Activated Sludge Sampling & Shipping Protocols

Objective: Target the **aerobic stage** of wastewater treatment plants using activated sludge processes. Take samples from a minimum of **4** plants for each geographic site (e.g. a large city), and a minimum of **3** samples per plant. Such sample design will allow reasonable statistical test and null model analysis.

1. Field sampling (see Appendix I for detail)

- Complete the sampling data sheet (Appendix II) for each plant.
- Take well-mixed samples (>20 mL each) from at least 3 different positions of the aerobic zone in the same aeration tank, e.g. in the front, middle and end part.

2. Sample preparation and storage (see Appendix I for detail)

- For your lab or your collaborators who have centrifuge and freezer:
  - Keep mixed liquor samples on ice and transport to lab within 24 hours.
  - Shake up the 20-mL sample and divide into aliquots (e.g. 3 x 3 mL + 10mL).
  - Centrifuge the samples at 15,000 *g* for 10 min.
  - Discard the supernatant and preserve the pellets at -80°C (or -20ºC for short term).
  - A collaborator should send frozen pellets with enough dry ice and ice packs to your lab by 24-hour or 48-hour shipping.
- For your collaborators who cannot centrifuge the samples
  - Send mixed liquor samples with enough ice packs to your lab as quickly as possible. It is recommended to send out within 12 hours after sampling, by 24-hour shipping.

3. Send us frozen pellets or DNA.

- Option 1: Extract and send DNA. This is better to avoid unexpected variations.
  - Use the pellet from 3-mL mixed liquor to extract DNA by identical protocol (see Appendix III).
  - Store DNA in 1xTE buffer at -80°C. Store the rest pellets as backup at -80°C.
  - Before shipping, contact Joy Van Nostrand (joy.vannostrand@ou.edu) and Daliang Ning (ningdaliang@ou.edu).
  - Keep DNA in TE buffer and send with enough dry ice and ice packs to Joe's Lab by 2 days shipping. Please avoid arrival during the weekend. See Appendix IV for international shipping notes.
- Option 2: Send frozen pellets if they can surely arrive within 48 hours or faster.
  - Ask Joy Van Nostrand (joy.vannostrand@ou.edu) or Daliang Ning (ningdaliang@ou.edu) for special import permit and detail notes.
  - Send the frozen pellets with enough dry ice and ice packs to Jeo's lab by overnight or 48-hour shipping. Please avoid arrival during the weekend. See Appendix IV for international shipping notes.

Joe's Lab
Recipient: Joy Van Nostrand Phone: +1-405-325-4403
Address: 101 David L Boren Blvd SRTC 2030, Norman, OK, USA 73019

Please feel free to contact Daliang Ning (ningdaliang@ou.edu) if you have any questions or suggestions.

Appendix I Detailed Protocol for Sampling and Shipping

In short, please i) complete a sampling data sheet, ii) take 3 mixed liquor samples from the aerobic zone of one aeration tank in a plant using activated sludge process*, iii) and transport samples to lab.

* If a site has less than 4 plants, please take more samples per plant (e.g. 6 samples from 2 tanks in one plant) to ensure at least 12 samples per site.
** If a plant has different activated sludge processes (e.g. conventional plug flow and oxidation ditch), it is recommended to take 3 samples from each process.

Detail procedures

If you have centrifuge and freezer

1. Preparation before sampling
   (1) Preparation for sampling:
   Sampling Data Sheet (1/plant), a cooler (or foam box) with enough ice,
   50-mL tubes (sterile, 1/sample), a sampler (if not available in the plant),
   a wash bottle containing clean water, gloves, pen and marker pen.
   * pH/DO/temperature meters (if available).
   (2) Preparation for centrifuge and storage:
   15mL, 1.5mL or 5mL tubes (sterile, for 3mL and 10mL sample),
   Parafilm and/or sealed plastic bags as secondary container,
   Centrifuge, Freezer, pipettor and tips.
2. Sampling
   (3) Fill the Sampling Data Sheet (provided) as fully as possible.
   ** You may also complete the Data Sheet after sending back samples and email to us.*
   (4) Figure out sampling positions.
   Typical sampling positions in an aeration tank are in the front, middle and end part of aerobic zone. If you can only reach one position of a tank, please take 3 sequential samples with 30-min interval.
   (5) Take out a 50-mL tube, make a label on the top and side of the tube by a marker pen.
   (6) Rinse the sampler by mixed liquor at the position. Take about 1 liter mixed liquor.
   (7) Shake or stir this 1-L mixed liquor and then transfer >20 mL to the tube.
   (8) Cap the tube tightly and chill on the ice. Wash the sampler by clean water.
   (9) Fill the sampling information in Sampling Data Sheet. Attention to sample labels.
   * Measure DO/pH/temperature if the meters are available.
   (10) Go to next position and repeat step (5)~(9).
   (11) Keep samples on ice and transport to lab within 24 hours.
3. Centrifuge and storage
   (12) Shake up a sample, divide into 3x 3mL and 1x 10mL and discard the rest. Make labels.
   * Keep the tubes on ice to keep liquor samples cold. Sample labels are important.
   (13) Centrifuge at 4°C, 15,000 g for 10 min. Discard the supernatant.
   ** If the centrifuge cannot be set at 4°C, you need to keep mixed liquor samples at room*

temperature instead of chilling on ice when sampling, and centrifuge within 3 hours after sampling.

(14) If you will ship pellets, you may use Parafilm to seal the cap of each tube, or put tubes of one sample into one plastic bag to avoid unexpected spillage and cross contamination when shipping. Then preserve the pellets at -80°C (-20°C is applicable for a short-term storage).

4. Packing and shipping

(15) Prepare tools
Carton box, foam box as cooler, enough dry ice and some frozen ice packs, wadded paper, other files required by courier.
* If shipping only takes 2 days or less, you may just use dry ice without ice packs. Consult a courier for notes, proper documentation and labeling requirements when using dry ice.

(16) Pack samples into a foam box, surround the samples by ice packs and put most dry ice on the top of the samples. Fill any empty space with wadded paper.

(17) Band the foam box and outer packaging (cardboard) tightly by tape. But DO NOT SEAL DRY ICE, it may explode when turning to gaseous carbon dioxide.

(18) Paste required dry ice label and mark "This Side Up" and "Freeze Upon Arrival" on the outside of carton box.

(19) Send as soon as possible after packing. Let the recipient know once you send.

If your collaborator cannot centrifuge, he/she may collect and send mixed liquor samples to your lab as follows.

1. Preparation before sampling and shipping

(1) Please take samples and send them on Monday or Tuesday.

(2) Preparation for sampling
Sampling Data Sheet (1/plant), a cooler (or foam box) with enough ice,
50-mL tubes (sterile, 1/sample), sealed plastic bags (1 bag/sample),
a sampler (if not available in the plant), a wash bottle containing clean water,
gloves, pen and marker pen.
* pH/DO/temperature meters (if available).

(3) Preparation for shipping
Carton box, foam box as cooler, ice packs, wadded paper, other files required by courier.
Freeze enough ice packs (-20°C, overnight is ideal) before sampling.
If necessary, schedule the courier previously to **ensure sending within 12 hours after sampling**.

2. Sampling

(4) Fill the Sampling Data Sheet (provided) as fully as possible.
** You may also complete the Data Sheet and email to us after sending back samples. Because timing is important for mixed liquor samples.*

(5) Figure out sampling positions.
Typical sampling positions in an aeration tank are in the front, middle and end part of aerobic zone. If you can only reach one position of a tank, please take 3 sequential samples with 30-min interval.

(6) Take out a 50-mL tube, make a label on the top and side of the tube by a marker pen.
(7) Rinse the sampler by mixed liquor at the position. Take about 1 liter mixed liquor.
(8) Shake or stir this 1-L mixed liquor and then transfer >20 mL to the tube.
(9) Cap the tube tightly, put it into a plastic bag, and chill on the ice. Wash the sampler by clean water.
(10) Fill the sampling information in Sampling Data Sheet. Attention to sample labels.
* Measure DO/pH/temperature if the meters are available.
(11) Go to next position and repeat step (6)~(10).

3. Packing and shipping:

(12) Pack samples into a foam box, surround the samples by frozen ice packs and put one ice pack on the top of the samples. Fill any empty space with wadded paper.
* If using dry ice, you may freeze the samples before packing and put the dry ice on the top of the samples.
(13) Band the foam box and outer packaging (cardboard) tightly by tape. DO NOT SEAL DRY ICE, it may explode when turning to gaseous carbon dioxide.
(14) Mark "This Side Up" and "Refrigerate Upon Arrival" on the cardboard.
(15) Send within 12 hours after sampling. Let the recipient know once you send.

See Appendix IV for international shipping notes before shipping samples to USA.

Appendix II Sampling Data Sheet

1- Plant ID, contact information and Location			
Name of WWTP			
WWTP ID			
Office Phone		**Fax**	
Technician name		**Email**	
Address			
Longitude		**Latitude**	
Air temperature	**Mean annual _____ Range _____**		

2- Basic information			
Age of Plant		Designed Capacity (m^3/d)	
Actual Influent Flow Rate (m^3/d)			
Influent: BOD_____(COD_____) NH_4-N_____ TN_____ TP_____		Effluent: BOD_____(COD_____) NH_4-N_____ TN_____ TP_____	
Industrial wastewater in influent:		□ No □ Yes, percentage_____% □ unknown	
Sludge Age (SRT) (Days)			
HRT in the whole plant		HRT in each aeration tank	
Volume of aeration tanks (total, each)			
Recycling Ratio(return sludge flow/influent flow)			
*If the sampled tank is SBR: Discharge Volume_____ Volume exchange ratio_____ Cycle time_____ =Fill time_____+React/Settle time_____+Draw time_____+Idle.			
Nitrification? Yes or No		Denitrification? Yes or No	
Activated Sludge Process Type [1]			
Nitrification Process Type [2]			
Denitrification Process Type [3]			

3- Sampling Information	
Sample Collection Date & Time (MM-DD-YY hr:min)	____-____-_____ ____:____
Information of Sampled Aeration Tank 1# Process Type_____	
Influent of this tank	BOD_____(COD_____), NH_4-N_____, TN_____,TP_____
MLSS Temperature (°C)	*When sampling_____; Winter_____Summer_____*
Dissolve Oxygen (DO, mg/L)	*When sampling_____; Yearly average_____*
MLSS pH (Units)	*When sampling_____; Yearly average_____*

Sample Label	DO (if available)	Sampling Position

Welcome additional notes and documents to clarify the answers.

Additional Form in case of more than 3 samples from this plant

Information of Sampled Aeration Tank 2# **Process Type_____**		
Influent of this tank	BOD_____(COD_____), NH_4-N_____, TN_____,TP_____	
MLSS Concentration (mg/l)	*When sampling_____; Yearly average_____*	
MLSS Temperature (°C)	*When sampling_____; Winter_____Summer_____*	
Dissolve Oxygen (mg/L)	*When sampling_____; Yearly average_____*	
MLSS pH (Units)	*When sampling_____; Yearly average_____*	
Sample Label	DO (if available)	Sampling Position
Information of Sampled Aeration Tank 3# **Process Type_____**		
Influent of this tank	BOD_____(COD_____), NH_4-N_____, TN_____,TP_____	
MLSS Concentration (mg/l)	*When sampling_____; Yearly average_____*	
MLSS Temperature (°C)	*When sampling_____; Winter_____Summer_____*	
Dissolve Oxygen (mg/L)	*When sampling_____; Yearly average_____*	
MLSS pH (Units)	*When sampling_____; Yearly average_____*	
Sample Label	DO (if available)	Sampling Position
Information of Sampled Aeration Tank 4# **Process Type_____**		
Influent of this tank	BOD_____(COD_____), NH_4-N_____, TN_____,TP_____	
MLSS Concentration (mg/l)	*When sampling_____; Yearly average_____*	
MLSS Temperature (°C)	*When sampling_____; Winter_____Summer_____*	
Dissolve Oxygen (mg/L)	*When sampling_____; Yearly average_____*	
MLSS pH (Units)	*When sampling_____; Yearly average_____*	
Sample Label	DO (if available)	Sampling Position

Additional Notes:

Notes about Process Type in the Sampling Data Sheet

Please try to use the Process names below to help us get consistent information. You are welcome to discuss with Daliang Ning (ningdaliang@ou.edu) to figure out the type name.

[1] List of Active Sludge Process Type:

□ Conventional Plug Flow □ Complete Mix □ Step Aeration
□ Sequencing Batch Reactor (SBR) □ Pure oxygen □ Contact Stabilization
□ Extended Aeration □ Deep shaft □ Deep tank
□ Oxidation ditch □ Step feed
□ Kraus Process (digested sludge added to return sludge) □ Other_____

[2] List of Nitrification Process Type:

□ Combined carbon oxidation and nitrification (the same unit/tank, process as [1])
□ Separate-stage nitrification:
□ Active sludge process for nitrification_____(select a type from [1])
□ Trickling Filter □ Rotating Biological Contactor □ Other_____

[3] List of Denitrification Process Type:

□ A^2/O (3-stage PhoRedox Process. Anaerobic/Anoxic/Oxic, return sludge back to Anaerobic, MLSS from Oxic to Anoxic)
□ A^2OAO (5-stage PhoRedox Process/Modified Bardenpho Process. Anaerobic/Anoxic/Oxic/Anoxic/Oxic, return sludge back to Anaerobic, MLSS from 1^{st} Oxic to 1^{st} Anoxic)
□ MLE (Modified Ludzack Ettinger Process. Anoxic/Oxic, return sludge and MLSS back to Anoxic)
□ UCT (University of Cape Town system. Anaerobic/Anoxic/Oxic, return sludge back to Anoxic, MLSS from Oxic to Anoxic and from Anoxic to Anaerobic)
□ VIP (Virginia Initiative Plant system. Anaerobic/Anoxic/Oxic, sludge back to head of Anoxic, MLSS from Oxic to head of Anoxic and from end of Anoxic to Anaerobic)
□ Oxidation ditches
□ Cyclical nitrogen removal (CNR, switching the aerators on and off)
□ Other_____
(Wuhrmann process, Ludzack Ettinger process, Bardenpho process, Modified UCT, Schreiber process, BioDenipho, etc.)

Appendix III DNA Extraction Protocol

In 2014, we will all use Powersoil® DNA Isolation Kit to extract DNA from activated sludge.

Materials

(1) Powersoil® DNA Isolation Kit (MOBIO Catalog# 12888-50 or 12888-100)

http://www.mobio.com/soil-dna-isolation/powersoil-dna-isolation-kit.html

A manufacture protocol will be provided in the Kit

(2) Vortex Genie® 2 Vortex (MOBIO Catalog# 13111-V or 13111-V-220)

http://www.mobio.com/vortex-and-vortex-adapters/vortex-genie-2-vortex.html

(3) Vortex adapter for 1.5~2 mL tubes (MOBIO Catalog# 13000-V1-24)

http://www.mobio.com/vortex-and-vortex-adapters/vortex-adapters-for-vortex-genie-2.html

(4) 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA.Na_2, pH=8, sterile, DNase free)

Prepare by yourself or purchase a qualified solution (e.g. 100x TE from Sigma, Catalog# T9285-100ML, http://www.sigmaaldrich.com/catalog/product/sigma/t9285?lang=en&region=US)

Notes in addition to manufacture protocol:

(1) For each sample, use a pellet from 3 mL mixed liquor to extract DNA by one prep of the kit.

(2) Once take the pellet out of freezer, use the bead solution in a PowerBead tube (provided in the kit) to melt, resuspend and transfer the pellet to a PowerBead tube.

(3) Extract 12 samples per round. Always place 12 bead tubes on the Vortex evenly and vortex at maximum speed for 10 min. If you are dealing with less than 12 samples, put some fake bead tubes to ensure 12 bead tubes on the vortex.

Because the number of tubes on the vortex and vortex time can influence lysis efficiency. Let us do it in the same way to minimize the differences between labs.

(3) **DO NOT use solution C6** but use 100 µL 1x TE buffer to elute DNA from the filter at the Step 20 in the manufacture protocol.

* C6 in the kit contains no EDTA. To avoid DNA degradation, we use TE instead of C6.

(4) In the end, use Nanodrop to check the DNA quality and quantity.

260/280 ~1.8, 260/230≥1.7, DNA amount > 3 μg (>5 µg is ideal)

(5) Seal each tube by Parafilm or put each one into a sealed plastic bag.

(6) Store DNA at -80°C (If not available, -20°C may be OK for a short-time storage).

Detailed Shipping Notes for International Samples

Here are some common notes. It is highly recommended to consult the shipping company and relevant departments in your school/country for formal regulations and requirements. A colleague who has sent frozen samples with dry ice to USA is also helpful.
Ask Daliang Ning (ningdaliang@ou.edu) for highlighted files in following text.

- **Export license and some certification**
  - Many countries take items including viruses, bacterium, toxins etc. very seriously. Please check whether you need to apply for an export permit before preparing to ship samples. Usually, sludge pellet samples need export license, while DNA samples do not.
  - Some countries or regions may ask for certification or agreement to meet the law of toxic or hazardous substance control. You may check it with relevant departments.
- **Import Permit for sludge pellet samples or formal letter for DNA samples**
  - For sludge pellet samples:
    The USDA has strict regulations regarding transporting soils/sludge into the US from foreign countries. I will send you the import permits once you need. Both of these ("Permit to receive soil" and "Soil sample label") should be attached to the outside of the shipping container.
  - For DNA samples:
    You need a letter on your institution's letterhead with the following or similar text:
    "

    To Whom It May Concern:

    The enclosed samples are purified nucleic acid (DNA) samples and are for research purposes only. They are non-toxic, non-pathogenic, and non-infectious.
    They belong to the category of Microbially Produced Materials (USDA category number 1110) for which no import permit is required.
    They will be analyzed for DNA sequence composition for a research project in the University of Oklahoma, Norman, OK, USA.
    "

    This letter should be included with any customs or export paperwork that needs to be included.
    You also need a Toxic Substance Control Act Certification (TSCA). When you are ready to ship, please ask Daliang (ningdaliang@ou.edu) or Joy (joy.vannostrand@ou.edu) for a signed Toxic Substance Control Act Certification. You need to put the date and the tracking number into the form
- **Commercial Invoice**

  - In most cases, you must state declared value for customs with the appropriate country currency, even if your shipment contains only samples or research materials and it is not for resale. Ask an experienced people or the shipping company for the source of applicable commercial invoice. We will provide an example "commercial invoice".
  - You are usually required to submit three signed commercial invoices.
- **Labels on the package (provided)**
  - "Dry Ice Label" is required.
    Minimum dimensions: 100 x 100mm, Symbol (Seven vertical stripes in upper half): Black, Background: White.
  - Following markings around the "Dry Ice Label" are required:
    - The net quantity of dry ice, in kilograms.
    - Name and address of both the sender and recipient.
  - "Freeze upon arrival" and "upward" labels are recommended.
- **FedEx Air Waybill (reference provided)**
  - We will prove an example of "Air Waybill". Dry ice information is required.
  - Choose 2-day shipping option.
- **Additional notes for packing with dry ice**
  ***** Sludge samples must be packed with dry ice. If it is really difficult to use dry ice, you should send DNA samples with enough ice packs in the Styrofoam cooler. In this case, please check that all files have nothing about dry ice and do not use the dry ice label.**
  - Dry ice releases carbon dioxide gas which can build up enough pressure to rupture the packaging. You must ensure the packaging you use allows the release of this pressure to prevent rupturing the package.
    For example; do not use steel drums or jerricans as outer packaging, and do not place dry ice within sealed plastic bags.
  - A Styrofoam cooler within a carton box works well as insulation. Ensure the Styrofoam IS NOT sealed to be airtight.
  - The samples should be in leak-proof containers, e.g. tubes sealed by Parafilm or in sealed bags. DO NOT freeze dry or thaw the DNA samples. TE buffer, dry ice and/or ice packs can protect DNA when shipping.
  - Pack the frozen DNA samples into the Styrofoam box, surround the samples by ice packs and put most dry ice on the top of the samples. Fill any empty space with wadded newspaper. It may need 9 pound dry ice for one package. Please ask the dry ice seller for suggestion about 2-day shipping.
  - Make sure the outside of the shipping container and the sample containers are clean and free of sludge or soil.
- **Pasting on the outside of package**

- Please check following files/labels are pasted on the outside of package:
  Export license and/or certification, import permit for sludge pellets or formal letter and TSCA for DNA samples, commercial invoice, dry ice label with markings, Air Waybill and freeze upon arrival label.

  If you use a shipping company to ship the soils, please make sure they know the permits need to be on the outside of the package. We have had packages returned to the sender because the permit was on the inside of the box instead of the outside.

It is very important to follow these instructions and regulations in your country. If your shipment is stopped at customs, the USDA can deny entry to any packages and the samples will be returned to you.

The package should be shipped directly to Joy at this address:
Joy D. Van Nostrand
Institute for Environmental Genomics, University of Oklahoma
101 David L Boren Blvd, SRTC 2030
Norman, OK 73019