Evaluation of Antibiotic Resistance in a Pharmaceutical Wastewater of China

and

a Novel Nitritation-Anommmox Process for Autotrophic Nitrogen Removal

Jiane Zuo, Ph D, Professor
School of Environment, Tsinghua University

Oct. 12, 2015
• Introduction of Tsinghua University;
• Antibiotic Resistance in Wastewater;
• Novel Nitritation-Anammox Process
• Summary
Location of Tsinghua University
Campus of Tsinghua University

Total area: 392.4 ha
Beautiful Campus

- Spring Water
- Winter Campanile
- Campus River
- Summer Blossoms
- Autumn Leaves
- Corridors in the President Office
School of Environment

School of Law

School of Medicine

School of Life Science
# Statistics of Tsinghua University (2014)

<table>
<thead>
<tr>
<th>Students in total</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undergraduates</td>
<td>15,692 (1420*)</td>
</tr>
<tr>
<td>Master students</td>
<td>18,296 (951*)</td>
</tr>
<tr>
<td>Ph.D students</td>
<td>11,249 (291*)</td>
</tr>
</tbody>
</table>

*: International students
# Statistics of Tsinghua University (2014)

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faculty and Staff</td>
<td>5,985</td>
</tr>
<tr>
<td>Members of Chinese Academy of Science</td>
<td>43</td>
</tr>
<tr>
<td>Members of Chinese Academy of Engineering</td>
<td>33</td>
</tr>
<tr>
<td>Full Professors</td>
<td>1,442</td>
</tr>
<tr>
<td>Associate Professors</td>
<td>2,138</td>
</tr>
</tbody>
</table>
● Introduction of Tsinghua University;
● Antibiotic Resistance in Wastewater;
● Novel Nitritation-Anammox Process
● Summary
Background

- Problem of antibiotic resistant bacteria (ARB) and resistance genes (ARG) has received increasing concern

  - Low concentrations of antibiotics in the environment can induce resistance to bacteria in long term
  - ARB and ARG have been detected in different environments

- ARB and ARG in the environment
  - Direct way by drinking, swimming, etc.
  - Indirect way by food chain transmission

- Human acquired antibiotic resistance – Increasing difficulty of curing disease
Background

- **Pathways of antibiotics entering the environments**

  - Antibiotic producing wastewater → Municipal Sewage → Hospitals
  - Antibiotic producing wastewater treatment plant
  - Municipal wastewater treatment plant
  - Medical wastewater treatment plant
  - Discharge of Effluents
  - Landfill
  - Agricultural Activity
  - Soil
  - Surface Water
  - Ground Water
Background

- **Pathways of antibiotics entering the environment**

An important source of antibiotics: **Wastewater Discharge**

- **Antibiotic producing wastewater**
  - Antibiotic producing wastewater treatment plant
  - Municipal Sewage
    - Municipal wastewater treatment plant
    - Medical wastewater treatment plant
  - Landfill
    - Agricultural Activity
      - Soil
        - Surface Water
        - Underground Water
Background

- **Focusing on antibiotic producing wastewater in China**
  - Antibiotic producing wastewater contains very high concentrations of antibiotics
  - China is now one of the world’s largest antibiotic manufacturing countries, discharging 540 million tons of pharmaceutical producing wastewater in 2013.

  - *China Statistical Yearbook on Environment -2014*
Background

Objectives

- Obtain a general understanding to the levels of antibiotic resistance in municipal wastewater, cephalosporin producing wastewater and receiving river
- Investigate the effect of cephalosporin producing wastewater discharge on receiving river
- Explore the influence of wastewater treatment on antibiotic resistance
Material and Method

● Sampling sites

- The cephalosporin producing wastewater treatment plant and municipal wastewater treatment plant were located in A city, China - 20 km apart.
- Both of the two wastewater treatment plants use the two-stage biological oxidation processes
- Wastewater were sampled from the equalization tanks and effluents of the final sedimentation tanks of the two wastewater treatment plants, respectively
Background

- River sediment samples that receive cephalosporin wastewater effluent were taken at the upstream 200 m and downstream 2000 m from the outfall, respectively.

- Wastewater samples were labeled as:
  - Municipal wastewater influent (MWI)
  - Municipal wastewater effluent (MWE)
  - Raw cephalosporin producing wastewater (RCPW)
  - Treated cephalosporin producing wastewater (TCPW)
  - Upstream river sediment (URS)
  - Downstream river sediment (DRS)
Material and Method

- **Antibiotic susceptibility test - Etest**
  
  - Step 1. Isolation and Identification of *Enterobacter*
    
    - *Enterobacter* was chosen as the indicator microorganism
    
    - pure colonies were identified by the API 20E system (bioMerieux, France)
**Material and Method**

- **Antibiotic susceptibility test - Etest**
  - Step 1. Isolation and Identification of *Enterobacter*
    - *Enterobacter* was chosen as the indicator microorganism
    - pure colonies were identified by the API 20E system (bioMerieux, France)
  - Step 2. Antibiotic susceptibility tests of isolates using E-test strips (bioMerieux, France)
    - Each *Enterobacter* was tested its minimum inhibition concentration (MIC) to seven antibiotics
      - Aminoglycosides: Gentamicin (GTM)
      - Quinolones: Levofloxacin (LVX)
      - Sulfonamides potentiator: Trimetroprim (TMP)
      - β-lactam class:
        - Semi-synthetic penicillins: Ampicillin (AMP), Amoxicillin (AMC)
        - Cephalosporins: Cefuroxime (CXM), Ceftriaxone (CTR)
      - Semisynthetic penicillins:
        - Cephalosporins: Cefuroxime (CXM), Ceftriaxone (CTR)
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    - Aminoglycosides: Gentamicin (GTM)
Material and Method

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    - pure colonies were identified by the API 20E system (bioMerieux, France)
  - Step 2. Antibiotic susceptibility tests of isolates using E-test strips (bioMerieux, France)
    - Each Enterobacter was tested its minimum inhibition concentration (MIC) to seven antibiotics
  - Step 3. Testing output
    - MICs were interpreted according to Clinical and Laboratory Standards Institute standards, and Enterobacter was divided into “sensitive” or “resistant” category accordingly.

Percentage of antibiotic resistant Enterobacteriaceae

\[
\text{Percentage} = \frac{\text{Number of antibiotic resistant Enterobacteriaceae}}{\text{Total number of tested Enterobacteriaceae}}
\]
Material and Method

- **Quantification of $bla_{TEM-2}$ gene - SYBR Green I real-time qPCR**
  - Step 1. Distracitron of total DNA
    - using MoBio PowerWater DNA Isolation Kit
  - Step 2. qPCR (7900, Applied Biosystems, U.S.)
    - $bla_{TEM-2}$ (Gene bank No. HQ162131.1)
    - Primer sequence:
      - $bla_{TEM-2}$-F: 5’-AAGCCATACCAAAACGACG-3’
      - $bla_{TEM-2}$-R: 5’-TTTATCCGCCTCCATCCA-3’
      - 16SrRNA-F: 5’-CCTTGAGGATGTTGGGTA-3’
      - 16SrRNA-R: 5’-CGTTTGGAGATTAGCG-3’
    - The $R^2$ value of the 6-point standard curve (10-fold serial dilutions, 109 to 104 copy numbers) for qPCR was 0.995
    - The amplification efficiency of $bla_{TEM-2}$ was 96.9%
Material and Method

Quantification of $bla_{\text{TEM-2}}$ gene - SYBR Green I real-time qPCR

- Step 1. Distraction of total DNA
  - using MoBio PowerWater DNA Isolation Kit
- Step 2. qPCR (7900, Applied Biosystems, U.S.)

**Reaction system**
- 10 µL of 2×SYBR qPCR Mastermix (Roche, Switzerland)
- 0.4 µL of each primer (10 µM)
- 1 µL of template DNA
- 8.2 µL of ddH$_2$O

**Reaction procedure**
- 95 °C, 2 min
- 40 cycles of 15s at 95 °C, 1 min at 60 °C
- extension at 60 °C and a fluorescence acquisition step at 60 °C
- final melting followed as: 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C.
Material and Method

Quantification of \( bla_{TEM-2} \) gene – SYBR Green I real-time qPCR

- Step 1. Distraciton of total DNA
  - using MoBio PowerWater DNA Isolation Kit

- Step 2. qPCR
  - The \( R^2 \) value of the 6-point standard curve (10-fold serial dilutions, 10^9 to 10^4 copy numbers) for qPCR was 0.995
  - The amplification efficiency of \( bla_{TEM-2} \) was 96.9%
## Result and Discussion

- **Antibiotic resistance levels of Enterobacteriaceae**

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Number of total heterotrophic bacteria (CFU/mL)</th>
<th>Number of Enterobacteriacea isolates</th>
<th>Percentage of antibiotic-resistant Enterobacteriacea (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GTM</td>
</tr>
<tr>
<td>RCPW</td>
<td>4.9×10⁸</td>
<td>79</td>
<td>13.9</td>
</tr>
<tr>
<td>TCPW</td>
<td>2.7×10⁵</td>
<td>94</td>
<td>12.8</td>
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<tr>
<td>MWI</td>
<td>2.8×10⁷</td>
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Result and Discussion

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<td>12.8 7.5 3.2 92.6 74.4 96.8 66.0</td>
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<tr>
<td>MWI</td>
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<td>14.0 16.1 11.8 18.3 25.8 10.8 9.7</td>
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- Removal efficiencies of total heterotrophic bacteria in both cephalosporin wastewater treatment plant and municipal wastewater treatment plant were 3-log approximately
Result and Discussion

- **Antibiotic resistance levels of Enterobacteriaceae**

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- Removal efficiencies of total heterotrophic bacteria in both cephalosporin wastewater treatment plant and municipal wastewater treatment plant were 3-log approximately
- Antibiotic resistant Enterobacteriaceae were popular in both cephalosporin producing wastewater and municipal wastewater
Result and Discussion

- Percentage of AMP-resistant, AMC-resistant, CXM-resistant and CTR-resistant Enterobacteriaceae in cephalosporin producing wastewater increased after wastewater treatment.
Percentage of GTM-resistant, LVX-resistant, AMP-resistant, AMC-resistant, CXM-resistant and CTR-resistant Enterobacteriaceae in municipal wastewater increased after wastewater treatment.
Result and Discussion

- Although bacteria were largely removed during the wastewater treatment process, in the remaining bacteria, the percentages of antibiotic resistant Enterobacteriaceae increased on the contrary.
- Microorganisms were further induced resistance to antibiotics during the wastewater treatment process.
Result and Discussion

- Percentages of β-lactam-antibiotic resistant Enterobacteriaceae were significantly higher than those of non-β-lactam-antibiotic resistant Enterobacteriaceae
- Cephalosporin producing wastewater: due to high concentration of cephalosporins
- Illustrated that cephalosporin residues induced the resistance to not only cephalosporins (CXM and CTR), but also other β-lactam antibiotics with similar chemical structure (AMP and AMC).
Municipal wastewater: implied that the consumption amount of β-lactam-antibiotics was more than that of the other three antibiotics
More than 50% of the Enterobacteriaceae were resistant to the 4 the tested β-lactam antibiotics (AMP, AMC, CXM and CTR)

Percentages of Enterobacteriaceae resistant to the β-lactam in cephalosporin producing wastewater were more than one time higher than those in municipal wastewater.
Result and Discussion

- **Presence of $bla_{TEM-2}$ gene**

![Bar graph showing concentration of $bla_{TEM-2}$ gene in different samples.](image)
**Result and Discussion**

- **Presence of** $\textit{bla}_{\text{TEM-2}}$ **gene**

  - **Removal efficiencies** of $\textit{blaTEM-2}$ gene by cephalosporin producing wastewater treatment plant and municipal wastewater treatment plant were $0.50\text{-log}$ and $0.55\text{-log}$, respectively.

  - Wastewater treatment plants performed much better in removing bacteria than in removing ARG.
Result and Discussion

• Presence of $bla_{\text{TEM-2}}$ gene

- Concentrations of $bla_{\text{TEM-2}}$ gene in cephalosporin producing wastewater were much higher than in municipal wastewater.
- Comparable with the percentages of $\beta$-lactam-antibiotic resistant Enterobacteriaceae in cephalosporin producing wastewater and municipal wastewater.
Abundance of \textit{bla}_{TEM-2} in raw cephalosporin producing wastewater was 100 times higher than that in municipal wastewater influent, while abundance of \textit{bla}_{TEM-2} in treated cephalosporin producing wastewater was 50 times higher than that in municipal wastewater effluent.
Abundance of $bla_{TEM-2}$ in the treated wastewater was higher than in raw wastewater in both treatment plants.
Abundance of \( \textit{bla}_{\text{TEM-2}} \) in the treated wastewater was higher than in raw wastewater in both treatment plants.
The discharge of RCPW resulted in a significant increase of β-lactam antibiotic and GTM-resistant Enterobacteriaceae in the receiving river sediment.
Result and Discussion

Concentration of \(bla_{TEM-2}\) gene...

\[
\begin{array}{c|c|c|c}
& \text{URS} & \text{DRS} & \text{URS} \\
\hline
\text{Concentration} & 10^2 & 10^6 & 10^8 \\
\end{array}
\]

\(bla_{TEM-2}\) copies/16S rRNA copies

\[
\begin{array}{c|c|c|c}
& \text{URS} & \text{DRS} & \text{URS} \\
\hline
\text{Concentration} & 10^{-3} & 10^{-4} & 10^{-5} \\
\end{array}
\]
Concentration of *bla*<sub>TEM-2</sub> gene increased in the downstream river sediment

Abundance of *bla*<sub>TEM-2</sub> gene increased in the downstream river sediment
Summary 1

- ARB and ARG were popular in both cephalosporin producing wastewater and municipal wastewater.
- In the investigated two wastewater treatment plants, the removal efficiencies of total heterotrophic bacteria and bla$_{TEM-2}$ gene were approximately 3-log and 0.5-log, respectively.
- The antibiotic resistant level of bacteria in the treated wastewater was significantly higher than in the raw wastewater.
- Percentages of Enterobacteriaceae resistant to the β-lactam antibiotics in cephalosporin producing wastewater were more than one time higher than those in municipal wastewater.
- The concentration and abundance of bla$_{TEM-2}$ gene in cephalosporin producing wastewater were higher than in municipal wastewater.
- Discharge of cephalosporin wastewater resulted in a significant increase of ARB and ARG in the receiving river sediment.
- Introduction of Tsinghua University;
- Antibiotic Resistance in Wastewater;
- Novel Nitritation-Anammox Process
- Summary
Enrichment of Anammox

We enriched the anammox bacteria from activated sludge and cultivated different froms of anammox sludge (anammox flocculent sludge, biofilm and granules).

The anammox sludge all showed characteristic red, and the nitrogen removing rate of the granular anammox reactor reached 18.0 kg N/(m$^3$ d), with total nitrogen removal rate around 89%.

Flocculent sludge  Biofilm  Granules
The FISH analysis also confirm that anammox bacteria consisted of the majority of the bacteria.

FISH images with FITC-labeled EUB338 probe (blue) and Cy3-labeled Amx368 probe (red)

Tab. The major probes used for FISH analyses

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Target</th>
<th>FA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>5'- GCT GCC TCC CGT AGG AGT -3'</td>
<td>Most bacteria</td>
<td>0-50</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>5'- GCA GCC ACC CGT AGG TGT -3'</td>
<td>Most bacteria</td>
<td>0-50</td>
</tr>
<tr>
<td>EUB338 III</td>
<td>5'- GCT GCC ACC CGT AGG TGT -3'</td>
<td>Most bacteria</td>
<td>0-50</td>
</tr>
<tr>
<td>Amx368</td>
<td>5'- CCT TTC GGG CAT TGC GAA -3'</td>
<td>anammox</td>
<td>15</td>
</tr>
</tbody>
</table>
Hydroxylamine addition

Nitritation and anammox process were combined into one single reactor. Hydroxylamine was added into the reactor, to enhance the nitritation and anammox process while inhibit nitrite oxidation process.

Addition of hydroxylamine could accelerate the start-up of reactor significantly and maintain the loading rate of the reactor around a relatively high level.

Tab. Comparison of the start-up time and nitrogen removing rate after start-up

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Time (d)</th>
<th>NRR(^d) after start-up (kg N m(^{-3}) d(^{-1}))</th>
<th>Original biomass</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-flow anaerobic sludge bed</td>
<td>246</td>
<td>0.11</td>
<td>Anammox granules and activated sludge</td>
<td>(Li and Sung 2015)</td>
</tr>
<tr>
<td>Bubble column continuous reactor</td>
<td>190</td>
<td>0.07</td>
<td>Anammox granules and AOB biomass</td>
<td>(Varas et al. 2015)</td>
</tr>
<tr>
<td>Up-flow biofilm reactor</td>
<td>169</td>
<td>0.35</td>
<td>Anammox biomass</td>
<td>(Cho et al. 2011)</td>
</tr>
<tr>
<td>Sequencing batch biofilm reactor</td>
<td>132</td>
<td>0.54</td>
<td>activated sludge</td>
<td>(Zhang et al. 2014)</td>
</tr>
<tr>
<td>Granular sludge bed reactor</td>
<td>52</td>
<td>0.77</td>
<td>Nitrifying sludge</td>
<td>(Wang et al. 2012)</td>
</tr>
<tr>
<td>Biofilm reactor (^b)</td>
<td>7</td>
<td>0.35</td>
<td>Anammox biomass and activated sludge</td>
<td>(Qiao et al. 2012)</td>
</tr>
<tr>
<td>Sequencing batch biofilm reactor</td>
<td>13</td>
<td>1.04</td>
<td>Anammox biofilm</td>
<td>This study</td>
</tr>
</tbody>
</table>
The addition of hydroxylamine could suppress the activity of NOB therefore decrease the production of nitrate and recover the reactor performance which was decrease by nitrate accumulation during long-term operation.
To determine the accurate variation of the population of the major bacteria involved in one-stage operation (AOB, AnAOB and NOB).

### Tab. The oligonucleotide sequences of the used primers for q-PCR analyses

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ - 3’)</th>
<th>Amplification size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOB amoA gene</td>
<td>amoA-1F GGGGTTTCTACTGGTGGT</td>
<td>550</td>
<td>Xiao et al. 2015</td>
</tr>
<tr>
<td></td>
<td>amoA-2R CCCCTCKGSAAGCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AnAOB hzsA gene</td>
<td>hzsA 1597F WTYGGKTATCARTATG</td>
<td>300</td>
<td>Harhangi et al. 2012</td>
</tr>
<tr>
<td></td>
<td>hzsA 1857R AAABGGYGAATCATARTGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitro-16S</td>
<td>Nitro-1198f ACCCTAGCAAATCTCAAAAAACCG</td>
<td>226</td>
<td>Daverey et al. 2013</td>
</tr>
<tr>
<td>Nitrospira 16S</td>
<td>NSR113F CCTGCTTCAGTTGCTACCG</td>
<td>165</td>
<td>Zeng et al. 2014</td>
</tr>
<tr>
<td></td>
<td>NSR1264R GTTTGCGACCCTTGTAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S</td>
<td>1055F ATGGCTGTCGTACGTTCGCTG</td>
<td>323</td>
<td>Zeng et al. 2014</td>
</tr>
<tr>
<td></td>
<td>1392R ACGGGCGGTTGTTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
q-PCR (quantitative polymerase chain reaction).

The addition of hydroxylamine would be beneficial for the growth and activity of AOB and AnAOB, while the population of NOB decreased with hydroxylamine addition.

The variation of population of AOB, AnAOB and NOB with presence of hydroxylamine.
The one-stage reactor was then fed with anaerobic digestion supernatant to figure out if it is available to treat the raw wastewater.

The addition of supernatant was adopted step by step. The influent ammonia was kept around 400 mg N/L while increasing the dosage of supernatant gradually (the organic compounds within supernatant increased accordingly). At the end of experiment, the ammonia in the influent was completely provided.

The different stage of the reactor operation
The reactor showed relatively stable throughout the operation, maintaining a nitrogen removing rate around 0.5 kg N/(m³ d), and the total nitrogen removal rate achieved 70 - 80%.

The sludge samples were also collected at different stage for GeoChip analyses.
GeoChip analyses

Reactor operation—Microbial community analysis—Mechanism exploration

Stable and efficient performance

Digestion supernatant
Increasing percentage

Geochip 4.0
Shift in microbial community

Combined nitritation-anammox

Nitrogen 23%
NXP 12.08%

C/N Ratio 19.59%

Unexplained 23.36%
**Functional Genes involved in Carbon cycling**

- Decrease in gene abundance
- No obvious TOC removal throughout the operation
- The organic carbons are mainly refractory organics—difficult to utilize.

![Graph](image)

*Fig. Relative abundance of the genes involved in complex carbon degradation*
Nitrogen cycling

- The genes involved in nitrogen cycling before/after supernatant addition were compared;
- Majority of these genes were also significantly decreased by introduction of supernatant;

The relative comparison of genes involved in nitrogen cycling before and after the addition of supernatant (Green color represents the decrease in gene abundance was significant. The grey-colored genes are not targeted by GeoChip 4.0. ***P<0.01, **P<0.05, *P<0.1.)
The functional genes for anammox process kept relatively stable;

The relative comparison of genes involved in nitrogen cycling before and after the addition of supernatant (Green color represents the decrease in gene abundance was significant. The grey-colored genes are not targeted by GeoChip 4.0. ***P<0.01, **P<0.05, *P<0.1.)
Correlation

- Relationship between Community Functional Structure and Reactor Performance

Matrix of Geochip Data

Matrix of Environment Variables

Correlation

<table>
<thead>
<tr>
<th>Inf. NH$_4^+$</th>
<th>Inf. NO$_2^-$</th>
<th>Inf. NO$_3^-$</th>
<th>DO</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/N</td>
<td>COD</td>
<td>HRT</td>
<td>pH</td>
</tr>
<tr>
<td>TOC</td>
<td>NLR</td>
<td>NRR</td>
<td>T</td>
</tr>
<tr>
<td>Eff. NH$_4^+$</td>
<td>Eff. NO$_2^-$</td>
<td>Eff. NO$_3^-$</td>
<td>Removal rate</td>
</tr>
</tbody>
</table>
Correlation

- Relationship between Community Functional Structure and Reactor Performance

- A strong relationship between whole microbial community structure and environmental variables

Fig. The similarity test between GeoChip and environmental variables.
7 key environmental variables were selected out from 16 potential variables through CCA analyses (Canonical correspondence analysis);
Key environmental variables

- ammonia, nitrite and nitrate in the influent, pH, Temperature, C/N ratio and TOC

CCA analyses
Contribution to the performance

- ammonia, nitrite and nitrate in the influent, pH, Temperature, C/N ratio and TOC

Nitrogen compounds
Parameters
C/N ratio
Contribution to the performance

- ammonia, nitrite and nitrate in the influent, pH, Temperature, C/N ratio and TOC

Variation partitioning analysis (VPA) were engaged to analyze the corresponding contribution of these variables groups to the reactor performance.
A total of 76.64% of community variations could be explained by these selected variables (this model is significant).

- The circles represent the specific variable groups by partitioning out the effects of the other groups.
- The squares represent the joint effect of the circles on either side of the square.
- The portion unexplained by any of the tested variables is represented by the rectangle at the bottom of the figure.
The C/N ratio showed significant contribution to the reactor performance and also intensive interaction with other two groups;

The key environmental factor shaping microbial community

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- The squares represent the joint effect of the circles on either side of the square.
- The portion unexplained by any of the tested variables is represented by the rectangle at the bottom of the figure.
Innovative reactor to achieve high-rate one-stage combined nitritation-anammox reactor maintaining stability during long-term operation.

<table>
<thead>
<tr>
<th></th>
<th>One-stage</th>
<th>Two-stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construction</td>
<td>One</td>
<td>Two</td>
</tr>
<tr>
<td>GHG release</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Nitrite release</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Engineering</td>
<td>Wide</td>
<td>Low</td>
</tr>
<tr>
<td>Loading</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Stability</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>
- **Aeration in the middle part**: Enhancement of AOB

  The bubbles would rise so that the concentration of dissolved oxygen differed in different parts of the reactor.

- **Anammox granules**: Efficient anammox activity

  The heavier anammox granules would only stay in the lower part of the reactor thus be well protected from oxygen inhibition.

- **On-off switch**: Inhibition of NOB

  AOB and NOB located in the flocculent sludge and the SRT of them could be controlled separately, regardless of AnAOB. The ‘On-off ’recycle of the flocculent between the aeration/anoxic condition was also beneficial for the select-out of NOB.
The anammox granules and flocculent sludge (mainly consisted of AOB and NOB) could separated from each other during pretty short time due to significant difference in settleability.
High-rate one-stage reactor

- The anammox granules stay in the lower part of the reactor throughout the operation.
- The interface between anammox granules and flocculent sludge was very clear especially in decanting phase.
# High-rate one-stage reactor

<table>
<thead>
<tr>
<th>Sludge form</th>
<th>TN removal rate</th>
<th>Nitrogen removing rate kg N/(m³d)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm</td>
<td>89%</td>
<td>0.3-0.4</td>
<td>Rosenwinkel et al (2005)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>82%</td>
<td>0.31-0.45</td>
<td>Furukawa et al (2006)</td>
</tr>
<tr>
<td>Flocculent sludge</td>
<td>85-90%</td>
<td>0.6</td>
<td>Wett (2007)</td>
</tr>
<tr>
<td>Flocculent sludge</td>
<td>88%</td>
<td>0.5</td>
<td>Joss et al (2009)</td>
</tr>
<tr>
<td>Flocculent sludge</td>
<td>89%</td>
<td>1.2</td>
<td>Abama et al (2010)</td>
</tr>
<tr>
<td>Flocculent sludge</td>
<td>73%</td>
<td>0.26</td>
<td>Desloover et al (2011)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>65-75%</td>
<td>1.1</td>
<td>Christensson et al (2011)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>70-80%</td>
<td>1.0</td>
<td>Ren et al (2014)</td>
</tr>
<tr>
<td>Granules</td>
<td>50-70%</td>
<td>2.0</td>
<td>Wang et al (2014)</td>
</tr>
<tr>
<td>Biofilm</td>
<td>70-85%</td>
<td>2.3</td>
<td>Zheng et al (2014)</td>
</tr>
<tr>
<td>Granules-Flocculent Sludge</td>
<td>75-80%</td>
<td>6.2</td>
<td>This Study</td>
</tr>
</tbody>
</table>
The anammox-based technologies especially one-stage combined nitritation-anammox process could be a promising alternative for biological nitrogen removal, including sidestream and mainstream treatment. The application of this process will be beneficial for energy efficiency of WWTPs.

The integrated molecular technologies especially metagenomic (GeoChip, for example) provide powerful and convenient approach for in-depth exploration of the complicated reactor system or ecosystem.

More attempts and application of these technologies would be expected in the near future!
Thank you for your attention!