AD Sludge Sampling & Shipping Protocols (Quick-view Page, version 20190307)

Objective: Target the **anaerobic digestion (AD) process,** mainly focus on **conventional AD treating sludge** in or from **municipal** wastewater treatment plants.

1. Field sampling (see Appendix I for details)

- **Time points**: We asked for at least one batch of samples in summer (one-time-point). It is recommended to take 4 batches (seasonally) or 2 batches (one in summer, the other in winter) of samples. Monthly or weekly sampling are welcome, but please let us know in advance. Additional sampling when the digester experiences upsets (poor performance) is highly recommended.
- **Minimum sample number**: It is recommended to sample **≥4** plants each site (a large city or a region) if applicable. Sample number requested from each plant is as below.

Position	Sample number each plant each time point
Anaerobic digestor (primary digestor) sludge	**≥3** (one sample each digestor if applicable)
Feed sludge	**≥1**
Anaerobic digestor influent (after pretreatment)	**≥1** if there is pretreatment
Co-digestion material(s)	**≥1** if applicable
Secondary digester sludge	**≥1** if applicable

- At each time point:
  - Complete the data sheet (Appendix II) for each plant.
  - Take samples (~45 mL each) from each digester (primary digester). If need multiple samples from one digester, take them from different positions or with 10~30 min interval.
  - Take a representative sample (~45 mL) of feed sludge, as well as co-digestion material, pretreatment effluent, and secondary digester sludge if applicable, see appendix I.

2. Sample preparation and preservation (see Appendix I for details)

- For collaborators who cannot centrifuge the samples
  - Send the 45-ml samples with enough ice packs to Zhou lab or a regional coordinator's lab as quickly as possible. Please send within 12 hours after sampling, by 24-hour shipping.
- For collaborators who have centrifuge and freezer:
  - Keep samples on ice and transport to lab within 24 hours.
  - Stir the 45-mL sample and take 3 aliquots (3 x 1.5 mL) from each sample.
    If you cannot measure some physical-chemical properties on the data sheet, please keep the remained 40-mL samples in a freezer (-80°C or -20ºC).
  - Centrifuge the 1.5-mL samples at 15,000 *g* for 10 min.
  - Discard the supernatant and preserve the pellets in a freezer (-80°C or -20ºC).
  - If you can, please send frozen pellets and the 40-ml samples with enough dry ice to Zhou lab or a regional coordinator lab by 24- or 48-hour shipping.
  - If you cannot ship sludge samples with dry ice, you may extract DNA using the pellets from 1.5-mL samples (see Appendix III for protocol). Store purified DNA in 1xTE buffer at -80°C. Store the rest pellets as backup at -80°C. Keep DNA in buffer and send to Zhou lab or a regional coordinator lab with enough ice packs by 2-day shipping.

3. Additional shipping notes:

- Please contact recipient before shipping, to avoid arrival at the weekend or any holiday.
- International shipping may need special documentation. See Appendix IV for details.

Zhou Lab Recipient: Joy Van Nostrand Phone: +1-405-325-4403
Address: 101 David L Boren Blvd SRTC 2030, Norman, OK, USA 73019
Contact: Daliang Ning (ningdaliang@ou.edu) and Joy Van Nostrand (joy.vannostrand@ou.edu)

Appendix I Detailed Protocol for Sampling and Shipping

Sampling Plan: Please provide at least one batch of samples in summer (**one-time-point**). If applicable, please provide 4 batches (one each season, so-called **seasonally**) or 2 batches (one in summer, the other in winter, so-called **summer & winter**) of samples. You are welcome to provide monthly or weekly samples for a year, but please communicate with Zhou lab (contact: Daliang Ning, ningdaliang@ou.edu) or a coordinator lab to let us get good preparation in advance. Additional sampling when the digester experiences upsets (poor performance) is strongly recommended (so-called **accidentally**).

Workflow:

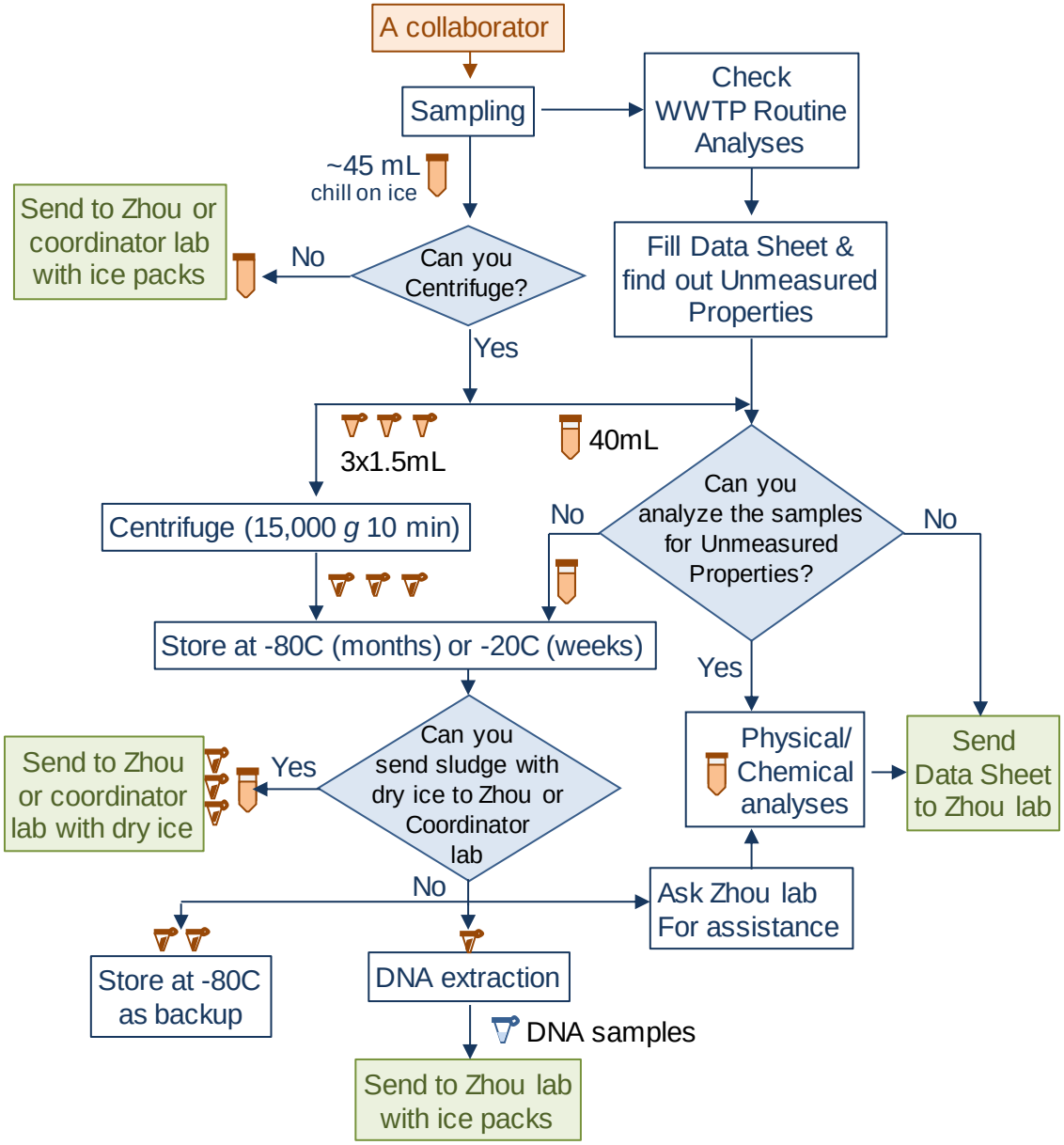


Figure 1 The scheme of sampling and shipping under different situations

Detailed procedure

Figure 1 shows the scheme of sampling and shipping under different situations. Detailed steps are described as below:

1. Preparation before sampling
   (1) Check with WWTP lab. It is better to take samples at the same day when WWTP will do routine physical chemical analyses for the digester.
   (2) Preparation for sampling:
   Sampling Data Sheet (1/digester), a cooler (or foam box) with enough ice,
   50-mL tubes (sterile, 1/sample), a sampler (~1 L), a bucket (for trash).
   a wash bottle containing clean water, gloves, pen and marker pen.
   pH meter, temperature meter.
   (3) Preparation for sample treatment, storage, and shipping:
   * If you CANNOT centrifuge (15,000 *g* for 1.5 mL tubes):
   Sealed plastic bags as secondary container (1/sample, to pack 50-mL tube),
   carton box, foam box as cooler, filling material (e.g. wadded newspaper),
   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.
   * If you CAN centrifuge (15,000 *g* for 1.5 mL tubes):
   1.5mL tubes (sterile), centrifuge, pipettor and tips;
   Freezer (-20°C for a week, -80°C for long-term);
   Carton box, foam box as cooler, enough frozen ice packs, filling material (e.g. wadded newspaper), dry ice is required if shipping sludge;
   Check Appendix IV and communicate with the courier for proper documentation and labeling requirements for your shipping.
   If you need to do DNA extraction, check Appendix III for necessary materials.
   If you need to do some physical chemical analyses, check standard methods for necessary materials.
   (4) Remember to contact the recipient before any shipping and avoid arrival at any weekend or holiday.

2. Sampling
   (1) Fill the Sampling Data Sheet (provided) as fully as possible.
   (2) Figure out sampling positions and record clearly on Data Sheet (Appendix II).

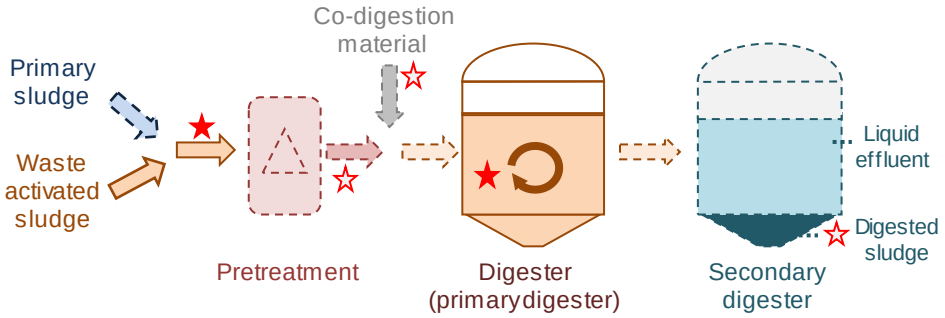


Figure 2 Typical sampling positions (stars) in anaerobic digestion system
The elements drawn in dash lines (e.g. pretreatment, co-digestion material, secondary digester) and relative sampling positions (unfilled stars) are highly recommended for those digestion systems if applicable.

Digester (or primary digester in two-stage digestion): *At the time point of global survey in summer*, our target is to ensure ≥3 digester samples per plant and ≥1 sample from each digester. Thus, in some cases (e.g. a plant has only one digester), we may need multiple samples from one digester. If so, please take them at different positions or with 10~30 min interval. *At the other time points if scheduled*, you may take only 1 sample from a selected digester in a selected plant.
Feed sludge: please take ≥1 sample of the feed sludge for the sampled digester(s) in each plant. It is required for the global survey in summer and recommended for other time points.
Co-digestion material: If applicable, please take a bag (50~100 g wet weight) or a tube (50 mL) of each co-digester material to the sampled digester(s) in each plant. It is highly desired for the global survey in summer but not required for other time points.
Digester influent: If applicable, take one sample of the influent of the sampled digester(s) in each plant, which is usually the effluent of pretreatment. Please clarify whether it is collected before or after mixed with co-digestion material on the Data Sheet (Appendix II). It is required for the global survey in summer and other time points.
Secondary digester: two-stage digestion will have a secondary digester. If applicable, take one sample of digested sludge from secondary digester in each plant. It is highly desired for the global survey in summer but not required for other time points

(3) At each sampling position, take out a 50-mL tube, make labels on the top and the side of the tube.
(4) Rinse the sampler by sludge at the position.
(5) Take about 1 liter sample and transfer 45 mL to the tube. Cap the tube tightly and chill on the ice.
(6) If there is online temperature and/or pH meter, record the temperature and/or pH when sampling. Otherwise, measure temperature and pH at the position if applicable, or in the 1-L sludge as soon as possible.
* The temperature in digester is higher than air temperature. Thus, if you measure

temperature in the 1-L sludge, please measure right after taking it from the digester and record the highest reliable temperature value.

(7) Wash the sampler by clean water.
(8) Go to next position and repeat step (5)~(9).
(9) Double check the sample list information on Data Sheet.
(10) Keep samples on ice and transport to lab within 24 hours.

If you cannot centrifuge:

3. Send 45-mL samples with ice packs:
   (1) Put each tube into a sealed plastic bag to avoid unexpected spillage and cross contamination when shipping.
   (2) 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.
   (3) Band the foam box and outer packaging (cardboard) tightly by tape.
   (4) Mark "This Side Up" and "Refrigerate Upon Arrival" on the cardboard.
   (5) Send within 12 hours after sampling and use 24-hour (or over-night) shipping. Let the recipient know once you send.

If you can centrifuge:

4. Centrifuge and storage:
   (1) Shake up a sample, take 3 x 1.5mL out and keep 40mL in the tube. Make labels.
   * Keep the tubes on ice to keep liquor samples cold. Sample labels are important.
   ** Keep the 40-ml samples for chemical analyses.
   (2) Centrifuge the 1.5-ml samples 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 the samples at room temperature instead of chilling on ice when sampling, and centrifuge within 3 hours after sampling.*
   (3) If you will ship frozen samples, you may put tubes of one sample into one plastic bag to avoid unexpected spillage and cross contamination when shipping. Then preserve the samples at -80°C (-20°C is applicable for a short-term storage).

If you can analyze the samples for unmeasured properties:

5. Physical chemical analyses.
   (1) If supernatant will be analyzed by IC (ion chromatography), please filter 10 mL water sample by 0.2 μm pore-size membrane and analyze as soon as possible. Samples which cannot be analyzed immediately should be preserved at 2~4°C dark place.
   (2) For other chemical analyses, the water sample should be pretreated (e.g. acidified by H_2SO_4 to pH<2) and preserved at 2~4°C dark place if you cannot measure right after sampling.

(3) Detailed sample preservation and analyses methods are suggested as below. The detected value should be calibrated by standards as QC, especially when using alternative methods or standard methods in your country.

Table 2 Standard methods for physical chemical analyses

Property	Preservation	Standard method	Alternative method
pH	Measure on site	ISO 10523:2008 (pH meter)	Reliable pH meter
Liquor temperature	Measure on site	Standard thermometer	
TS (total solid)	4°C, <7 days	APHA 2540 B (Dried at 103-105°C)	US EPA method 1684
VS (volatile solid)	4°C, <7 days	APHA 2540 E (Ignited at 550°C)	US EPA method 1684
TDS (total dissolved solid)	4°C, <7 days	APHA 2540 C (Dried at 180°C)	US EPA method 160.1
COD_{Cr}	H_2SO_4 pH<2, 4°C, <7 days or <28 days	ISO 6060:1989 (Dichromate method)	ISO 15705:2002 (Sealed tube method) HACH Method 8000
VFAs	0.2 μm filter, 4°C, <7 days (optional: pH>12)	APHA 5560D (Chromatography method)	
Acetate			
NH4-N	H_2SO_4 pH<2, 4°C, ASAP, <24 hours	ISO 7150-1:1984 (Salicylate method)	HACH Method 10205
TN	H_2SO_4 pH<2, 4°C, <1 week	ISO 11905-1:1997 (Peroxodisulfate method)	HACH Method 10208
Sulfate	4°C, <7 days	APHA 4500-SO_4^{2-} B (ion chromatography)	HACH Method 8051
Sulfide	pH>9, 4°C, <2 weeks	APHA 4500-S^{2-} D (Methylene Blue)	HACH Method 8131
(Optional) Heavy metals (except Cr^{VI})	pH<2 (by HNO_3), < 6 months (Hg <28 days)	US EPA method 6010C (ICP-AES for Al, Sb, As, Ba, Be, Cd, Cr, Co, Cu, Pb, Mn, Ni, Ag, Tl, Zn, etc.)	US EPA method 6020A (ICP-MS)

* A package of all standard methods will be available to any collaborators as needed.
** Equivalent standard methods in a collaborator's country or qualified alternative methods can be acceptable, but please communicate with Zhou lab in advance.

If you cannot determine the properties:

6. Send sludge samples with dry ice.
   (1) Put the tubes of each sample into a sealed bag to avoid unexpected spillage and cross contamination when shipping.
   (2) 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.
   (3) Band the foam box and outer packaging (cardboard) by tape. But DO NOT SEAL DRY ICE, it may explode when turning to gaseous carbon dioxide.
   (4) Paste required dry ice label and mark “This Side Up” and “Freeze Upon Arrival” on the outside of carton box.
   (5) Send as soon as possible after packing. Use 24-hour or 48-hour shipping. Let the recipient know once you send.

If you cannot send sludge samples with dry ice:

7. DNA extraction and shipping.
   (6) Use the pellets from 1.5-mL samples. For each sample, one tube of pellet (from 1.5 mL sludge) is usually enough. Use more if need. See Appendix III for detailed protocol.
   (7) Store purified DNA in 1xTE buffer at -80°C. Store the rest pellets as backup at -80°C.
   (8) Keep DNA in buffer and send with enough ice packs by 2-day shipping.
   Put each DNA sample into a sealed plastic bag to avoid unexpected spillage and cross contamination when shipping.
   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.
   Band the foam box and outer packaging (cardboard) tightly by tape.
   Mark "This Side Up" and "Refrigerate Upon Arrival" on the cardboard.
   Use 2-day shipping. Let the recipient know once you send.
   (9) Besides DNA extraction, since the 40-mL samples cannot be sent, please contact Zhou lab to figure out a way to determine the properties which are not measured by WWTP.

See Appendix IV for international shipping notes.

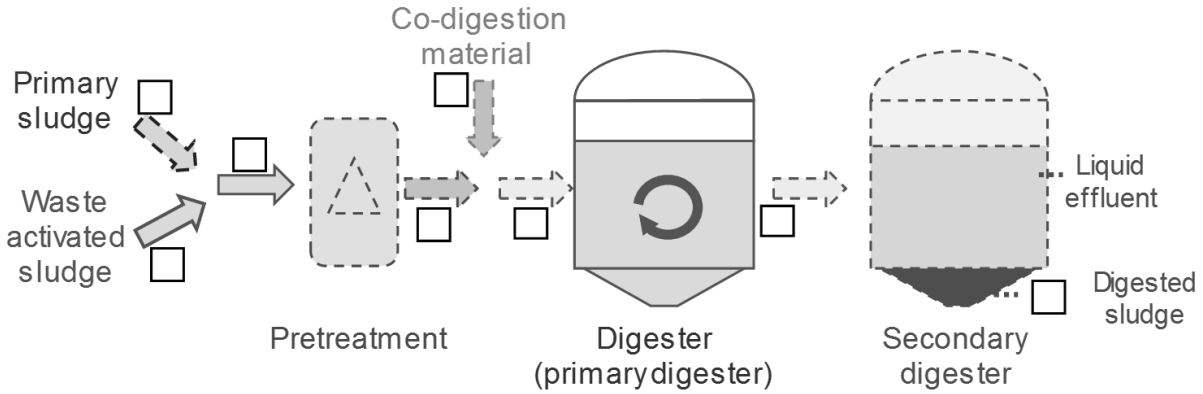
Appendix II Sampling Data Sheet for Each Plant

Sampling date*[1]		Investigator(s)	

1. Wastewater Treatment Plant Information

Name of WWTP[2]			
Sampling Plan	☐One time ☐Summer & Winter ☐Seasonally ☐Weekly ☐Accidentally		
Not to publish [3]*	☐Plant identity ☐Location ☐other_____		
Office Phone		Fax	
Technician name		Email	
Address			
Longitude*[4]		Latitude*	
Activated sludge	Process type(s)*[5]:	SRT*:	Days

2. Sampling Positions



If the chart above is not adequate/applicable, please draft below to indicate sample positions in this plant.

3. Feed Sludge Information

Parameters	Feed sludge [6]		Co-digest material(s): _____ [7]
	Primary sludge	Waste activated sludge	
Sample ID*			
Loading rate (m^3/d)*			
Temperature (°C)*			
pH*			
TSS (g/L)			
VSS (g/L)			
Other:			

4. Pretreatment Process Information

Pretreatment method(s)	Key parameter	Value	Note
	Temperature (°C)*		
	pH		

5. Anaerobic Digester(s) Information

Parameters	*(Primary)* Digester(s) ID			Secondary digester
	AD____#[8]	AD_____#	AD_____#	
Sample ID*				
Digester built year				
Digester Volume (m^3)*				
Designed Capacity (m^3/d)				
Digester Influent Rate (m^3/d)*				
Water Effluent Flow Rate (m^3/d)[9]				
Sludge Effluent Flow Rate(m^3/d)[9]				
Biogas production (m^3/d)**				
Methane in Biogas(v/v)**				
Digester SRT (days)*				
VSR(Volatile Solids Reduction)*[10]				

6. Sample Properties [11]

Measurement		**Digester Influent**[12]	**(*primary*) Digester**[13]			*Secondary digester*
Sample ID*						
Temperature (°C)*	W					
pH*	W					
TSS[4] (g/L)*	W					
VSS[5] (g/L)*	W					
Acetate (mg/L)*	F					
VFAs(total mg/L as Acetic acid)*	F					

W, measured in whole sample. F, measured in filtered supernatant.

Notes for Digester Influent[12]:_____

Additional property data

Measurement		**Digester Influent**	**(*primary*) Digester**[13]	*Secondary digester*
COD (mg/L)	W			
Total Nitrogen (mg/L)	W			
Total Dissolved Solids(g/L)	F	*N*		
NH_4-N (mg/L)	F			
Orthophosphate (mg/L)	F			
Sulfate (mg/L)	F		*N*	*N*
Sulfide (mg/L)	F	*N*		

VFAs (if available)		**Digester Influent**	**(*primary*) Digester**[13]	*Secondary digester*
Acetic acid	F			
Propanoic acid	F			
n-Butyric acid	F			
iso-Butyric acid	F			
n-Valeric acid	F			
iso-Valeric acid	F			
	F			
	F			
	F			

Metals (if available)		**(*primary*) Digester**[13]	*Secondary digester*
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		
	W		

W, measured in whole sample; F, measured in filtered supernatant; *N*, not necessary to measure.

Notes for Sampling Data Sheet

[1] The stars (*) indicate the parameters which must be collected or measured unless not exist. All parameter values should be on or very close to the sampling date, such that we can link them to the microbiome in the samples.

[2] If the anaerobic digestion system is treating sludge from multiple wastewater treatment plants (WWTPs), please collect the information of all WWTPs or several large WWTPs which count for the major (80%) sludge loading.

[3] By providing the samples and data, we consider you agree to publish the data provided or generated from the samples. Please let us know if you would like us to keep some part of the data confidential.

[4] Better to provide latitude and longitude on Google Map: *https://maps.google.com/*

[5] Please select activated sludge process type from the list below unless not applicable.

☐ Conventional plug flow	☐ Plug flow with step feed	☐ Anaerobic-aerobic (AO)
☐ Anaerobic–anoxic–aerobic (A^2O)	☐ Oxidation ditch	☐ Contact stabilization
☐ Extended aeration	☐ Pure oxygen	☐ Complete mix
☐ Sequencing batch reactor (SBR)	☐ Other_____	

[6] It is fine to just provide the parameters for mixed feed sludge you sampled.

[7] Please describe what is the co-digest material(s).

[8] Please input the numbering or ID of the digester.

[9] If the digester has both water (supernatant) and sludge effluents, please provide both.

[10] VSR is typical index to measure the removal rate of volatile solid (VSS removal rate). A classic method to calculate VSR is Van Kleeck equation:

$$VSR = \frac{VS_f - VS_b}{VS_f - (VS_f VS_b)} \times 100\%$$

where VS is the mass of volatile solids per unit mass of total solids, kg/kg; VS_f is the VS value of raw sludge fed to the digester, kg/kg; VS_b is the VS value of the digester effluent sludge (digested sludge), kg/kg.

[11] Cells with stars(*) are required, while the others are recommended. If WWTP could not provide, please send enough volume (40-50 ml each) of samples to Zhou lab or a coordinator lab for additional chemical analyses.

[12] Digester influent usually is not the feed sludge, but may be the effluent of some pretreatment and mixed with some other materials (for co-digestion). The influent sample may be analyzed before or after mixed with co-digestion material(s). Please clarify.

[13] Only need to measure one or a mixture of the three digester samples, unless they are quite different.

[14] Abbreviations list: SRT: Solid Retention Time. TSS: Total Suspended Solid. VSS: Volatile suspended solids. VFAs: Volatile Fatty Acids. COD: Chemical Oxygen Demand.

[15] You are appreciated to provide additional materials to clarify the anaerobic digestion system, pretreatment method, co-digestion materials, etc.

Appendix III DNA Extraction Protocol

We will all use DNeasy PowerSoil Kit to extract DNA from Anaerobic Digester samples.

Materials

(1) DNeasy PowerSoil Kit (QIAGEN, Catalog# 12888-50 or 12888-100)

https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/dneasy-powersoil-kit/#orderinginformation

A manufacture protocol will be provided in the Kit

(2) Vortex Genie® 2 Vortex (Scientific Industries)

https://www.scientificindustries.com/vortex-genie-2.html

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

https://www.qiagen.com/us/shop/automated-solutions/accessories/vortex-adapter/#orderinginformation

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

Prepare by yourself or prepare from a qualified commercial 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 1.5 mL sludge to extract DNA by one prep of the kit. Use more pellets if necessary.

(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.

(4) **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.

(5) 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)

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

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

Appendix IV 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. Zhou lab 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 if you used dry ice.
    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**
  **** If you send DNA samples, you do not need to use dry ice. Just use 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.**
  ***** Sludge samples must be packed with dry ice.**
  - 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 samples into the Styrofoam box, surround the samples by ice packs and put one ice pack and most dry ice (if used) on the top of the samples. Fill any empty space with wadded newspaper. It may need 7~9 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 if need,
    Import permit for sludge pellets or formal letter and TSCA for DNA samples,
    Commercial invoice
    Dry ice label with markings (if used dry ice)
    Air Waybill
    "Freeze upon arrival" label.

    If you use a shipping company to ship the sludge samples, 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