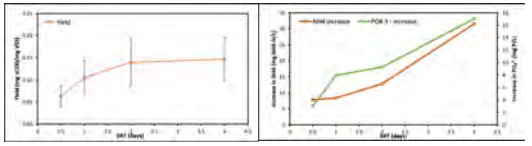


Lab scale CSTR ⟶ Temperature controlled at 20° C

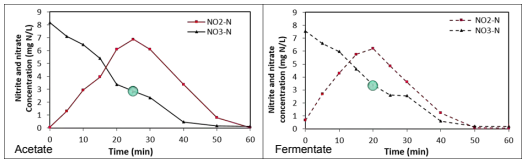
SRT management ⟶ Feeding and wasting primary sludge

Results

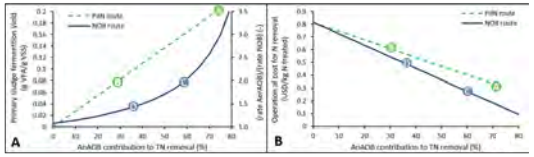
Comparison of yield and nutrient release with SRT



Kinetic test results show feasibility of fermentate to perform PdN with similar PdN set points (2-3mg N/L) like Acetate



Cost comparison coupled with AnAOB contribution for 4 different scenarios



(A) PdN+IFAS (B) PdN+Screen (C) NOB outselection+IFAS (D) NOB outselection+Screen

Conclusions

- ❑2 day SRT is preferable in terms of yield and nutrient added to the system
- ❑**$6.2M** out of $10M (62% of total operational cost) can be saved with PdN-AnAOB route and using fermentate as a carbon source

dc water is life

Attachment 6 • Kyle Brumfield et al., A comparative analysis of drinking water employing metagenomics

NOTE: The attached document is for informational purposes only. It has not been reviewed or endorsed by the NWRI Independent Science Advisory Panel.

A comparative analysis of drinking water employing metagenomics

Article *in* PLoS ONE · April 2020

DOI: 10.1371/journal.pone.0231210

7 authors, including:

Kyle Brumfield
University of Maryland, College Park
6 PUBLICATIONS **8** CITATIONS

PLOS ONE

RESEARCH ARTICLE

A comparative analysis of drinking water employing metagenomics

Kyle D. Brumfield[1,2], **Nur A. Hasan**[2,3], **Menu B. Leddy**[4], **Joseph A. Cotruvo**[5], **Shah M. Rashed**[1,3], **Rita R. Colwell**[1,2,3], **Anwar Huq**[1]*

1 Maryland Pathogen Research Institute, University of Maryland, MD, College Park, United States of America, **2** University of Maryland Institute for Advanced Computer Studies, University of Maryland, College Park, MD, United States of America, **3** CosmosID Inc., Rockville, MD, United States of America, **4** Essential Environmental and Engineering Systems, Huntington Beach, CA, United States of America, **5** Joseph Cotruvo and Associates LLC, Washington, DC, United States of America

* huq@umd.edu

Citation: Brumfield KD, Hasan NA, Leddy MB, Cotruvo JA, Rashed SM, Colwell RR, et al. (2020) A comparative analysis of drinking water employing metagenomics. PLoS ONE 15(4): e0231210. https://doi.org/10.1371/journal.pone.0231210

Editor: Christopher Staley, University of Minnesota Twin Cities, UNITED STATES

Received: October 4, 2019

Accepted: March 18, 2020

Published: April 9, 2020

Data Availability Statement: Ion Torrent metagenomic sequencing data for all samples, after respectively removing P. pnomenusa spiked sequences, are deposited in the NCBI Sequence Read Archive database under BioProject PRJNA575040. Additionally, this project establishes a new NCBI Taxonomy ID for sequencing reads associated with the drinking water metagenome as 2651591.

Funding: Research reported in this publication was funded by the National Science Foundation (NSF, https://nsf.gov) through the EAGER,

Abstract

The microbiological content of drinking water traditionally is determined by employing culture-dependent methods that are unable to detect all microorganisms, especially those that are not culturable. High-throughput sequencing now makes it possible to determine the microbiome of drinking water. Thus, the natural microbiota of water and water distribution systems can now be determined more accurately and analyzed in significantly greater detail, providing comprehensive understanding of the microbial community of drinking water applicable to public health. In this study, shotgun metagenomic analysis was performed to determine the microbiological content of drinking water and to provide a preliminary assessment of tap, drinking fountain, sparkling natural mineral, and non-mineral bottled water. Predominant bacterial species detected were members of the phyla *Actinobacteria* and *Proteobacteria*, notably the genera *Alishewanella*, *Salmonella*, and *Propionibacterium* in non-carbonated non-mineral bottled water, *Methyloversatilis* and *Methylibium* in sparkling natural mineral water, and *Mycobacterium* and *Afipia* in tap and drinking fountain water. Fecal indicator bacteria, i.e., *Escherichia coli* or *enterococci*, were not detected in any samples examined in this study. Bacteriophages and DNA encoding a few virulence-associated factors were detected but determined to be present only at low abundance. Antibiotic resistance markers were detected only at abundance values below our threshold of confidence. DNA of opportunistic plant and animal pathogens was identified in some samples and these included bacteria (*Mycobacterium* spp.), protozoa (*Acanthamoeba mauritaniensis* and *Acanthamoeba palestinensis*), and fungi (*Melampsora pinitorqua* and *Chryosporium queenslandicum*). Archaeal DNA (*Candidatus Nitrosoarchaeum*) was detected only in sparkling natural mineral water. This preliminary study reports the complete microbiome (bacteria, viruses, fungi, and protists) of selected types of drinking water employing whole-genome high-throughput sequencing and bioinformatics. Investigation into activity and function of the organisms detected is in progress.

COLLABORATIVE RESEARCH program under award number 1742869 [NAH, RRC, AH]. Support in the form of partial salaries for authors was provided from NSF to the University of Maryland, College Park, MD [KDB, AH], CosmosID Inc., Rockville, MD [NAH, SMR], Joseph Cotruvo and Associates LLC, Washington, DC [JAC], and Essential Environmental and Engineering Systems, Huntington Beach, CA [MBL]. The funding agency had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of funding agencies. The specific roles of these authors are articulated in the 'author contributions' section.

Competing interests: The authors declare the follow potential conflicts of interest with respect to the research, authorship and/or publication of this article: RRC is the founder of CosmosID, Inc., Rockville, MD and also a Professor at the University of Maryland; NAH and SMR are employees of CosmosID, Inc., Rockville, MD; JAC is the founder of Joseph Cotruvo and Associates LLC, Washington, DC; MBL is an employee of Essential Environmental and Engineering Systems, Huntington Beach, CA. The specific roles of these authors are articulated in the 'author contributions' section. This does not alter our adherence to PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors (https://journals.plos.org/plosone/s/competing-interests).

Introduction

Access to safe drinking water (DW) is considered a fundamental human right, yet it is estimated that globally more than two billion people suffer from a lack of safely managed DW services [1]. During the late nineteenth and early twentieth centuries, major cities in the U.S. adopted filtration and disinfection water treatment methods, significantly reducing mortality rates and incidence of disease associated with contaminated water [2]. Thereafter, waterborne disease outbreaks associated with conventional source water declined. Unfortunately, legionellosis, caused by inhalation of *Legionella* spp. contaminated aerosols from water distribution and plumbing, remains a concern since this disease accounts for roughly 60 percent of reported waterborne disease outbreaks in the U.S. and has emerged recently as a leading cause of reported deaths associated with contaminated water [3,4].

Under the Safe Drinking Water Act of 1974, the United States Environmental Protection Agency (USEPA) regulates public DW supplies. The United States Food and Drug Administration (USFDA), uses USEPA standards as the basis for regulating bottled water (BW) in interstate commerce. State enforcement of public DW standards protect against both naturally occurring and man-made contaminants in water entering a drinking water distribution system (DWDS) from municipal treatment facilities. The U.S. national drinking water Maximum Contaminant Level, under the Revised Total Coliform Rule [5], is less than one fecal coliform per 100 mL of water, in addition to filtration and disinfection requirements that depend upon the source. Certain bacterial and fungal species present in natural source water promote biodegradation of organic and inorganic matter, which can enhance biological stability and lower concentrations of micropollutants [6,7]. Other microorganisms pose potential health concerns. Municipal water treatment facilities eliminate or at least significantly reduce the number of pathogenic microorganisms in finished DW. Thus, municipal water is not expected to be sterile but must be microbially safe.

To limit microbial regrowth in finished DW, disinfectants, e.g., additional free chlorine or monochloramine, are added to the water prior to distribution, therefore residuals should be present in DW if they have not dissipated in transit [8,9]. However, such disinfectant residuals can introduce selective pressure, that may result in communities of disinfectant-resistant microorganisms [10–13]. For example, chlorination has been shown to greatly affect microbial community structure in DWDS [14]. Ridgway and Olson showed a possible selection for chlorine-tolerant microorganisms in chlorinated water as bacteria isolated from a chlorinated DWDS were more resistant to both combined and free forms of chlorine compared to bacteria isolated from an unchlorinated DWDS [13]. Differential resistance to monochloramine in bacterial populations has also been observed in certain genera detected in DWDS, including *Legionella*, *Escherichia*, and *Sphingomonas*, and *Mycobacterium* [12].

Furthermore, regrowth or after growth of microorganisms in treated DW, including BW, can occur [10,15–18]. Uncontrolled growth of bacteria, notably biofilm bacteria, in water mains and premise plumbing during delivery is well documented and can introduce operational issues within distribution systems, resulting in deterioration of color and taste or causing potential human hygiene problems [8,10,17,19,20]. Complex interactions also can develop between microorganisms and their environment and lead to metabolism of biologically available nutrients, particle deposition and sediment re-suspension, appearance of potential inhibitory substances, and biofilm formation. Microbial response to environmental conditions, notably temperature, also can contribute to changes in microbial water quality during distribution [8].

Thus, a major challenge is being able to measure the total microbial content of water accurately [21]. Traditionally, it has been assumed that indicator microorganisms provide adequate

assurance for the microbial safety of water. Culture-dependent methods are used to detect and enumerate indicator organisms and have been remarkably successful in improving DW quality and safety, but do not detect all microorganisms present in that water. Metagenomic analysis employing high-throughput sequencing coupled with bioinformatics has gained attention during the past decade, allowing detection, identification, and characterization of all microorganisms present in DWDS [22–24]. Inferences of infectious potential of detected microbial species is determined by detecting genes coding for pathogenic and metabolic properties [24,25]. Thus, bacteria, viruses, fungi, and protists now can be detected, identified to sub-species level, and characterized. A significant benefit is detection of microorganisms in water that were previously missed or not identified by culture-dependent methods [22,26,27]. Metagenomic surveys carried out by other investigators have provided evidence that ingested diet-borne components can have short- and long-term effects on the human microbiota [28–31]. Only a few studies have used high-throughput sequencing to analyze DWDS, and the complete microbiome of finished drinking waters is vastly understudied.

This preliminary investigation is the first to use detailed and highly sensitive shotgun metagenomic high-throughput sequence analysis to identify components of microbial communities in order to describe the microbiome of DW. Metagenomic analysis of DW samples collected from a municipal tap, public drinking fountain, and BW, including sparkling natural mineral, spring, artesian, and reprocessed tap was employed. The relative abundance of bacteria, fungi, protists, bacteriophages, and virulence-associated factors was determined to provide an initial metagenomic survey of the total microbial content, including microorganisms in the viable but non-culturable state.

Materials and methods

Sample collection and preparation

DW samples collected in this study, including label, water type, source, collection date, production date, best-before date, storage container characteristics, major treatment steps prior to bottling, total and free residual chlorine concentrations, and volume of water analyzed, are described in **Table 1**. While the date and time of municipal tap (sample E) and drinking fountain (sample F) water samples leaving the water treatment plant (WTP) is not known, date and site of collection are provided. To analyze the DW microbiome and reduce the effect of premise plumbing, municipal tap and drinking fountain water samples (E and F) were collected after flushing the source water faucets. The municipal tap water faucet was flushed for 10 min and drinking fountain water faucet for 20 min prior to collecting 40 L of water in sterile Nalgene carboys (Thermo Fisher Scientific, Waltham, MA, USA) treated previously with hydrochloric acid (10% v/v), ethanol (95% v/v), and autoclaved. The drinking fountain water sample F was collected from a non-filtered, non-refrigerated, stainless steel Halsey Taylor OVL-II E Single Fountain (Halsey Taylor, Oak Brook, IL, USA). Tap and drinking fountain water samples were transported to the laboratory in a cooler box with ice and processed within one hour of collection to prevent growth, which would result in changes to the microbial community composition. Municipal tap and drinking fountain water samples were collected from the same location in Maryland, third floor of a building containing copper plumbing and approximately four miles from the municipal WTP supplying water to this location. The WTP employs free chlorine to disinfect water. Free chlorine concentrations in the water leaving the WTP during the sampling periods were reported by the WTP (5/9/2018 = 1.8 mg/L; 5/10/2018 = 1.9 mg/L; 5/11/2018 = mg/L; 6/21/2018 = 2.4 mg/L; 6/22/2018 = 2.5 mg/L; 6/23/2018 = 2.4 mg/L). Dates of purchase of sparkling natural mineral BW (sample A) and three non-mineral BW samples, including spring (sample B), artesian (sample C), and reprocessed

Table 1. Drinking water samples included in the study.

Sample	Water Type	Source	Collection Date (M/D/Y)	Production Date (M/D/Y)	Best-Before Date (M/D/Y)	Storage Container (Color/Material)	Major Treatment Steps Prior to Bottling	Total Residual Chlorine (mg/L)	Free Residual Chlorine (mg/L)	Volume Analyzed (L)
A	Bottled Sparkling Natural Mineral Water	Commercial	6/24/2018	3/15/2018	3/15/2021	Green/Glass	Injection of natural CO_2			20
B	Bottled Spring Water	Commercial	5/11/2018	6/4/2018	12/31/2019	Clear/Plastic	Microfiltration; ultraviolet light and/or ozone disinfection			40
C	Bottled Artesian Water	Commercial	5/14/2018	1/15/2018	1/15/2020	Clear/Plastic	Microfiltration; ultraviolet light			9
D	Bottled Reprocessed Tap Water	Commercial	6/28/2018	5/30/2018	5/27/2019	Clear/Plastic	Reverse osmosis, ultraviolet light; ozone disinfection			40
E	Municipal Tap Water	Maryland, USA[a]	6/25/2018					0.78	0.65	40
F	Public Drinking Fountain Water	Maryland, USA[a]	5/16/2018					0.95[b]	0.775[b]	10

Respective sample label, water type, source, collection date, production date, best-before date, storage container characteristics, major treatment steps prior to bottling, total residual chlorine concentration, total free residual chlorine concentration, and volume of water concentrated are given.

[a] Municipal tap and drinking fountain DW samples (E and F) were collected from the same location in Maryland.

[b] Values represent an average of the total and free residual chlorine concentrations, respectively, collected on 5/7/2018 and 5/14/2018 from tap water near the sampling location.

https://doi.org/10.1371/journal.pone.0231210.t001

tap (sample D) water types are also provided. Different brands of BW (samples A-D) were selected for study as our intent was to obtain a generalized knowledge of the DW microbiome. The brands selected did not disclose the exact source of their bottled waters. All BW samples were stored unrefrigerated until time of purchase. BW samples were stored at room temperature (23˚C– 25˚C) out of direct sunlight for up to one week after purchase since it was not possible to process all samples at the same time. BW brands in the interstate commerce are required to adhere to the standard of quality set by the USEPA, which requires a total residual chlorine concentration of less than 4 mg/L in finished DW [32]. Across BW samples selected for this study, the annual bottled water quality reports provided from each respective brand measured the total residual chlorine concentrations of water prior to bottling, and across all samples, the chlorine levels were below the minimum reporting limit set by the USFDA of 0.1 mg/L. Residual disinfectant was measured using a Pocket Colorimeter™ II portable colorimeter (Hach, Loveland, CO, USA) in tap water from a building neighboring the sampling location through a shared distribution system on 5/7/2018 (total = 0.99 mg/L; free = 0.86 mg/L), 5/14/2018 (total = 0.91 mg/L; free = 0.69 mg/L), and 6/25/2018 (total = 0.78 mg/L; free = 0.65 mg/L). To represent the sampling event on May 11, 2018, the average of the total and free residual chlorine levels of May 7 and May 14 were taken (**Table 1**). As residual chlorine can cause complications during high-throughput sequencing, Safe Dchlor T20 sodium thiosulfate 20 mg tablets (Brim Technologies Inc., Randolph, NJ, USA) tablets were added to tap and drinking fountain water samples, per manufacturer's specifications for dechlorination.

All DW samples were concentrated by stepwise vacuum filtration at room temperature (23˚C– 25˚C) in sterile glass filtration units treated previously with hydrochloric acid (10% v/v), ethanol (95% v/v), and autoclaved. For each concentration, a total of 10 filters were used. Samples were passed through two 0.6 μm pore size polycarbonate Whatman Nuclepore Track-

Etch Membranes (Millipore Sigma, St. Louis, MO, USA) which trapped trace minerals and expedited downstream filtration. The filtrate was aseptically collected and consecutively passed through two 0.2 μm and six 0.1 μm pore size polycarbonate Whatman Nuclepore Track-Etch Membranes (Millipore Sigma, St. Louis, MO, USA). However, because each water type contained a variable mineral content, the volume of water filtered was dependent on whether the filter clogged. Accordingly, the volume of water analyzed from each sample before the membrane filters clogged can be found in **Table 1**. The total filtrate passed through the two 0.6 μm filter membranes was subsequently processed as described. The 10 filter membranes for each sample were stored at -80˚C until DNA preparation.

Heterotrophic bacterial enumeration

Heterotrophic plate counts (HPC) of total bacteria were performed for BW (samples A-D) and, prior to dechlorination, tap water (sample E) and drinking fountain water (sample F), by direct and diluted (1/10 and 1/100) spread plating on BD Difco™ R2A Agar (Fisher Scientific, Hampton, NH, USA), as previously described [33]. Incubation was at 24˚C, and colonies were counted every 24 hours, for seven days to determine the HPC.

DNA extraction and whole genome shotgun sequencing

Total DNA was isolated from the microbial biomass collected on all 10 filter membranes for each sample, using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA), with the following modifications for DNA extraction from filter membranes. The 10 filter membranes for each sample were cut into ribbons approximately 2 mm by 10 mm and evenly distributed amongst five ZymoBIOMICS™ Lysis Tubes, included in the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Final elution volume for each of the five preparations was 20 μl, and eluted DNA was pooled for each sample to 100 μl, respectively. DNA was purified using DNA Clean and Concentrator™-25 Kit (Zymo Research, Irvine, CA, USA), following manufacturer's instructions, with final elution volume of 50 μl.

Concentration of genomic dsDNA was measured using Qubit® dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on an Invitrogen Qubit® 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), which has a dsDNA quantification range of between 0.2 ng and 100 ng. Sparkling natural mineral BW and municipal tap and drinking fountain water samples yielded between 0.524–76.6 ng/μl of dsDNA (**Table 2**). However, dsDNA concentrations of spring, artesian, and reprocessed tap BW (samples B, C, and D) were below the limit of detection. To ensure sufficient genomic material was present in each sample that was required for subsequent library construction, 6.0 ng of *Pandoraea pnomenusa* KWW5 genomic DNA was added to samples B, C, and D. *P. pnomenusa*, serving as reference, is a Gram-negative bacterium of the family *Burkholderiaceae* and is frequently isolated from sputum of cystic fibrosis patients [34] and not expected to be present in finished DW in the USA. Genomic DNA used for spiking was prepared from pure cultures grown under standard conditions in BD Difco™ LB Broth, Miller (Luria-Bertani broth; Fisher Scientific, Hampton, NH, USA), with aeration at 30˚C overnight (16 hours) using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA), following manufacturer's instructions.

Genomic DNA libraries were constructed from the metagenomic samples and purified *P. pnomenusa* KWW5 genomic DNA, using the Thermo Fisher IonXpress Plus Fragment Library kit (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer's instructions, with slight modifications for low-input DNA. Metagenomic and *P. pnomenusa* KWW5 DNA libraries were enriched and barcoded using the IonXpress Barcode Adapter Kit (Thermo

Table 2. DNA Concentrations and sequencing statistics for samples included in the study as measured by FastQC.

Sample	Water Type	DNA Concentration (ng/μl)	Duplicate Reads (%)	Average GC Content (%)	Average Sequence Length (bp)	Total Sequences (Millions)
A	Bottled Sparkling Natural Mineral Water	0.524	38.0%	48.0%	186	10.5
B[a]	Bottled Spring Water	BDL	44.8%	63%	171	4.8
C[a]	Bottled Artesian Water	BDL	42.0%	63%	172	5.6
D[a]	Bottled Reprocessed Tap Water	BDL	54.2%	64%	187	30.0
E	Municipal Tap Water	76.6	27.4%	60%	146	19.9
F	Public Drinking Fountain Water	1.85	32.7%	54%	166	20.9
P. pnomenusa KWW5		68.0	38.7%	65%	153	6.7

[a] Metagenomic reads contain sequence from *Pandoraea pnomenusa KWW5;* after removing spiked reads, 0.3, 0.1, and 0.4 million total sequences reads remained for samples B, C, and D, respectively.

BDL, below detection limit.

https://doi.org/10.1371/journal.pone.0231210.t002

Fisher Scientific, Waltham, MA, USA) and 13 cycles of PCR amplification, following manufacturer's instructions. Resulting PCR products were purified using SPRIselect Reagent (Beckman Coulter, Indianapolis, IN, USA), following manufacturer's user guide for next-generation library construction, and eluted in 25 μl low Tris-EDTA (TE) buffer (Thermo Fisher Scientific, Waltham, MA, USA). Final libraries were quantified by qPCR using the Ion Library TaqMan fQuantification Kit (Thermo Fisher Scientific, Waltham, MA, USA), which targets adapter sequences on each Ion Torrent library fragment. Sequencing was performed on an Ion S5 XL Semiconductor Sequencer (Ion Torrent, Thermo Fisher Scientific, Waltham, MA, USA) to generate 200 bp sequence reads, following manufacturer's instructions. Operations and quality control associated with high-throughput sequencing, including a negative sequencing control, consisting of nuclease-free water, and a sequencing standard, i.e., ZymoBIOMICS^{TM} Microbial Community Standard (Zymo Research, Irvine, CA, USA), were done at CosmosID Inc. (CosmosID Inc., Rockville, MD, USA). Metagenomic samples were sequenced with an average of 1.5 x 10^7 (min = 4.8 x 10^6; max = 3.0 x 10^7) sequence read depth across samples (**Table 2**). The low DNA input observed in BW samples B and C resulted in a slightly lower number of reads, 4.8 x 10^6 and 5.6 x 10^6, respectively, compared to the number of reads observed in samples A, D, E, and F and that employed in similar metagenomic investigations employing Ion Torrent chemistry [26].

Metagenomic sequencing analyses

General sequencing statistics for all samples and mean sequence quality distribution, as measured by FastQC (v.0.11.6) [35], are detailed in **Table 2**. Base-calling error probabilities (P) were evaluated using Phred Quality Score (Q), defined by: $Q = -10log_{10}(P)$. Residual primer and adapter content were trimmed using the Joint Genome Institute Bestus Bioinformatics Decontamination Using Kmers (BBDuk) tool (v.38.07) [36] with a previously defined read quality trimming threshold [26]. Reads were trimmed from both ends until the mean quality value across each base position in the reads for all sample read libraries were above a Phred Quality Score of 17 for at least 80% of the read lengths, i.e., probability of correct base call was at least 98%. After quality trimming, the average Ion Torrent sequencing read lengths across libraries were between 146 bp and 186 bp.

To remove spiked *P. pnomenusa* KWW5 sequences from the metagenomic sample read libraries, the single *P. pnomenusa* KWW5 read library was assembled using the St. Petersburg genome assembler (SPAdes) software (v.3.12.0) [37] and options '—iontorrent', required when assembling Ion Torrent data, '—s', to specify a single read library, '—careful', to reduce the number of misassemblies, and '—cov-cutoff auto', to remove potentially mis-assembled low coverage contigs. The Translated Basic Local Alignment Search Tool (TBLASTX) was used to search the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) genome database using the largest contig (450,794 bp) from the KWW5 assembly as query sequence against *P. pnomenusa* published genomes. A subject database was built locally from the top five genome nucleotide sequences identified (GenBank Accession Numbers: CP015371.1, CP009553.3, CP006900.2, CP006938.2, CP007506.3) and the KWW5 draft assembly (94 contigs, scaffold sequence total = 5.504×10^6 bp, $L_{50} = 2.774 \times 10^5$ bp). Raw metagenomic sample reads were mapped to the local *P. pnomenusa* database using the Burrows-Wheeler Aligner Maximal Exact Match (BWA-MEM) algorithm with default parameters from the Burrows-Wheeler Alignment Tool (v.0.7.17-r1188) [38]. Mapped reads were removed from the read libraries using SEQTK (v.1.3-r106) [39]. Successful removal of *Pandoraea pnomenusa KWW5* genomic sequences was confirmed by mapping the unmapped read datasets against the local *P. pneomenusa* database as previously mentioned. A further quality assurance was performed by manually inspecting the total list of detected organisms following subsequent metagenomic analysis for incidence of the genus *Pandoraea*—which was not detected.

Unassembled metagenomic sequencing reads, with *P. pnomenusa* sequences removed, were analyzed as previously described [26,40–42] using the CosmosID Metagenomics Cloud Application [43] to achieve microbial identification to species, subspecies, and/or strain level and quantification of microorganism relative abundance. Analogously, antibiotic and virulence-associated genes present in each sample were identified by querying unassembled sequence reads against GenBook®, a proprietary series of extensive databases curated by CosmosID Inc. (CosmosID Inc., Rockville, MD, USA). Briefly, the platform uses a data-mining k-mer algorithm to disambiguate sequencing reads into the discrete genomes or genes comprising the particular sequences. The GenBook® databases are composed of over 150,000 microbial genomes and gene sequences representing over 15,000 bacterial, 5,000 viral, 250 protozoan, and 1,500 fungal species, as well as over 5,500 antibiotic resistant and virulence-associated genes. All metagenomic analyses were performed using a filtered dataset with default parameters of the CosmosID Metagenomics Cloud Application [44].

Relative abundance of bacterial taxa in each sample was used for principal coordinate analysis (PCoA), employing Bray-Curtis distance measure [45]. Analysis of community virulome and virome was achieved by identifying virulence genes and viruses based on percent coverage as a function of gene-specific k-mer frequency in each sample. Sunburst visualizations and a heatmap of organism specific k-mer relative abundance (percentage) for each sample, were generated using Krona [46] and Morpheus [47], respectively. All datasets used to generate sunburst visualizations and heatmap were normalized by reducing the total list of detected microbial species less than 0.5% relative abundance in each sample to represent 'other' microorganisms. *Acidovorax* spp. NO-1 (GenBank Accession Number: HM357240.1) was detected in sample A and *Plasmodium falciparum* FCC-2/Hainan (GenBank Accession Number: ABGW00000000.1) was detected in sample E. Following quality control, *Acidovorax* spp. NO-1 was removed from the list of detected microorganisms in sample A, but *Plasmodium falciparum* FCC-2/Hainan was not removed from the list of detected microorganisms in sample E because the detected relative abundance was less than 0.5% and was included as 'other' microorganisms.

Results

Total bacterial culture count

HPC performed employing R2A medium yielded growth for artesian BW at a concentration of 1.92 x 10^4 CFU/mL, after incubation for 96 hr at 24˚C. The other BW samples did not yield growth, even after incubation for up to seven days at 24˚C. The abundance of total heterotrophic bacteria for municipal tap water and drinking fountain water was 7.3 x 10^4 CFU/mL and 7.8 x 10^3 CFU/mL, respectively, after incubation for 72 hr at 24˚C.

Metagenomics of drinking water samples

A total of six DW samples were collected in this study including municipal tap water, water from a drinking fountain, sparkling natural mineral BW, and three non-mineral BW samples. Volumes of up to 40 L of water were analyzed and sources and descriptions of each sample are provided in **Table 1**. Shotgun metagenomic sequencing, using total DNA prepared from the six DW samples, generated approximately 9.84 x 10^7 reads across the raw sequence libraries. The spiked *Pandoraea pnomenusa KWW5* genomic sequences were removed from the sequencing libraries of spring, artesian, and reprocessed tap BW samples, yielding 5.88 x 10^7 high-quality metagenomic sequences, with number of sequencing reads between samples ranging from 1 x 10^5 reads in artesian BW (sample C) to 2.09 x 10^7 reads in drinking fountain water (sample F) (**Table 2**).

Core bacterial communities of sparkling natural mineral BW, non-mineral BW (samples B, C and D), municipal tap water, and drinking fountain water were analyzed by three-dimensional Principal Coordinates Analysis (PCoA) using the Bray-Curtis dissimilarity index (**Fig 1**), where distance between points indicates degree of difference in bacterial DNA sequence composition. That is, points clustered more closely have similar microbiome composition. Each water type contained a relatively distinct bacterial composition across samples examined in this study. Non-mineral BW samples treated by microfiltration or reverse osmosis (samples B, C, and D) clustered together. Municipal tap water and drinking fountain water clustered more closely, compared to the other samples. Sparkling natural mineral BW (sample A) contained a bacterial composition unlike that of the other DW samples.

Relative abundance of bacterial species in each DW sample was analyzed by principal coordinate analysis using Bray-Curtis distance measure. Distance between points indicates degree of difference in bacterial DNA sequence composition, ranging from zero (samples share the same species abundances) to one (samples contain completely different species abundances). The percent of variation explained by each axis is indicated. Black circles are used to demonstrate distinct clustering observed across water types, i.e., municipal tap water clustered with drinking fountain water and the non-mineral bottled water samples clustered together, respectively. Blue square: sparkling natural mineral water, sample A; green circle: bottled non-mineral water, samples B, C, and D; red star: municipal tap water, sample E; yellow triangle: public drinking fountain water, sample F.

Bacteria, archaea, fungi, and protozoa identified by DNA characterization are shown in Krona plots, representing relative abundance of microbial species detected in sparkling natural mineral BW (Fig 2), non-mineral BW, showing gamma-diversity, i.e., total species diversity, among spring, artesian, and reprocessed tap water (Fig 3), municipal tap water (Fig 4), and public drinking fountain water (Fig 5). Interactive Krona plots used to generate Figs 2–5 are available in the Supporting Information (S1 File). The heatmap in Fig 6 depicts relative abundance of microbial species detected in all DW samples.

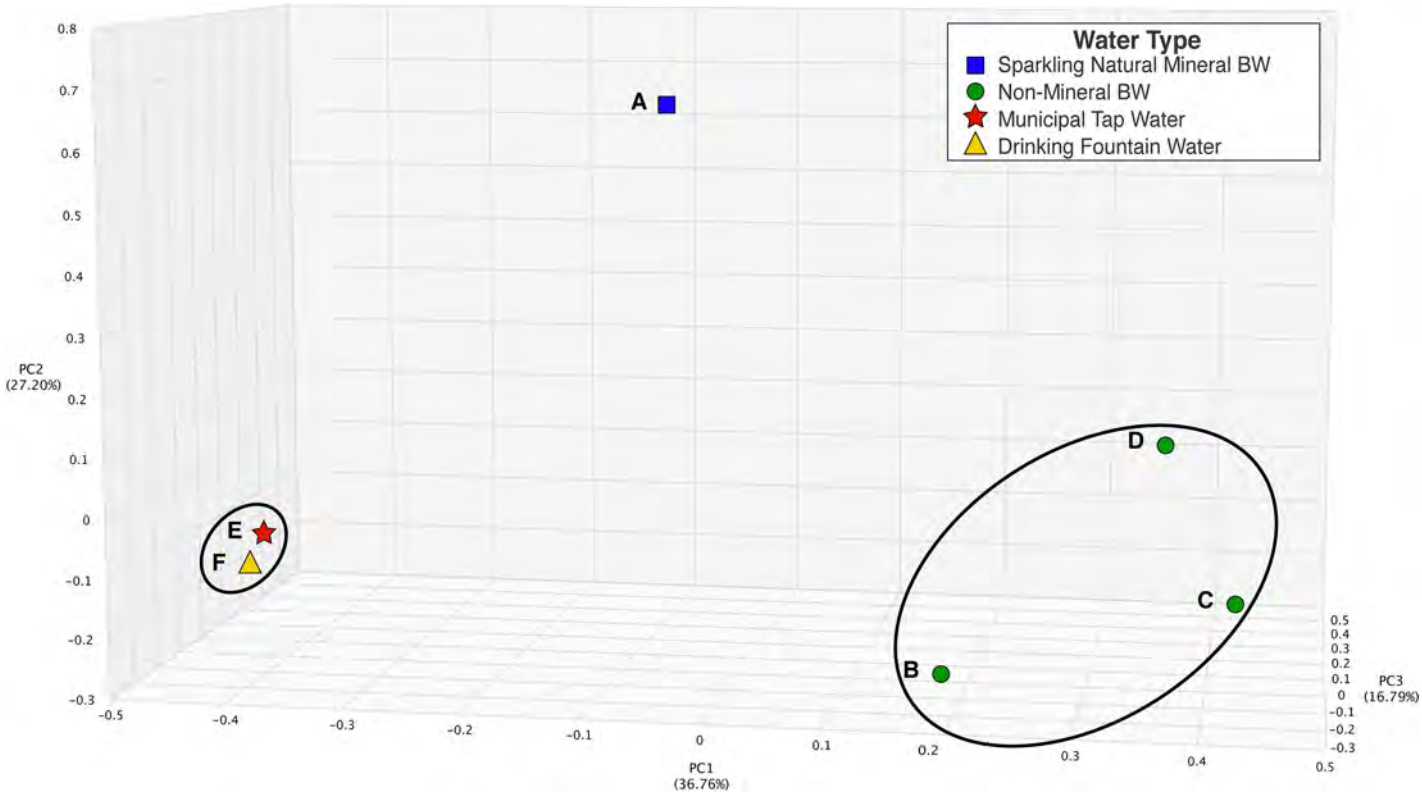


Fig 1. Principal coordinate analysis of bacterial communities in drinking water microbiomes.

https://doi.org/10.1371/journal.pone.0231210.g001

Dominant bacterial phyla detected include Gram-positive *Actinobacteria* and Gram-negative *Proteobacteria* in all samples. The majority of the *Alphaproteobacteria* was detected in municipal tap and drinking fountain water (Fig 4 and Fig 5), *Betaproteobacteria* in sparkling natural mineral BW (Fig 2), and *Gammaproteobacteria* in other types of non-mineral BW (Fig 3). *Deltaproteobacteria* were not detected. *Rhizobiales* were common to both municipal tap and drinking fountain water (Fig 4 and Fig 5). *Burkholderiales* were dominant in sparkling natural mineral BW (Fig 2) and *Alteromonadales* and *Enterobacteriales* in other types of non-mineral BW (Fig 3). *Propionibacterium* was detected in both sparkling natural mineral BW (Fig 2) and non-mineral BW (Fig 3).

Afipia birgiae and *Novosphingobium subterraneum* accounted for 24% and 13% of the relative sequencing read abundance, respectively, in municipal tap water. *Methylobacterium* spp. were detected in in both municipal tap and drinking fountain water, while *Sphingobium* spp. were unique to municipal tap water. *Mycobacterium* spp. were dominant in drinking fountain water, at 74% relative abundance (Fig 5), and also detected in spring BW and municipal tap water (Fig 6).

Opportunistic *Mycobacterium* spp. detected were primarily plant and animal pathogens, with *M. kansasii* the most abundant non-tuberculosis mycobacterium (NTM), detected at 68% of the total sequencing read abundance in drinking fountain water and lower abundance in municipal tap water and spring BW at 7% and 18%, respectively. *Mycobacterium intracellulare* was detected in municipal tap and drinking fountain water, and *Mycobacterium avium* and *Mycobacterium indicus pranii* in drinking fountain water, and *Mycobacterium parascrofulaceum*, an NTM and non-MAC (*Mycobacterium avium* Complex) organism, in both tap and drinking fountain water.

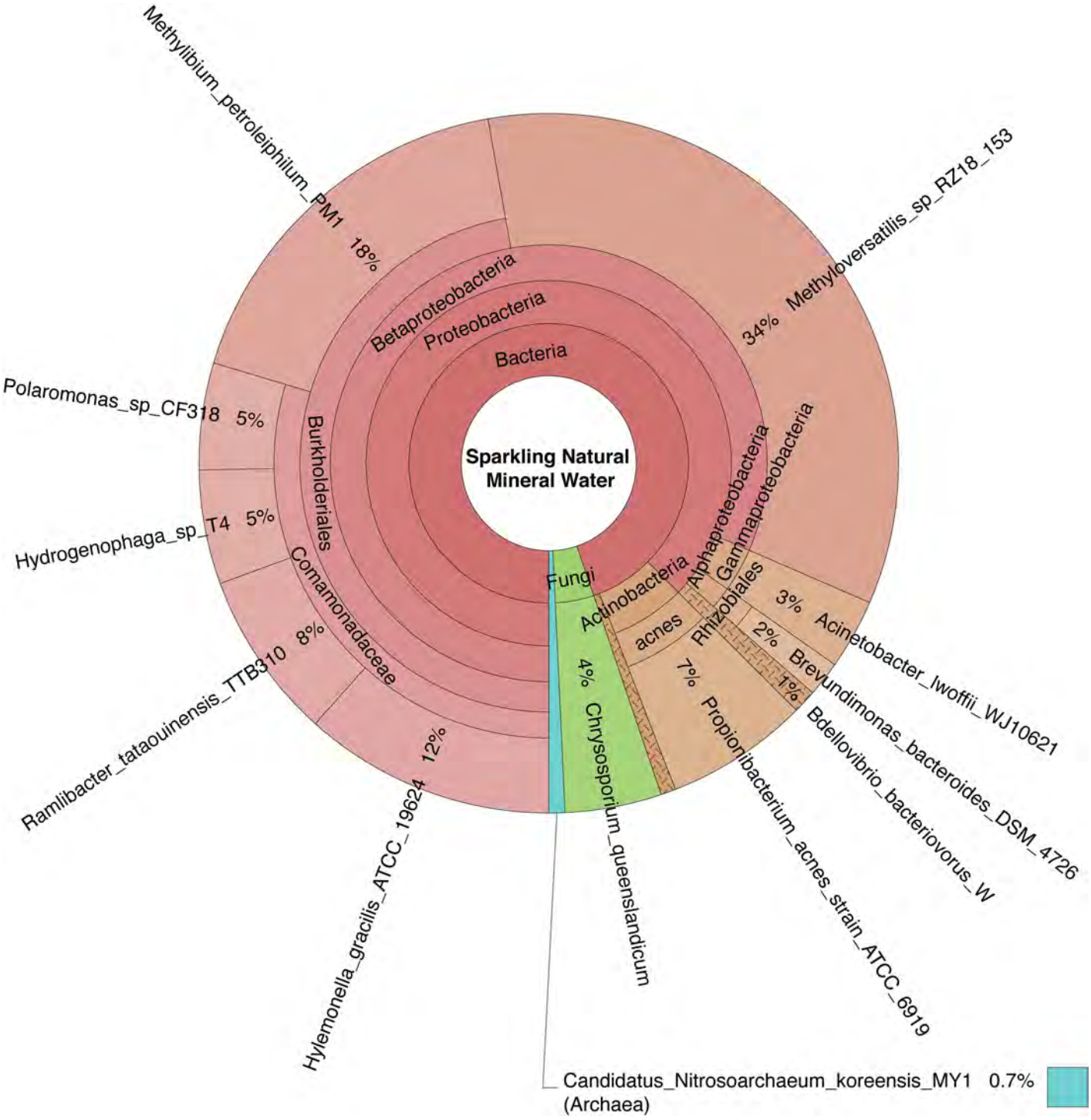


Fig 2. Krona plot of bottled sparkling natural mineral water microbiome. Species composition percentages are displayed as the normalized proportion of organism specific k-mers observed relative to the total microbial species diversity detected in the sample. Red, bacteria; green, fungi; purple, protozoa; teal, archaea.

https://doi.org/10.1371/journal.pone.0231210.g002

BW samples showed less species richness and diversity than tap and drinking fountain water samples, with fewer bacterial species detected. *Bradyrhizobium japonicum*, *Mycobacterium kansasii*, *and Afipia birgiae* were detected in spring BW and municipal and drinking

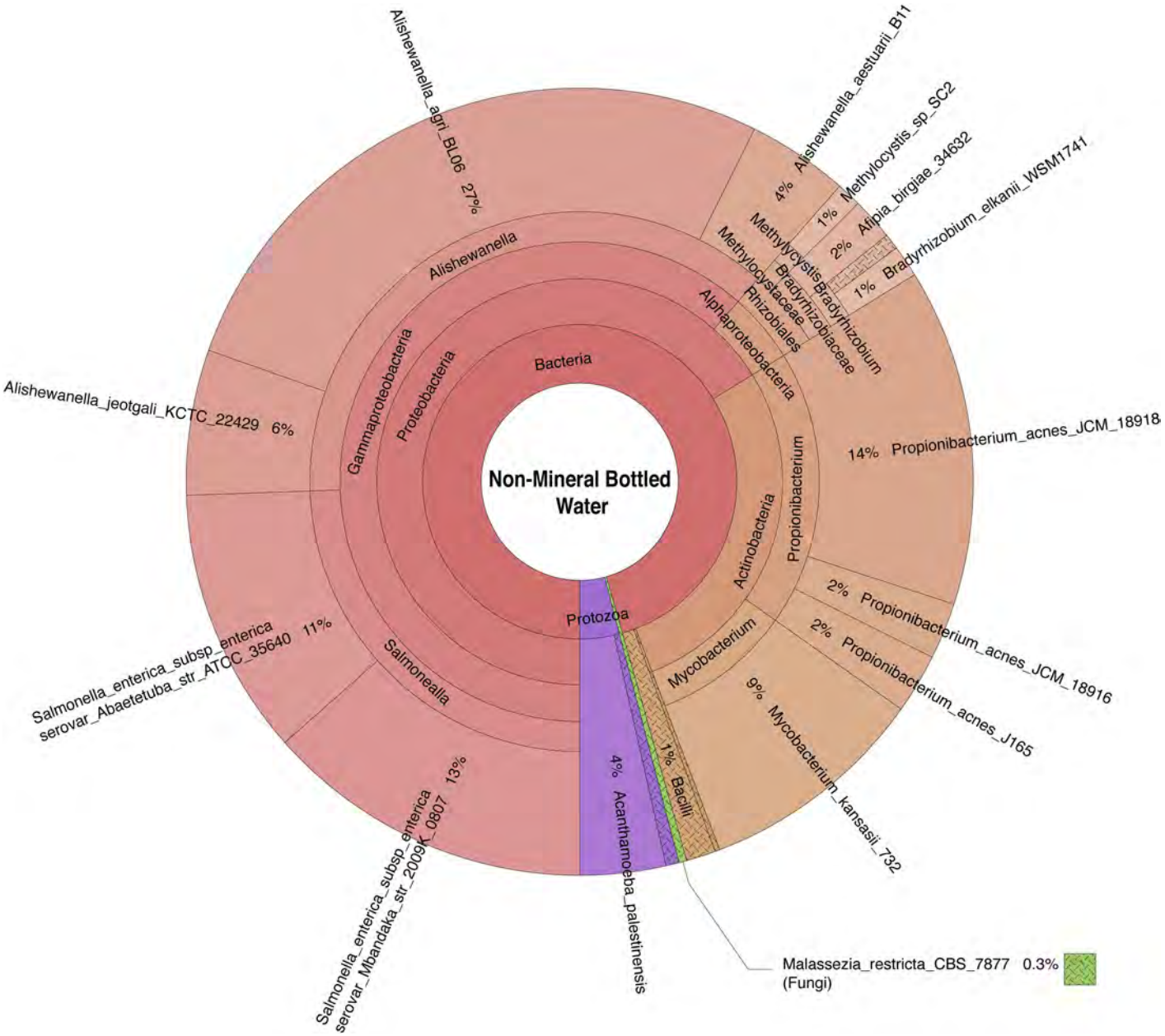


Fig 3. Krona plot of normalized bottled non-mineral water, including spring, artesian, and reprocessed tap water sample microbiomes. Species composition percentages are displayed as average number of organism specific k-mers detected, normalized to represent the proportion of organism specific k-mers observed relative to total microbial species diversity detected. Red, bacteria; green, fungi; purple, protozoa; teal, archaea.

https://doi.org/10.1371/journal.pone.0231210.g003

fountain water. *Methylocystis* spp. were detected in spring BW and drinking fountain water (Fig 6). Different strains of *Propionibacterium acnes* were detected in each of the BW samples but not in sparkling natural mineral BW or municipal tap and drinking fountain water (Fig 6). *Alishewanella* spp. were most common in artesian BW but also detected in spring BW (Fig 6). *Salmonella enterica* subspp. *enterica* serovars Abaetetuba and Mbandaka and were detected in spring BW and reprocessed tap BW, respectively (Fig 6). As *Salmonella enterica* subspp. *enterica* are important opportunistic bacteria, further validation of these strain calls was performed by visualizing read coverage of *Salmonella enterica* subspp. *enterica* serovar Mbandaka str.

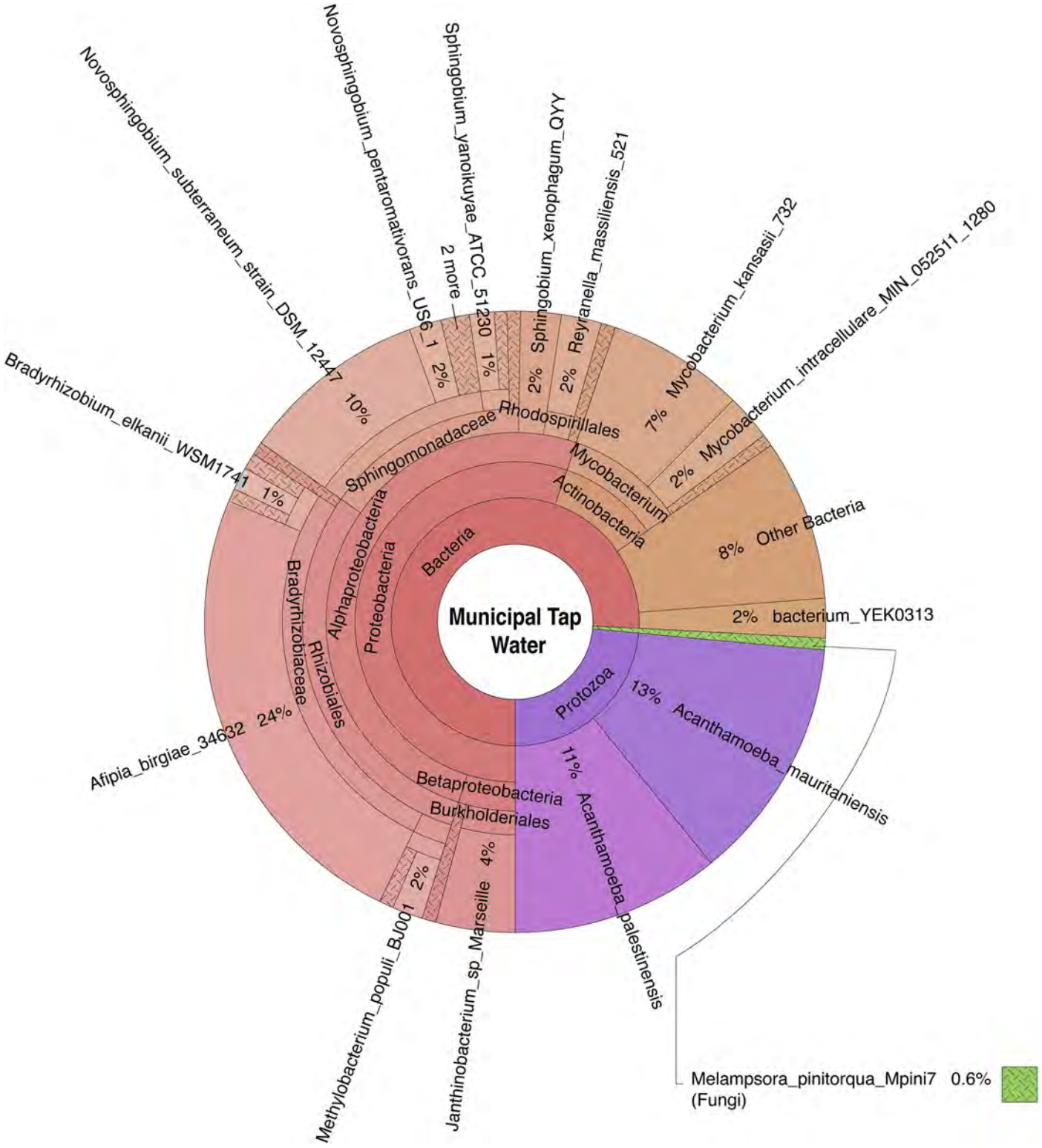


Fig 4. Krona plot of municipal tap water microbiome. Species composition percentages are displayed as the normalized proportion of organism specific k-mers observed relative to total microbial species diversity detected in the sample. Red, bacteria; green, fungi; purple, protozoa; teal, archaea.

https://doi.org/10.1371/journal.pone.0231210.g004

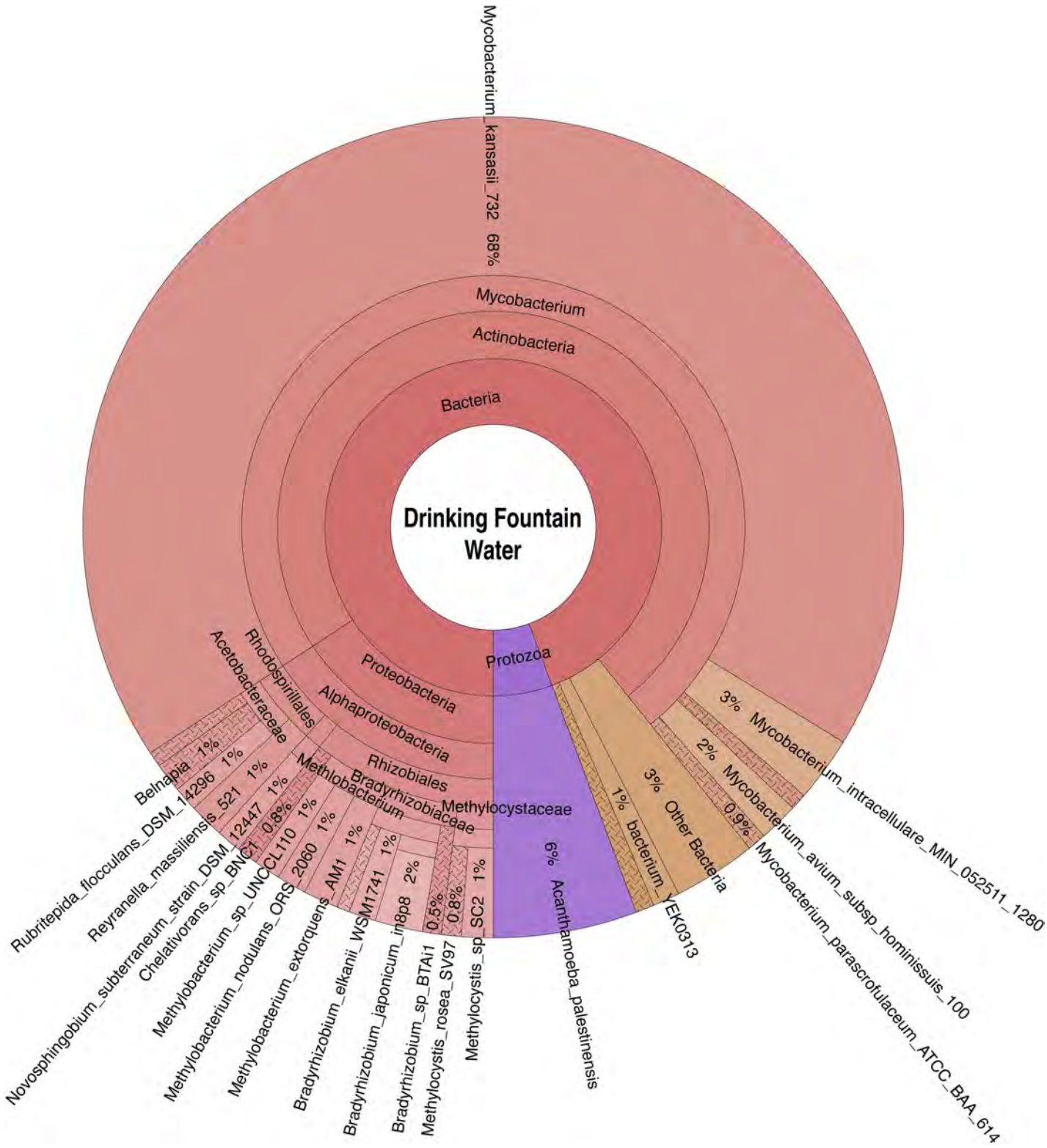


Fig 5. Krona plot of public drinking fountain water microbiome. Species composition percentages are displayed as the normalized proportion of organism specific k-mers observed relative to the total microbial species diversity detected in the sample. Red, bacteria; green, fungi; purple, protozoa; teal, archaea.

https://doi.org/10.1371/journal.pone.0231210.g005

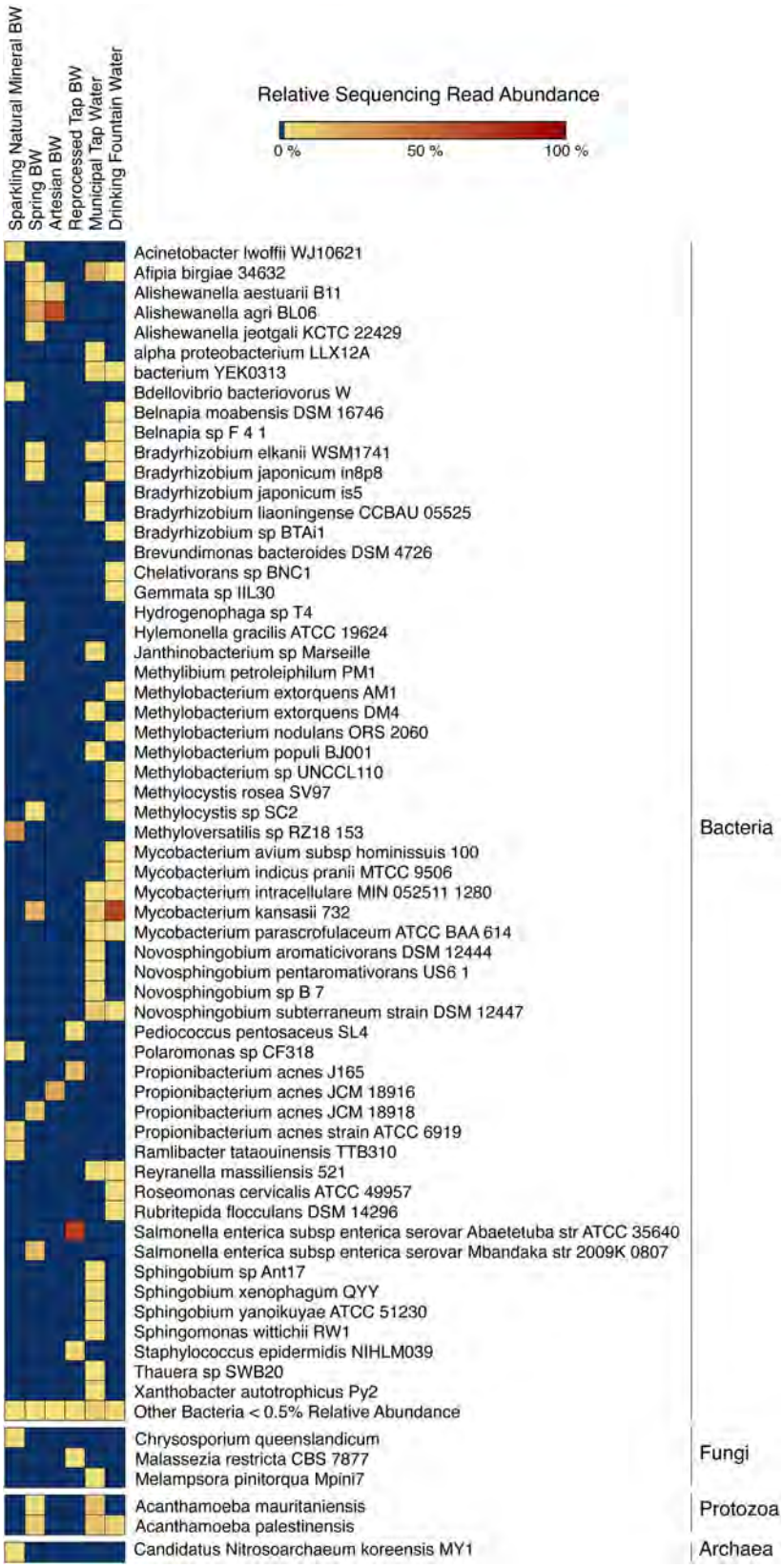

Sparkling Natural Mineral BW
Spring BW
Artesian BW
Reprocessed Tap BW
Municipal Tap Water
Drinking Fountain Water
Relative Sequencing Read Abundance
0 %
50 %
100 %
Acinetobacter lwoffii WJ10621
Afipia birgiae 34632
Alishewanella aestuarii B11
Alishewanella agri BL06
Alishewanella jeotgali KCTC 22429
alpha proteobacterium LLX12A
bacterium YEK0313
Bdellovibrio bacteriovorus W
Belnapia moabensis DSM 16746
Belnapia sp F 4 1
Bradyrhizobium elkanii WSM1741
Bradyrhizobium japonicum in8p8
Bradyrhizobium japonicum is5
Bradyrhizobium liaoningense CCBAU 05525
Bradyrhizobium sp BTAi1
Brevundimonas bacteroides DSM 4726
Chelativorans sp BNC1
Gemmata sp IIL30
Hydrogenophaga sp T4
Hylemonella gracilis ATCC 19624
Janthinobacterium sp Marseille
Methylibium petroleiphilum PM1
Methylobacterium extorquens AM1
Methylobacterium extorquens DM4
Methylobacterium nodulans ORS 2060
Methylobacterium populi BJ001
Methylobacterium sp UNCCL110
Methylocystis rosea SV97
Methylocystis sp SC2
Methyloversatilis sp RZ18 153
Mycobacterium avium subsp hominissuis 100
Mycobacterium indicus pranii MTCC 9506
Mycobacterium intracellulare MIN 052511 1280
Mycobacterium kansasii 732
Mycobacterium parascrofulaceum ATCC BAA 614
Novosphingobium aromaticivorans DSM 12444
Novosphingobium pentaromativorans US6 1
Novosphingobium sp B 7
Novosphingobium subterraneum strain DSM 12447
Pediococcus pentosaceus SL4
Polaromonas sp CF318
Propionibacterium acnes J165
Propionibacterium acnes JCM 18916
Propionibacterium acnes JCM 18918
Propionibacterium acnes strain ATCC 6919
Ramlibacter tataouinensis TTB310
Reyranella massiliensis 521
Roseomonas cervicalis ATCC 49957
Rubritepida flocculans DSM 14296
Salmonella enterica subsp enterica serovar Abaetetuba str ATCC 35640
Salmonella enterica subsp enterica serovar Mbandaka str 2009K 0807
Sphingobium sp Ant17
Sphingobium xenophagum QYY
Sphingobium yanoikuyae ATCC 51230
Sphingomonas wittichii RW1
Staphylococcus epidermidis NIHLM039
Thauera sp SWB20
Xanthobacter autotrophicus Py2
Other Bacteria < 0.5% Relative Abundance
Chrysosporium queenslandicum
Malassezia restricta CBS 7877
Melampsora pinitorqua Mpini7
Acanthamoeba mauritaniensis
Acanthamoeba palestinensis
Candidatus Nitrosoarchaeum koreensis MY1
Bacteria
Fungi
Protozoa
Archaea

Fig 6. Heatmap of relative abundance of bacterial, fungal, protozoan, and archaeal species DNA in drinking water microbiomes. Species composition percentages are displayed as the normalized proportion of the microorganism specific k-mers observed in each sample relative to the total microbial species diversity of the sample. Color gradient key displays the scale of relative abundance percentages. Sample A, bottled sparkling natural mineral water; sample B, bottled spring water; sample C, bottled artesian water; sample D, bottled reprocessed tap water; sample E, municipal tap water; sample F, public drinking fountain water.

https://doi.org/10.1371/journal.pone.0231210.g006

2009K-0807 (NCBI GenBank Accession Number: AMRS00000000.1) and *Salmonella enterica* subspp. *enterica* serovar Abaetetuba str. ATCC 35640 (NCBI Reference Sequence: NZ_CP007532.1) for samples B and D, respectively (S1 Fig).

Sparkling natural mineral water (sample A) appears to have a distinctive microbiome compared to other samples examined in this study (Fig 6). The dominant bacterial species identified were *Methyloversatilis RZ18 153* and *Methylobium petroleiphilum*, accounting for 36% and 19% of the relative microbial species diversity. Bacterial species of the family *Comamonadaceae* were detected in sparkling natural mineral BW. *Candidatus Nitrosoarchaeum koreensis* of the Archaeal TACK (*Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota* and *Korarchaeota*) superphylum was detected at less than 1% and not in any other DW samples.

Fungi and protists were detected but only at low relative abundance (Fig 6). Fungi detected include *Chrysosporium queenslandicum* in sparkling natural mineral BW, *Malassezia restricta* in reprocessed tap BW, and *Melampsora pinitorqua* in municipal tap water. *Acanthamoeba palestinensis* was detected in all DW samples except artesian and reprocessed tap BW. *Acanthamoeba mauritaniensis* was detected in spring BW and municipal tap water.

Genes associated with virulence were detected in some of the DW samples above the predefined metagenomic dataset filtering criteria (Table 3). Virulence-associated genes were not detected in artesian and reprocessed BW samples, and only at low abundance in other BW samples, including the genes *Proteus mirabilis tnpA* in spring BW and *Salmonella infantis tnpR* in sparkling natural mineral BW. Virulence coding genes were more common in municipal tap and drinking fountain water samples, e.g., *Klebsiella pneumoniae tnpA* and *Pseudomonas aeruginosa GI 3342496* and *Enterobacter aerogenes tniB*. Antibiotic resistance coding genes were not detected at a frequency to meet the predefined confidence levels set by the metagenomic analysis.

Viruses were detected at a very low abundance and all were dsDNA bacteriophages (Table 4). These included *Salmonella* bacteriophages *vB_SemP_Emk* and *Fels-2* in spring BW and *Staphylococcus* bacteriophage *PvL108* and *Pseudomonas* bacteriophage *Pf1* in reprocessed tap BW and none in any of the other DW samples.

Table 3. Number of unique sequencing reads associated with bacterial virulence genes detected in the metagenomic analysis of drinking water DNA.

Sample	Water Type	Source Organism	Virulence Gene	Gene Function	Number of Unique Reads
A	Bottled Sparkling Natural Mineral Water	*Salmonella infantis*	*tnpR*	Resolvase	142
B	Bottled Spring Water	*Proteus mirabilis*	*tnpA*	Transposase	60
C	Bottled Artesian Water				0
D	Bottled Reprocessed Tap Water				0
E	Municipal Tap Water	*Klebsiella pneumoniae*	*tnpA*	Transposase	54958
		Pseudomonas aeruginosa	GI 3342496	Methyl-accepting chemotaxis-like protein	5913
		Enterobacter aerogenes	*tniB*	Transposase	264
F	Public Drinking Fountain Water	*Pseudomonas aeruginosa*	GI 3342496	Methyl-accepting chemotaxis-like protein	4003
		Klebsiella pneumoniae	*tnpA*	Transposase	463

https://doi.org/10.1371/journal.pone.0231210.t003

Table 4. Number of bacteriophage sequencing reads detected by metagenomic analysis drinking water DNA.

Sample	Water Type	Bacteriophages	Gene Function	Number of Unique Reads
A	Bottled Sparkling Natural Mineral Water			0
B	Bottled Spring Water	Salmonella phage vB_SemP-Emek	O-antigen modification [48,49]	147
		Salmonella phage Fels-2	Cell lysis [50]	470
C	Bottled Artesian Water			0
D	Bottled Reprocessed Tap Water	Staphylococcus phage PvL108	Transposase [51]	18
		Pseudomonas phage Pf1	Filamentous bacteriophage [52]	34
E	Municipal Tap Water			0
F	Public Drinking Fountain Water			0

https://doi.org/10.1371/journal.pone.0231210.t004

Discussion

Total viable bacterial counts

Currently, HPC is used to measure overall bacteriological quality of DW. Reprocessed tap BW in the U.S. interstate commerce is usually purified using a variety of steps, including conventional coagulation, flocculation, sedimentation, distillation, microfiltration, ozonation, reverse osmosis, and ultraviolet (UV) light treatment, to ensure the finished product meets USFDA standards derived from USEPA national DW standards—which does not intend for the final product to be sterile [53]. Having a high HPC in BW does not necessarily correlate with poor quality water, and heterotrophic regrowth in BW upon storage is common [18,54–56] due to the lack of a residual disinfectant being present [15]. Artesian BW, the only BW sample in this study to yield a positive HPC, did not contain added disinfectant residual but the sample had been collected post-treatment (**Table 1**), and the possibility of introduction during the bottling process cannot be ruled out. No residual disinfectants were present in the artesian BW sample included in this study, and the HPC(1.92×10^4 CFU/ml) was similar to heterotrophic bacterial counts obtained in tap water collected from an intensive care unit (2.4×10^4 CFU/ml) [22] and is within the HPC magnitude (10^4 CFU/ml) observed in DWDS when the residual chlorine is less than 0.1 mg/L [57].

Microbial diversity of drinking water

Bacterial phyla detected in DW (**Figs 2–5**) were similar to those commonly detected in municipal DWDS [22,58–61] and natural mineral BW [18,62], with *Proteobacteria* the most abundant. However, *Actinobacteria* was also detected in all DW samples examined.

A culture-independent study focused on the microbiota of DWDS, using 16S rRNA sequencing [22], identified *Alpha-* and *Beta-proteobacteria* subclasses as dominant bacterial communities of a water distribution network, but determined the Gammaproteobacteria subclass represented a relative abundance of less than 1%. Other studies of the microbiome of natural mineral water reported *Alpha-*, *Beta-*, and G*ammaproteobacteria* at moderate abundance [22,59,62]. In the study reported here, *Alphaproteobacteria* was dominant in municipal tap and drinking fountain water (**Figs 4 and 5**), *Betaproteobacteria* in sparkling natural mineral BW (**Fig 2**), and *Gammaproteobacteria* a relative majority in non-mineral BW (**Fig 3**).

Bacterial genera detected in municipal tap and drinking fountain water samples were similar to those frequently detected in DWDS [22,58–60]. *Mycobacterium* spp. were dominant in spring BW. *Mycobacteria* are commonly resistant to ozone- and chlorine-based disinfectants, two primary methods used to treat DW [63]. Biofilm production of *Mycobacteria* in DWDS has also been observed [10,61,64]. Some *Mycobacterium* spp. are opportunistic pathogens.

Mycobacterium spp. are divided into two major categories: 1) causative agent of tuberculosis, including *M*. *tuberculosis*, *M*. *africanum*, and *M*. *canettii*, which are spread through the air and rarely detected in water and 2) NTM, including those responsible for MAC, the cause of many diseases in animals [65] and occasionally associated with pulmonary disease in humans, primarily *M*. *avium* and *M*. *intracellulare* [66]. Environmental NTM, i.e., *M*. *avium*, *M*. *kansasii*, and *M*. *xenopi*, are frequently isolated from DW and hospital water distribution systems, and resistance to chlorine, biofilm formation, and commensal relationships with amoeba has been recognized as a factor contributing to their persistence in DWDS [67]. Aerosols are the major route of dissemination of NTM, which is important because a number of NTM are spore forming—which may contribute to their persistence in the environment [68]. Hypersensitivity pneumonitis has been traced to the presence of NTM in shower heads [69]. In the USA, higher concentrations of NTM have been reported in DWDS disinfected with monochloramine than in DWDS disinfected by chlorination [70]. Haig and colleagues used a high-throughput approach to determine that greater water age, i.e., combined DWDS residence time and home plumbing stagnation time, is associated with a greater relative abundance of *M*. *avium*, and DW from locations closer to WTPs contain more diverse NTM spp. [71]. The WTP supplying water to the municipal tap and drinking fountain water samples included in this study use chlorine disinfection methods, yet presence of NTM was detectable. These findings point to the difficulty of eradicating NTM from premise plumbing, as consequence of their disinfectant-resistance and formation of biofilm [69], and highlight the importance of continued microbiological surveillance of DWDS.

Other genera detected in the municipal tap and drinking fountain water samples were *Afipia* and *Bradyrhizobium*, both common to the natural environments, specifically soil and water. However, it was recently demonstrated that the genus *Bradyrhizobium* is a common contaminant [72], including the 1000 Human Genomes Project [73]. It is possible that contamination of these bacteria was from the ultra-pure water used for DNA extraction and library preparation, since these organisms have an affinity for nitrogen flushed water [74].

Alishewanella was detected in spring and artesian BW samples. The genus *Alishewanella* belongs to the family *Alteromonadacease* and has been isolated from tidal flats [75], lakes [76], landfill soils [77], and fermented foods [78]. *Salmonella* was present in the reprocessed tap BW sample (**S1 Fig**) but at low abundance. Recognition of *Salmonella* outbreaks associated with fresh produce is relevant [79–81], and *Salmonella* spp. have been shown to survive and multiply in BW [82]. However, as can be seen from **Table 1**, the BW samples were collected post-treatment and considered finished DW. While *Salmonella* spp. were detected by DNA sequence analysis, growth on R2A was negative suggesting very low number of cells in the sample. Nonetheless, presence of *Salmonella* spp., particularly *S*. *enterica* subspp. *enterica* serovars Mbandaka and Abaetetuba, is important from a public health perspective as both serovars have been traced to culture confirmed *Salmonella* infections in the USA recently [83].

Methylibium spp. detected in sparkling natural mineral BW are hydrocarbon degrading organisms known to metabolize toluene, a solvent in many coatings used to protect municipal DW storage tanks [84]. *Methyloversatilis* spp. RZ18-153 was detected in sparkling natural mineral BW and is capable of utilizing single carbon (C_1) compounds as sole source of energy [85]. *Methyloversatillis* spp. play an important role in H_2/CO_2-based membrane biofilm reactors that incorporate diffusions of H_2 and CO_2 to remove perchlorate [86] and may be naturally occurring or introduced via injection of natural CO_2 into the sparkling natural mineral water.

E. *coli* and the *enterococcus* group, a subgroup of fecal *streptococci* including *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus gallinarum*, and *Enterococcus avium*, are widely accepted as indicators of biological quality of DW [87,88]. Bacterial genera, e.g., *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Escherichia*, have also been used as indicators of total coliform

bacteria as they inhabit the intestinal tract of warm-blooded animals, but also soil, water, grain, and vegetation [89]. Fecal indicator and coliform bacteria have been reported in DWDS [90,91] and BW [92,93]. In the current study, no fecal indicator bacteria, i.e., *Escherichia coli* or *enterococci*, were detected in any of the DW samples analyzed. However, a transposase virulence factor coding for *Klebsiella pneumoniae*, a total coliform bacterium, was detected in municipal tap and drinking fountain water samples (**Table 3**). Transposases have potential to promote horizontal gene transfer across bacteria [94,95], and because *Klebsiella pneumoniae* was not detected in these samples, these genes might be indicative of horizontal gene transfer.

Prevalence of bacteria over archaea observed in this study is in agreement with previous reports [18,62,96]. *Candidatus nitrosoarchaeum* of the archaeal domain was detected only in sparkling natural mineral BW (**Fig 2**). *Candidatus nitrosarchaeum* is a very small rod-shaped archaea (diameter 0.3–0.5 μm and length 0.6–1.0 μm) that plays an important role in global nitrogen and carbon cycling [97]. This organism may occur more widely in DW than currently known, since other studies reporting on the microbiome of water employed 0.45 μm [82] or 0.2 μm [22,26] pore size filter membranes to concentrate the water samples before DNA extraction. These archaea would pass through those relatively large pore size filters. In this study, 0.1 μm pore size filter membranes were employed, making it possible to detect the *Candidatus nitrosarchaeum*.

Viruses and bacteriophages dominate the biosphere and have been reported to be present in some treated DW supplies [98]. Viruses are extremely host specific, and most phages can only infect a subset of bacterial species [99]. Some viruses, e.g., adenovirus, enterovirus, hepatitis A and E viruses, norovirus, and rotavirus, can cause a variety of human infections, including acute gastroenteritis [100]. In prokaryotes, the majority of viruses possess dsDNA genomes, while in eukaryotes, RNA viruses comprise the majority of the virome [101]. No known eukaryotic viruses were detected in any of the DW samples, and the bacteriophages that were detected were dsDNA viruses (**Table 3**). Specifically, the class II *Pseudomonas* phage Pf1, which can infect only those bacteria bearing retractile pili and not known to infect eukaryotes [52], was detected in reprocessed tap BW but in none of the other samples. Certain bacteriophages are important in overall microbial community structure and also major drivers of bacterial evolution [102]. Detection of *Salmonella* phages (vB_SemP_Emek and Fels-2) in spring BW provide confirmation of the presence of *Salmonella* spp.

Bacteriophages were readily detected in BW samples, but only below the limit of confidence in the municipal tap water sample and not in drinking fountain water (**Table 4**). Other studies have detected bacteriophages in municipal DW at high abundance. Méndez and colleagues found the concentration of bacteriophages outnumbered bacteria in metropolitan DW samples treated by chlorination while bacteria are detected more frequently than bacteriophages in springs, household water wells and rural water supplies [103]. A similar study done on chlorinated DW across three Canadian cities and determined the concentrations of bacteria were variable but all DW samples contained bacteriophages [104]. It is evident that bacteria and the phages that infect them respond differently to chlorination and other abiotic environmental influence in DWDS and finished DW. Additional microbiome investigation is needed to elucidate these complex interactions in DW.

Fungi can survive some water treatments and enter the DWDS post-treatment. Many fungal species survive in oligotrophic environments and are capable of growth by attaching to substrates that promote production of biofilm on pipe surfaces in DWDS [105,106]. Certain fungi, e.g., *Aspergillus* and *Candida*, pose serious health concerns for hospitals and health institutions, particularly immunocompromised patients [107,108]. Presence of fungi in DWDS and BW have been reported previously [109–111].

Chrysosporium queenslandicum, detected in the sparkling natural mineral BW sample belongs to the family *Onygenaceae*, and is not known to be a human pathogen. It has been

used to hydrolyze keratinous debris and recycle poultry waste [112]. *Malassezia restricta*, found in the reprocessed tap BW sample is common to human skin and is a member of a group of yeasts detected in non-culture-based epidemiological studies [113]. *Melampsora pinitorqua* in the municipal tap water sample is a fungal parasite, known to induce pine twist rust in certain plant species [114].

Acanthamoeba spp. are ubiquitous free-living amoebae and function as predators, controlling microbial communities. These protists are common in the environment and previously have been detected in some domestic tap water samples [115], mineral BW, and laboratory distilled water [116]. In this study, *Acanthamoeba palestinensis* was detected in all DW samples except artesian and reprocessed tap BW. *Acanthamoeba mauritaniensis* was detected in spring BW and municipal tap water. *Acanthamoeba* spp. exist in two primary stages, one as a dormant cyst, and the other as an actively feeding and dividing trophozoite [116]. Under certain conditions, they have been recognized as opportunistic pathogens, which can be fatal or invalidating in humans and other animals, causing keratitis in immunocompetent individuals and cutaneous infection or granulomatous amoebic encephalitis in immunocompromised individuals [117,118]. Furthermore, *Acanthamoeba* spp., including *A. palestinensis* and *A. mauritaniensis*, have been shown to harbor opportunistic pathogens, particularly *Legionella* spp. [119,120], and are important to public health.

We observed prevalence of bacterial virulence genes to be higher in tap and drinking fountain water samples compared to BW samples (**Table 3**). It is possible that the low abundance of virulence factors detected in BW samples in this study can be attributed to a low amount of DNA for sequencing. A larger sample size and volume of water would allow for sequencing with higher coverage and yield a more complete characterization of microorganisms. Other studies employing molecular techniques have detected virulence factors in DW, including the DWDS [121,122], point of use tap water [123], artesian well water [124], mineral bottled water [124], and non-mineral bottled water [125].

Throughout this investigation, we were able to detect antibiotic resistance genes (ARG) at abundance levels below the limit of confidence. That is, ARGs were not present after implementing the confidence threshold. This finding differs from other molecular studies of DW where specific pathogenic bacteria harboring ARGs were detected in DWDS [126,127], bottled mineral water [128,129], and non-mineral BW [130]. Recent reports suggest that chlorination during treatment and distribution may enhance antibiotic resistance [13,14,131]. Stamps and colleagues demonstrated that during water treatment, microfiltration and reverse osmosis is effective in removing whole cells and transmissible genetic elements, including ARGs [121]. While the presence of antibiotic resistance genes in DWDS is important to public health, it is likely that the absence of antibiotic genes in DWDS is underreported. Collectively, these findings illustrate the need for microbiological monitoring of DW and DWDS to ensure water quality and safety.

Overall, this preliminary study reports increased bacterial species diversity in DW compared to previous findings where amplicon-sequencing and culture-dependent methods were employed. The whole genome metagenomic method employed in this study, despite total biological material recovered from BW being extremely low in concentration, provided a very rich set of useful information and new insight into DW microbiology warranting further assessments, relative to the public health significance of the non-traditional microbes present in various types of DW, and their relationships to the presence of indicator microorganisms.

Limitations

With current status of whole DNA metagenomic sequencing technologies, investigators cannot conclude viability or infectious potential of the detected microorganisms. However, these

approaches can utilize total DNA to detect accurately and identify all microorganisms in a sample, including bacteria, viruses, fungi, and protists, to sub-species level and characterize them. Primarily during concentration, there was variability in the volume of water analyzed. The dominant organisms detected in each sample would likely still be dominant if a larger sample volume was analyzed. During this investigation, a novel "DNA-spiking" approach was developed to increase the material used for DNA sequencing of samples with low input matrices. We chose to use purified genomic DNA of an unrelated organism, *Pandoraea pnomenusa* KWW5. Future metagenomic studies of samples with low biological content, e.g., water treated with reverse osmosis at water treatment facilities, may need to spike with human DNA or synthetic DNA of known DNA sequence that could be more effectively removed from the sequencing libraries. It is likely that the microbial communities detected in municipal tap and BW samples are water system, treatment, source, and possibly even seasonally specific. Further studies with water types would provide comprehensive analysis of the microbiome of DW.

Conclusions

Whole DNA metagenomic sequencing and bioinformatics can be used effectively to study the autochthonous microbial community of DW and provide a powerful method for extracting new information on the quality of finished DW. Although they are valuable operational tools, the shortcomings of culture-based and indicator methods are well acknowledged, yielding only limited information on the microbiology of DWDS. This study provides an assessment of all microorganisms, bacteria, fungi, protists, and bacteriophages, present in various types of DW and allows an improved understanding of the microbial community structure of finished DW. This preliminary analysis, by applying whole genome metagenomics to determine the microbial composition of finished DW, has yielded new information on the microbial species composition of several drinking waters. Further investigation to address quantitative data will include additional samples and types of DW, and this work is in progress.

Supporting information

S1 Fig. Bottled water samples B and D sequencing coverage plots of *Salmonella enterica* subspp. *enterica*. (A) Bottled spring water (sample B) and (B) Bottled reprocessed tap water (sample D) sequencing reads were mapped against the genomes of *Salmonella enterica* subspp. *enterica* serovars Mbandaka (NCBI GenBank Accession Number: AMRS00000000.1) and Abaetetuba (NCBI Reference Sequence: NZ_CP007532.1), respectively, using the BWA-MEM algorithm with default parameters from the Burrows-Wheeler Alignment Tool [38].
(TIF)

S1 File. Interactive Krona plots used to generate Figs 2–5.
(HTML)

Acknowledgments

We sincerely thank Dr. Peiying Hong of King Abdullah University of Science and Technology for providing DNA of *Pandoraea pnomenusa* KWW5 used as a DNA-spiking agent in this project.

Author Contributions

Conceptualization: Kyle D. Brumfield, Nur A. Hasan, Menu B. Leddy, Joseph A. Cotruvo, Shah M. Rashed, Rita R. Colwell, Anwar Huq.

Data curation: Kyle D. Brumfield, Nur A. Hasan, Shah M. Rashed.

Formal analysis: Kyle D. Brumfield, Nur A. Hasan.

Funding acquisition: Nur A. Hasan, Rita R. Colwell, Anwar Huq.

Investigation: Kyle D. Brumfield.

Methodology: Kyle D. Brumfield, Nur A. Hasan, Menu B. Leddy, Joseph A. Cotruvo, Shah M. Rashed, Anwar Huq.

Project administration: Kyle D. Brumfield, Nur A. Hasan, Rita R. Colwell, Anwar Huq.

Resources: Anwar Huq.

Software: Kyle D. Brumfield, Nur A. Hasan.

Supervision: Nur A. Hasan, Menu B. Leddy, Joseph A. Cotruvo, Rita R. Colwell, Anwar Huq.

Validation: Kyle D. Brumfield, Nur A. Hasan.

Visualization: Kyle D. Brumfield.

Writing – original draft: Kyle D. Brumfield.

Writing – review & editing: Kyle D. Brumfield, Nur A. Hasan, Menu B. Leddy, Joseph A. Cotruvo, Shah M. Rashed, Rita R. Colwell, Anwar Huq.

References

1. WHO. Progress on Drinking Water, Sanitation and Hygiene. Launch version July 12 Main report Progress on Drinking Water, Sanitation and Hygiene. Geneva; 2017.
2. Cutler DM, Miller G. The Role of Public Health Improvements in Health Advances: The Twentieth-Century United States. Demography. 2005; 42: 1–22. https://doi.org/10.1353/dem.2005.0002 PMID: 15782893
3. Cotruvo J. Legionella, Legionellosis, and Regrowth of Microorganisms in Drinking Water Quality and Contaminants Guidebook. First. CRC Press; 2018.
4. Adams DA, Thomas KR, Jajosky RA, Foster L, Baroi G, Sharp P, et al. Summary of Notifiable Infectious Diseases and Conditions—United States, 2015. MMWR Morb Mortal Wkly Rep. 2017; 64: 1–143. https://doi.org/10.15585/mmwr.mm6453a1
5. United States Environmental Protection Agency. Drinking Water Requirements for States and Public Water Systems: Revised Total Coliform Rule and Total Coliform Rule. 2013 [cited 3 Oct 2019]. Available: https://www.epa.gov/dwreginfo/revised-total-coliform-rule-and-total-coliform-rule
6. Deshmukh R, Khardenavis AA, Purohit HJ. Diverse Metabolic Capacities of Fungi for Bioremediation. Indian J Microbiol. 2016; 56: 247–264. https://doi.org/10.1007/s12088-016-0584-6 PMID: 27407289
7. WHO. Guidelines for Drinking-Water Quality. Fourth. Geneva; 2017.
8. Prest EI, Hammes F, Loosdrecht MCM Van. Biological Stability of Drinking Water: Controlling Factors, Methods, and Challenges. Front Cell Infect Microbiol. 2016; 7: 1–24. https://doi.org/10.3389/fmicb.2016.00045
9. Gillespie S, Lipphaus P, Green J, Parsons S, Weir P, Juskowiak K, et al. Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry. Water Res. 2014; 65: 224–234. https://doi.org/10.1016/j.watres.2014.07.029 PMID: 25123436
10. Waak MB, Hozalski RM, Hallé C, Lapara TM. Comparison of the microbiomes of two drinking water distribution systems—With and without residual chloramine disinfection. Microbiome. 2019; 7: 1–14. https://doi.org/10.1186/s40168-018-0604-3
11. Gomez-Smith CK, LaPara TM, Hozalski RM. Sulfate Reducing Bacteria and Mycobacteria Dominate the Biofilm Communities in a Chloraminated Drinking Water Distribution System. Environ Sci Technol. 2015; 49: 8432–8440. https://doi.org/10.1021/acs.est.5b00555 PMID: 26098899
12. Chiao T-H, Clancy TM, Pinto A, Xi C, Raskin L. Differential resistance of drinking water bacterial populations to monochloramine disinfection. Environ Sci Technol. 2014; 48: 4038–4047. https://doi.org/10.1021/es4055725

13. Ridgway HF, Olson BH. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Appl Environ Microbiol. 1982; 44: 972 LP– 987. Available: http://aem.asm.org/content/44/4/972.abstract

14. Shi P, Jia S, Zhang X-X, Zhang T, Cheng S, Li A. Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. Water Res. 2013; 47: 111–120. https://doi.org/10.1016/j.watres.2012.09.046 PMID: 23084468

15. Shifat-e-raihan K, Hasan A, Hossain M, Alam J, Hossain MN, Nesha M, et al. Heterotrophic Plate Count (HPC) of the Commercially Available Bottled Water in Dhaka, Bangladesh. 2017. https://doi.org/10.9790/1959-0605092327

16. Hammes F, Berney M, Wang Y, Vital M, Köster O, Egli T. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Res. 2008; 42: 269–277. https://doi.org/10.1016/j.watres.2007.07.009 PMID: 17659762

17. Prest EI, Hammes F, Kötzsch S, van Loosdrecht M. CM, Vrouwenvelder JS. A systematic approach for the assessment of bacterial growth-controlling factors linked to biological stability of drinking water in distribution systems. Water Supply. 2016; 16: 865–880. https://doi.org/10.2166/ws.2016.001

18. Singer GA, Lesaulnier CC, Loy A, Le Coz X, Niggemann J, Dittmar T, et al. Bottled aqua incognita: microbiota assembly and dissolved organic matter diversity in natural mineral waters. Microbiome. 2017; 5: 1–17. https://doi.org/10.1186/s40168-016-0209-7

19. El-Chakhtoura J, Prest E, Saikaly P, van Loosdrecht M, Hammes F, Vrouwenvelder H. Dynamics of bacterial communities before and after distribution in a full-scale drinking water network. Water Res. 2015; 74: 180–190. https://doi.org/10.1016/j.watres.2015.02.015 PMID: 25732558

20. Fish K, Osborn AM, Boxall JB. Biofilm structures (EPS and bacterial communities) in drinking water distribution systems are conditioned by hydraulics and influence discolouration. Sci Total Environ. 2017; 593–594: 571–580. https://doi.org/10.1016/j.scitotenv.2017.03.176 PMID: 28360007

21. Hull NM, Ling F, Pinto AJ, Albertsen M, Jang HG, Hong PY, et al. Drinking Water Microbiome Project: Is it Time? Trends Microbiol. 2019; 27: 670–677. https://doi.org/10.1016/j.tim.2019.03.011 PMID: 31031092

22. Farhat M, Alkharsah KR, Alkhamis FI, Bukharie HA. Metagenomic study on the composition of culturable and non-culturable bacteria in tap water and biofilms at intensive care units. J Water Health. 2018; 72–83. https://doi.org/10.2166/wh.2018.213

23. Saleem F, Mustafa A, Kori JA, Hussain MS, Kamran Azim M. Metagenomic Characterization of Bacterial Communities in Drinking Water Supply System of a Mega City. Microb Ecol. 2018; 76: 899–910. https://doi.org/10.1007/s00248-018-1192-2 PMID: 29691611

24. Douterelo I, Calero-Preciado C, Soria-Carrasco V, Boxall JB. Whole metagenome sequencing of chlorinated drinking water distribution systems. Environ Sci Water Res Technol. 2018; 4: 2080–2091. https://doi.org/10.1039/C8EW00395E

25. Dai D, Rhoads WJ, Edwards MA, Pruden A. Shotgun Metagenomics Reveals Taxonomic and Functional Shifts in Hot Water Microbiome Due to Temperature Setting and Stagnation. Frontiers in Microbiology. 2018. p. 2695. Available: https://www.frontiersin.org/article/10.3389/fmicb.2018.02695 https://doi.org/10.3389/fmicb.2018.02695 PMID: 30542327

26. Roy MA, Arnaud JM, Jasmin PM, Hamner S, Hasan NA, Colwell RR, et al. A Metagenomic Approach to Evaluating Surface Water Quality in Haiti. Int J Environ Res Public Health. 2018; 15: 2211. https://doi.org/10.3390/ijerph15102211

27. Waldor MK, Tyson G, Borenstein E, Ochman H, Moeller A, Finlay BB, et al. Where Next for Microbiome Research? PLoS Biol. 2015; 1–9. https://doi.org/10.1371/journal.pbio.1002050

28. Tilg H, Moschen AR. Food, Immunity, and the Microbiome. Gastroenterology. 2015; 148: 1107–1119. https://doi.org/10.1053/j.gastro.2014.12.036 PMID: 25575570

29. Knight R, Callewaert C, Marotz C, Hyde ER, Debelius JW, McDonald D, et al. The Microbiome and Human Biology. Annu Rev Genomics Hum Genet. 2017; 18: 65–86. https://doi.org/10.1146/annurev-genom-083115-022438 PMID: 28375652

30. Boddu RS, Divakar K. Metagenomic Insights into Environmental Microbiome and Their Application in Food/Pharmaceutical Industry BT—Microbial Biotechnology: Volume 2. Application in Food and Pharmacology. In: Patra JK, Das G, Shin H-S, editors. Singapore: Springer Singapore; 2018. pp. 23–38. https://doi.org/10.1007/978-981-10-7140-9_2

31. Vangay P, Johnson AJ, Ward TL, Al-Ghalith GA, Shields-Cutler RR, Hillmann BM, et al. US Immigration Westernizes the Human Gut Microbiome. Cell. 2018; 175: 962–972.e10. https://doi.org/10.1016/j.cell.2018.10.029 PMID: 30388453

32. EPA. §141.65 Maximum residual disinfectant levels. 2001 [cited 6 Feb 2020]. Available: https://www.ecfr.gov/cgi-bin/text-idx?SID=44ae4bf9f100f5c557a2d6d4dce37ca3&node=sp40.23.141.g&rgn=div6#se40.25.141_165

33. Reasoner DJ. Heterotrophic plate count methodology in the United States. Int J Food Microbiol. 2004; 92: 307–315. https://doi.org/10.1016/j.ijfoodmicro.2003.08.008 PMID: 15145589

34. Coenye T, Falsen E, Hoste B, Ohlen M, Goris J, Govan JR, et al. Description of Pandoraea gen. nov. with Pandoraea apista sp. nov., Pandoraea pulmonicola sp. nov., Pandoraea pnomenusa sp. nov., Pandoraea sputorum sp. nov. and Pandoraea norimbergensis comb. nov. Int J Syst Evol Microbiol. 2000; 50 Pt 2: 887–899. https://doi.org/10.1099/00207713-50-2-887

35. Andrews SC. FastQC. 2019 [cited 2 Oct 2019]. Available: https://github.com/s-andrews/FastQC

36. Institute JG. BBMap short read aligner, and other bioinformatics tools. 2020.

37. Kulikov AS, Prjibelski AD, Tesler G, Vyahhi N, Sirotkin A V., Pham S, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol. 2012; 19: 455–477. https://doi.org/10.1089/cmb.2012.0021 PMID: 22506599

38. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. Genomics. 2013; 00: 1–3.

39. Li H. Seqtk. 2019 [cited 2 Oct 2019]. Available: https://github.com/lh3/seqtk

40. Lax S, Smith DP, Hampton-marcell J, Owens SM, Shogan BD, Weiss S, et al. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science (80-). 2012; 12702: 1048–1052.

41. Ponnusamy D, Kozlova E V, Sha J, Erova TE, Azar SR, Fitts EC. Cross-talk among flesh-eating Aeromonas hydrophila strains in mixed infection leading to necrotizing fasciitis. Proc Natl Acad Sci. 2016; 113: 722–727. https://doi.org/10.1073/pnas.1523817113 PMID: 26733683

42. Connelly S, Fanelli B, Hasan NA, Kaleko M, Colwell RR. Oral Metallo-Beta-Lactamase Protects the Gut Microbiome From Carbapenem-Mediated Damage and Reduces Propagation of Antibiotic Resistance in Pigs. Front Microbiol. 2019; 10: 1–12. https://doi.org/10.3389/fmicb.2019.00001

43. CosmosID. CosmosID Metagenomics Cloud. 2019 [cited 2 Oct 2019]. Available: https://app.cosmosid.com/login

44. CosmosID. Bioinformatics: Filtering. 2019. Available: https://docs.cosmosid.com/docs/filtering

45. Bray JR, Curtis JT. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecol Monogr. 1957; 27: 325–349. https://doi.org/10.2307/1942268

46. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics. 2011; 12: 385. https://doi.org/10.1186/1471-2105-12-385 PMID: 21961884

47. Broad Institute. Morpheus. 2019 [cited 2 Oct 2019]. Available: https://software.broadinstitute.org/morpheus/

48. Karpe YA, Kanade GD, Pingale KD, Arankalle VA, Banerjee K. Genomic characterization of Salmonella bacteriophages isolated from India. Virus Genes. 2016; 52: 117–126. https://doi.org/10.1007/s11262-015-1269-7 PMID: 26757942

49. Ho N, Lingohr EJ, Villegas A, Cole L, Kropinski AM. Genomic Characterization of Two New Salmonella Bacteriophages: vB_SosS_Oslo and vV_SemP_Emek. Agron Agroecol. 2012; 10: 32.

50. Erickson M, Newman D, Helm RA, Dino A, Calcutt M, French W, et al. Competition among isolates of Salmonella enterica ssp. enterica serovar Typhimurium: Role of prophage/phage in archived cultures. FEMS Microbiol Lett. 2009; 294: 37–44. https://doi.org/10.1111/j.1574-6968.2009.01554.x PMID: 19493006

51. Ma XX, Ito T, Chongtrakool P, Hiramatsu K. Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant Staphylococcus aureus strains isolated in Japanese hospitals from 1979 to 1985. J Clin Microbiol. 2006; 44: 4515–4527. https://doi.org/10.1128/JCM.00985-06 PMID: 17050818

52. Hill DF, Short NJ, Perham RN, Petersen GB. DNA sequence of the filamentous bacteriophage Pf1. J Mol Biol. 1991; 218: 349–364. https://doi.org/10.1016/0022-2836(91)90717-k PMID: 2010913

53. USP. <1231> WATER FOR PHARMACEUTICAL PURPOSES. Available: https://hmc.usp.org/sites/default/files/documents/HMC/GCs-Pdfs/c1231_1SUSP40.pdf

54. Diduch M, Polkowska Z, Namieśnik J. The role of heterotrophic plate count bacteria in bottled water quality assessment. Food Control. 2016; 61: 188–195. https://doi.org/10.1016/j.foodcont.2015.09.024

55. Ansa EDO, Andoh AH, Banu RA, Ansa GA. Photoreactivation of total heterotrophic bacteria in bottled drinking water after inactivation with pulsed ultra-violet light. Ghana J Sci. 2017; 57: 57–64.

56. Charnock C, Hagen RX, Nguyen TN-T, Vo LT. Diversion and phylogenetic relatedness of filterable bacteria from Norwegian tap and bottled waters. J Water Health. 2019; 17: 295–307. https://doi.org/10.2166/wh.2019.284 PMID: 30942779

57. Lu W, Zhang X-J. Factors Affecting Bacterial Growth in Drinking Water Distribution System 1. Biomed Environ Sci. 2005; 18: 137–140. PMID: 16001834

58. Baron JL, Vikram A, Duda S, Stout JE, Bibby K. Shift in the microbial ecology of a hospital hot water system following the introduction of an on-site monochloramine disinfection system. PLoS One. 2014; 9: 1–9. https://doi.org/10.1371/journal.pone.0102679

59. Williams MM, Domingo JWS, Meckes MC, Kelty CA, Rochon HS. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. 2004; 96: 954–964. https://doi.org/10.1111/j.1365-2672.2004.02229.x

60. Poitelon J-B, Duguet J, Prestel E, Lespinet O, Dubow MS. Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. Water Res. 2009; 43: 4197–4206. https://doi.org/10.1016/j.watres.2009.07.020 PMID: 19665751

61. Perrin Y, Bouchon D, Delafont V, Moulin L, Héchard Y. Microbiome of drinking water: A full-scale spatio-temporal study to monitor water quality in the Paris distribution system. Water Res. 2019; 149: 375–385. https://doi.org/10.1016/j.watres.2018.11.013 PMID: 30471533

62. Loy A, Beisker W, Meier H. Diversity of bacteria growing in natural mineral water after bottling. Appl Environ Microbiol. 2005; 71: 3624–3632. https://doi.org/10.1128/AEM.71.7.3624-3632.2005 PMID: 16000770

63. Taylor RH, Falkinham III JO, Norton CD, LeChevallier MD. Chlorine, Chloramine, Chlorine Dioxide, and Ozone Susceptibility of. Appl Environ Microbiol. 2000; 66: 1702–1705. https://doi.org/10.1128/aem.66.4.1702-1705.2000 PMID: 10742264

64. Falkinham III JO, Norton CD, LeChevallier MD. Factors In uencing Numbers of. Society. 2001; 67: 1225–1231. https://doi.org/10.1128/AEM.67.3.1225

65. Thoen C, Karlson AG. Mycobacterial Infections in Animals. Rev Infect Dis. 2019; 3: 960–972.

66. Jagielski T, Minias A, Ingen J Van, Rastogi N, Brzostek A. Methodological and Clinical Aspects of the Molecular Epidemiology of Mycobacterium tuberculosis and Other Mycobacteria. Clin Microbiol Rev. 2016; 29: 239–290. https://doi.org/10.1128/CMR.00055-15 PMID: 26912567

67. Vaerewijck MJM, Huys G, Palomino JC, Swings J, Portaels F. Mycobacteria in drinking water distribution systems: Ecology and significance for human health. FEMS Microbiol Rev. 2005; 29: 911–934. https://doi.org/10.1016/j.femsre.2005.02.001 PMID: 16219512

68. Percival SL, Williams DW. Chapter Nine—Mycobacterium. In: Percival SL, Yates M V, Williams DW, Chalmers RM, Gray NFBT-M of WD (Second E, editors. London: Academic Press; 2014. pp. 177–207. https://doi.org/10.1016/B978-0-12-415846-7.00009-3

69. Falkinham JO. Current Epidemiologic Trends of the Nontuberculous Mycobacteria (NTM). Curr Environ Heal Reports. 2016; 3: 161–167. https://doi.org/10.1007/s40572-016-0086-z

70. Donohue MJ, Mistry JH, Donohue JM, O'Connell K, King D, Byran J, et al. Increased Frequency of Nontuberculous Mycobacteria Detection at Potable Water Taps within the United States. Environ Sci Technol. 2015; 49: 6127–6133. https://doi.org/10.1021/acs.est.5b00496 PMID: 25902261

71. Haig S-J, Kotlarz N, LiPuma JJ, Raskin L. A High-Throughput Approach for Identification of Nontuberculous Mycobacteria in Drinking Water Reveals Relationship between Water Age and Mycobacterium avium Bailey Tim van der Wielen, Paul MJL, editor. MBio. 2018; 9: e02354–17. https://doi.org/10.1128/mBio.02354-17 PMID: 29440575

72. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014; 12: 1–12. https://doi.org/10.1186/1741-7007-12-1

73. Laurence M, Hatzis C, Brash DE. Common contaminants in next-generation sequencing that hinder discovery of low-abundance microbes. PLoS One. 2014; 9: e97876–e97876. https://doi.org/10.1371/journal.pone.0097876 PMID: 24837716

74. Kulakov LA, McAlister MB, Ogden KL, Larkin MJ, O'Hanlon JF. Analysis of bacteria contaminating ultrapure water in industrial systems. Appl Environ Microbiol. 2002; 68: 1548–1555. https://doi.org/10.1128/AEM.68.4.1548-1555.2002 PMID: 11916667

75. Roh SW, Nam Y-D, Chang H-W, Kim K-H, Kim M-S, Oh H-M, et al. Alishewanella aestuarii sp. nov., isolated from tidal flat sediment, and emended description of the genus Alishewanella. Int J Syst Evol Microbiol. 2009; 59: 421–424. https://doi.org/10.1099/ijs.0.65643-0 PMID: 19196789

76. Tarhriz V, Nematzadeh G, Vahed SZ, Hejazi MA, Hejazi MS. Alishewanella tabrizica sp. nov., isolated from qurugöl lake. Int J Syst Evol Microbiol. 2012; 62: 1986–1991. https://doi.org/10.1099/ijs.0.031567-0 PMID: 22003035

77. Kim MS, Jo SK, Roh SW, Bae JW. Alishewanella agri sp. nov., isolated from landfill soil. Int J Syst Evol Microbiol. 2010; 60: 2199–2203. https://doi.org/10.1099/ijs.0.011684-0 PMID: 19897613

78. Kim M-S, Roh SW, Nam Y-D, Chang H-W, Kim K-H, Jung M-J, et al. Alishewanella jeotgali sp. nov., isolated from traditional fermented food, and emended description of the genus Alishewanella. Int J Syst Evol Microbiol. 2009; 59: 2313–2316. https://doi.org/10.1099/ijs.0.007260-0 PMID: 19620373

79. Jackson BR, Griffin PM, Cole D, Walsh KA, Chai SJ. Outbreak-associated Salmonella enterica Serotypes and Food Commodities, United States, 1998–2008. Emerg Infect Dis. 2013; 19: 1239–1244. https://doi.org/10.3201/eid1908.121511 PMID: 23876503

80. Proctor ME, Hamacher M, Tortorello MLOU, Archer JR, Davis JP. Multistate Outbreak of Salmonella Serovar Muenchen Infections Associated with Alfalfa Sprouts Grown from Seeds Pretreated with Calcium Hypochlorite. 2001; 39: 3461–3465. https://doi.org/10.1128/JCM.39.10.3461

81. Greene S, Daly E, Talbot E, Demma L, Holzbauer S, Patel N, et al. Recurrent multistate outbreak of Salmonella Newport associated with tomatoes from contaminated fields, 2005. Epidemiol Infect. 2008; 157–165. https://doi.org/10.1017/S095026880700859X PMID: 17475091

82. Ahmed W, Hasan R, Ashraf W, Goonetilleke A, Toze S, Gardner T. Fecal indicators and bacterial pathogens in bottled water from Dhaka, Bangladesh. Brazilian J Microbiol. 2013; 44: 97–103. https://doi.org/10.1590/s1517-83822013005000026

83. CDC. National Enteric Disease Surveillance: Salmonella Annual Report, 2016. 2018 [cited 14 Jan 2020]. Available: https://www.cdc.gov/nationalsurveillance/pdfs/2016-Salmonella-report-508.pdf

84. WHO. Toluene in Drinking-water: Background document for development of WHO Guidelines for Drinking-water Quality. Heal (San Fr. 2004;2. Available: https://www.who.int/water_sanitation_health/dwq/chemicals/toluene.pdf

85. Smalley NE, Taipale S, De Marco P, Doronina N V., Kyrpides N, Shapiro N, et al. Functional and genomic diversity of methylotrophic Rhodocyclaceae: Description of Methyloversatilis discipulorum sp. nov. Int J Syst Evol Microbiol. 2015; 65: 2227–2233. https://doi.org/10.1099/ijs.0.000190 PMID: 26231539

86. Li H, Zhou L, Lin H, Zhang W, Xia S. Nitrate effects on perchlorate reduction in a H2/CO2-based biofilm. Sci Total Environ. 2019; 694: 133564. https://doi.org/10.1016/j.scitotenv.2019.07.370 PMID: 31400688

87. Odonkor ST, Ampofo JK. Escherichia coli as an indicator of bacteriological quality of water: an overview. Microbiol Res (Pavia). 2013; 4: 2. https://doi.org/10.4081/mr.2013.e2

88. Scott TM, Rose JB, Jenkins TM, Farrah SR, Lukasik J. Microbial Source Tracking: Current Methodology and Future Directions. Appl Environ Microbiol. 2002; 68: 5796 LP– 5803. https://doi.org/10.1128/AEM.68.12.5796–5803.2002

89. Cabral JPS. Water microbiology. Bacterial pathogens and water. Int J Environ Res Public Health. 2010/10/15. 2010; 7: 3657–3703. https://doi.org/10.3390/ijerph7103657 PMID: 21139855

90. Stillo F, MacDonald Gibson J. Exposure to Contaminated Drinking Water and Health Disparities in North Carolina. Am J Public Health. 2016; 107: 180–185. https://doi.org/10.2105/AJPH.2016.303482 PMID: 27854523

91. Chen Z, Yu D, He S, Ye H, Zhang L, Wen Y, et al. Prevalence of Antibiotic-Resistant Escherichia coli in Drinking Water Sources in Hangzhou City. Frontiers in Microbiology. 2017. p. 1133. Available: https://www.frontiersin.org/article/10.3389/fmicb.2017.01133 PMID: 28670309

92. Mills K, Golden J, Bilinski A, Beckman AL, McDaniel K, Harding AS, et al. Bacterial contamination of reusable bottled drinking water in Ecuador. J Water, Sanit Hyg Dev. 2017; 8: 81–89. https://doi.org/10.2166/washdev.2017.064

93. Pant ND, Poudyal N, Bhattacharya SK. Bacteriological quality of bottled drinking water versus municipal tap water in Dharan municipality, Nepal. J Heal Popul Nutr. 2016; 35: 17. https://doi.org/10.1186/s41043-016-0054-0

94. Cuecas A, Kanoksilapatham W, Gonzalez JM. Evidence of horizontal gene transfer by transposase gene analyses in Fervidobacterium species. PLoS One. 2017; 12: e0173961. Available: https://doi.org/10.1371/journal.pone.0173961 PMID: 28426805

95. Luo P, Jiang H, Wang Y, Su T, Hu C, Ren C, et al. Prevalence of mobile genetic elements and transposase genes in Vibrio alginolyticus from the southern coastal region of China and their role in horizontal gene transfer. Int Microbiol. 2012; 15: 201–210. https://doi.org/10.2436/20.1501.01.172 PMID: 23844479

96. França L, Lopéz-lopéz A, Rosselló-móra R, Costa MS. Microbial diversity and dynamics of a groundwater and a still bottled natural mineral water. 2015; 17: 577–593. https://doi.org/10.1111/1462-2920.12430

97. Jung M-Y, Islam MA, Gwak J-H, Kim J-G, Rhee S-K. Nitrosarchaeum koreense gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon member of the phylum Thaumarchaeota

isolated from agricultural soil. Int J Syst Evol Microbiol. 2018; 68: 3084–3095. https://doi.org/10.1099/ijsem.0.002926 PMID: 30124400

98. Borchardt MA, Spencer SK, Kieke BA, Lambertini E, Loge FJ. Viruses in nondisinfected drinking water from municipal wells and community incidence of acute gastrointestinal illness. Environ Health Perspect. 2012; 120: 1272–1279. https://doi.org/10.1289/ehp.1104499 PMID: 22659405

99. Flores CO, Meyer JR, Valverde S, Farr L, Weitz JS. Statistical structure of host-phage interactions. Proc Natl Acad Sci U S A. 2011/06/27. 2011; 108: E288–E297. https://doi.org/10.1073/pnas.1101595108 PMID: 21709225

100. Gall AM, Mariñas BJ, Lu Y, Shisler JL. Waterborne Viruses: A Barrier to Safe Drinking Water. PLOS Pathog. 2015; 11: e1004867. https://doi.org/10.1371/journal.ppat.1004867 PMID: 26110535

101. Koonin E V, Dolja V V, Krupovic M. Origins and evolution of viruses of eukaryotes: The ultimate modularity. 2018; 2–25. https://doi.org/10.1016/j.virol.2015.02.039.Origins

102. Jorch G, Kluge S, König F, Markewitz A, Notz K, Parvu V, et al. Phages and the Evolution of Bacterial Pathogens: from Genomic Rearrangements to Lysogenic Conversion. Microbiol Mol Biol Rev. 2010; 68: 1–40. https://doi.org/10.1128/MMBR.68.3.560

103. Méndez J, Audicana A, Cancer M, Isern A, Llaneza J, Moreno B, et al. Assessment of drinking water quality using indicator bacteria and bacteriophages. J Water Health. 2004; 2: 201–214. https://doi.org/10.2166/wh.2004.0018 PMID: 15497816

104. Palmateer GA, Dutka BJ, Janzen EM, Meissner SM, Sakellaris M. COLIPHAGES AND BACTERIOPHAGES IN CANADIAN DRINKING WATERS. Water Int. 1990; 15: 157–159. https://doi.org/10.1080/02508069008687132

105. Hageskal G, Knutsen AK, Gaustad P, Hoog GS De, Skaar I. Diversity and Significance of Mold Species in Norwegian Drinking Water. Appl Environ Microbiol. 2006; 72: 7586–7593. https://doi.org/10.1128/AEM.01628-06 PMID: 17028226

106. Doggett MS. Characterization of fungal biofilms within a municipal water distribution system. Appl Environ Microbiol. 2000; 66: 1249–1251. https://doi.org/10.1128/aem.66.3.1249-1251.2000 PMID: 10698803

107. Pfaller MA, Diekema DJ. Rare and Emerging Opportunistic Fungal Pathogens: Concern for Resistance beyond Candida albicans and Aspergillus fumigatus J Clin Microbiol. 2004; 42: 4419 LP– 4431. https://doi.org/10.1128/JCM.42.10.4419–4431.2004

108. Sonigo P, Toni A De, Reilly K. Defra A REVIEW OF FUNGI IN DRINKING WATER AND THE IMPLICATIONS FOR. 2011. Available: http://dwi.defra.gov.uk/research/completed-research/reports/DWI70-2-255.pdf

109. Ribeiro A, Machado AP, Kozakiewicz Z, Ryan M, Luke B, Buddie AG, et al. Fungi in bottled water: a case study of a production plant. Rev Iberoam Micol. 2006; 23: 139–144. https://doi.org/10.1016/s1130-1406(06)70033-x PMID: 17196019

110. Cabral D, Fernández Pinto VE. Fungal spoilage of bottled mineral water. Int J Food Microbiol. 2002; 72: 73–76. https://doi.org/10.1016/s0168-1605(01)00628-6 PMID: 11843415

111. Novak Babic M, Gunde-cimerman N, Vargha M, Tischner Z. Fungal Contaminants in Drinking Water Regulation? A Tale of Ecology, Exposure, Purification and Clinical Relevance. Int J Environ Res Public Health. 2017; 114: 636. https://doi.org/10.3390/ijerph14060636

112. Kumawat TK, Sharma A, Bhadauria S. Chrysosporium queenslandicum: a potent keratinophilic fungus for keratinous waste degradation. Int J Recycl Org Waste Agric. 2017; 6: 143–148. https://doi.org/10.1007/s40093-017-0162-x

113. Gaitanis G, Magiatis P, Hantschke M, Bassukas ID, Velegraki A. The Malassezia genus in skin and systemic diseases. Clin Microbiol Rev. 2012; 25: 106–141. https://doi.org/10.1128/CMR.00021-11 PMID: 22232373

114. Martinsson O. The influence of pine twist rust (Melampsora pinitorqua) on growth and development of Scots pine (Pinus sylvestris). Eur J For Pathol. 1985; 15: 103–110. https://doi.org/10.1111/j.1439-0329.1985.tb00873.x

115. Choi SH, Cho MK, Ahn SC, Lee JE, Lee JS, Kim DH, et al. Endosymbionts of Acanthamoeba isolated from domestic tap water in Korea. Korean J Parasitol. 2009; 47: 337–344. https://doi.org/10.3347/kjp.2009.47.4.337 PMID: 19967080

116. Ferro S, Coppellotti O, Roncucci G, Ben Amor T, Jori G. Photosensitized inactivation of Acanthamoeba palestinensis in the cystic stage. J Appl Microbiol. 2006; 101: 206–212. https://doi.org/10.1111/j.1365-2672.2006.02893.x PMID: 16834608

117. CDC. Parasites—Acanthamoeba—Granulomatous Amebic Encephalitis (GAE); Keratitis Information for Specific Groups. 2017 [cited 22 Aug 2019]. Available: https://www.cdc.gov/parasites/acanthamoeba/index.html#asterisktwo

118. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as Agents of Disease in Humans. Clin Microbiol Rev. 2003; 16: 273 LP– 307. https://doi.org/10.1128/CMR.16.2.273–307.2003

119. Anand CM, Skinner AR, Malic A, Kurtz JB. Interaction of L. pneumophilia and a free living amoeba (Acanthamoeba palestinensis). J Hyg (Lond). 1983; 91: 167–178. https://doi.org/10.1017/s0022172400060174

120. Dobrowsky PH, Khan S, Khan W. Resistance of Legionella and Acanthamoeba mauritaniensis to heat treatment as determined by relative and quantitative polymerase chain reactions. Environ Res. 2017; 158: 82–93. https://doi.org/10.1016/j.envres.2017.06.003 PMID: 28609649

121. Stamps BW, Leddy MB, Plumlee MH, Hasan NA, Colwell RR, Spear JR. Characterization of the Microbiome at the World ' s Largest Potable Water Reuse Facility. 2018; 9: 2435. https://doi.org/10.3389/fmicb.2018.02435

122. Yu C-P, Chu K-H. Molecular quantification of virulence gene-containing Aeromonas in water samples collected from different drinking water treatment processes. Environ Monit Assess. 2011; 176: 225–238. https://doi.org/10.1007/s10661-010-1578-1 PMID: 20632090

123. Sen K, Rodgers M. Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilities: a PCR identification. J Appl Microbiol. 2004; 97: 1077–1086. https://doi.org/10.1111/j.1365-2672.2004.02398.x PMID: 15479425

124. Silva MEZ da, Filho IC, Endo EH, Nakamura CV, Ueda-Nakamura T, Filho BPD. Characterisation of potential virulence markers in Pseudomonas aeruginosa isolated from drinking water. Antonie Van Leeuwenhoek. 2007/11/24. 2008; 93: 323–334. https://doi.org/10.1007/s10482-007-9209-8 PMID: 18038252

125. Edberg SC, Gallo P, Kontnick C. Analysis of the Virulence Characteristics of Bacteria Isolated from Bottled, Water Cooler, and Tap Water. Microb Ecol Health Dis. 1996; 9: 67–77. https://doi.org/10.3109/08910609609166445

126. Sanganyado E, Gwenzi W. Antibiotic resistance in drinking water systems: Occurrence, removal, and human health risks. Sci Total Environ. 2019. https://doi.org/10.1016/j.scitotenv.2019.03.162

127. Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, et al. Prevalence of Antibiotic Resistance in Drinking Water Treatment and Distribution Systems. Appl Environ Microbiol. 2009; 75: 5714 LP–5718. https://doi.org/10.1128/AEM.00382-09

128. Falcone-Dias MF, Vaz-Moreira I, Manaia CM. Bottled mineral water as a potential source of antibiotic resistant bacteria. Water Res. 2012/04/12. 2012; 46: 3612–3622. https://doi.org/10.1016/j.watres.2012.04.007 PMID: 22534119

129. Falcone-Dias MF, Centrón D, Pavan F, Moura AC da S, Naveca FG, de Souza VC, et al. Opportunistic Pathogens and Elements of the Resistome that Are Common in Bottled Mineral Water Support the Need for Continuous Surveillance. PLoS One. 2015; 10: e0121284. Available: https://doi.org/10.1371/journal.pone.0121284 PMID: 25803794

130. Hernandez Duquino H, Rosenberg FA. Antibiotic-resistant Pseudomonas in bottled drinking water. Can J Microbiol. 1987; 33: 286–289. https://doi.org/10.1139/m87-049 PMID: 3594309

131. Jia S, Shi P, Hu Q, Li B, Zhang T, Zhang X-X. Bacterial Community Shift Drives Antibiotic Resistance Promotion during Drinking Water Chlorination. Environ Sci Technol. 2015; 49: 12271–12279. https://doi.org/10.1021/acs.est.5b03521 PMID: 26397118