GWMC Weekly Sampling Protocols

5th edition to implement, Apr 24, 2015

Based on 4st edition, Jan 21, 2015, revised according to additional suggestions on Feb, 13, 2015

Objective: Target the **aerobic stage** of wastewater treatment plants using activated sludge processes. Take a minimum of **1 sludge sample** from **1** plant for each geographic site (e.g. a large city) **weekly** for **one year**.

1. Field sampling (see Appendix I for detail)

- Take samples weekly at nearly the same time of a day and the same day of a week.
  * Please avoid Monday and weekend. Recommend 10:00~11:59 am on every Wednesday.
  It is important to keep the interval the same for mathematics.
- Complete the sampling data sheet (Appendix II).
- Usually, take 15 mL mixed liquor samples for Microbiome analyses from the outlet of the aeration tank.
- If WWTP can share their results of a parameter, you do not need to measure the parameter by your own. If not, please take samples to measure the parameter.

2. Sample preparation, analyses and storage

- Keep mixed liquor samples on ice and transport to lab ASAP (within 12 hours).
- Shake up the mixed liquor sample and divide into aliquots (e.g. 4 x 1.5 mL + 9 mL).
- Centrifuge at 15,000 *g* for 10 min, discard the supernatant and preserve pellets at -80°C.
- If a required parameter is not available from WWTP, please measure it. See Appendix I for methods.

3. Send us frozen pellets or DNA.

After one-year sampling:

- Option 1: Extract and send DNA. This is better to avoid unexpected variations.
  - Use the pellets from two 1.5-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.
  - Keep DNA in TE buffer and send with enough ice packs to Joe's Lab by 2 days shipping.
- Option 2: Send the frozen pellets with enough dry ice and ice packs to Jeo's lab by overnight or 48-hour shipping.
- Before shipping, contact Joy Van Nostrand (joy.vannostrand@ou.edu) and Daliang Ning (ningdaliang@ou.edu). Please avoid arrival during the weekend. See Appendix IV for international shipping notes.

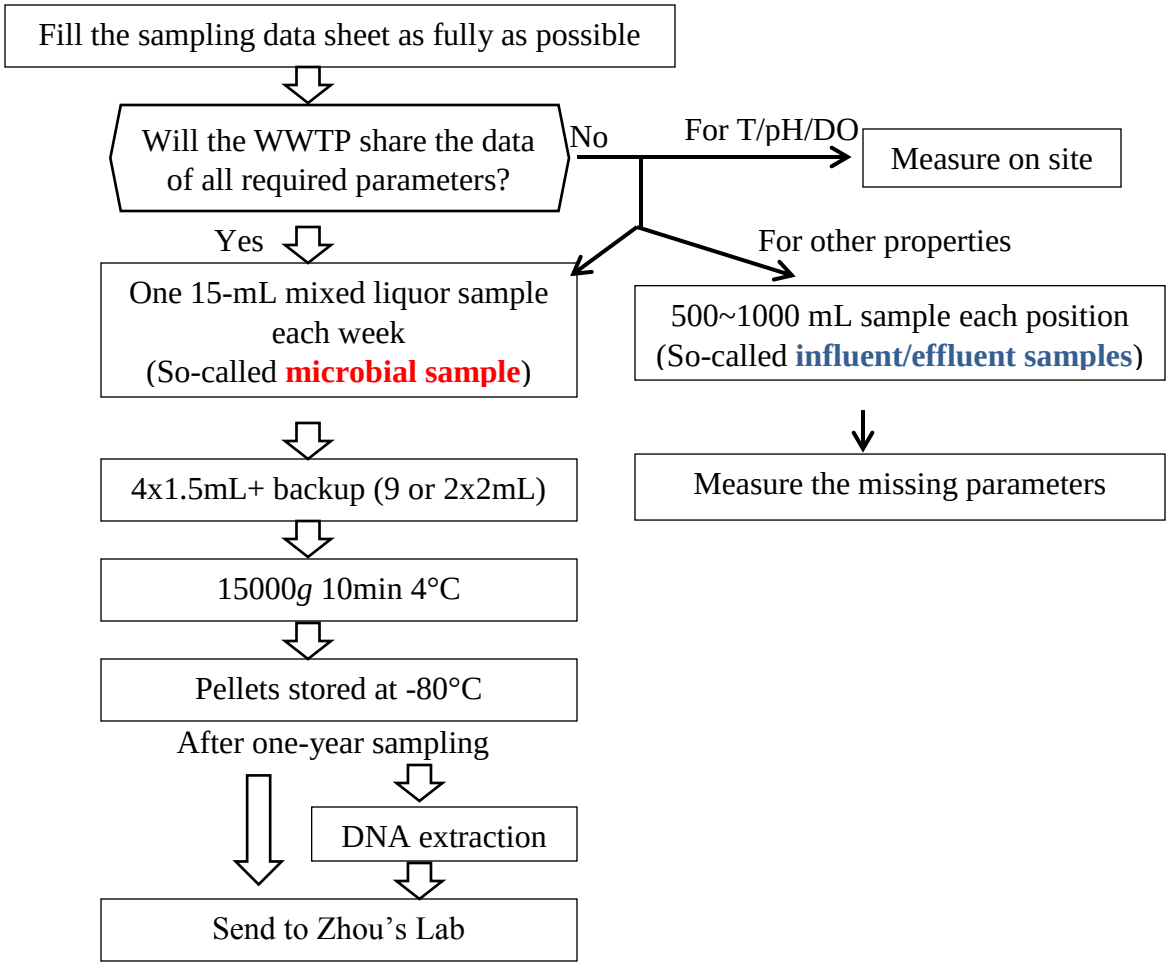
Joe's Lab
Recipient: Joy Van Nostrand Phone: +1-405-325-4403
Address: 101 David L Boren Blvd SRTC 2030, Norman, OK, USA 73019

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

Generally, please i) complete the sampling data sheet, ii) take 1 mixed liquor sample from the selected aeration tank in a plant *, iii) centrifuge to get the pellets and store at -80C, iv) after one-year sampling, extract DNA and/or send to Zhou's lab.

* One sample each week is minimum requirement. More samples, tanks, and plants for comparison and theory test are surely welcome.



Detail procedures

1. Preparation before sampling

(1) Preparation for sampling:
Sampling Data Sheet (1/plant), a cooler (or foam box) with enough ice,
15-mL tubes (sterile, 1 per mixed liquor sample), sampler (if not available in the plant),
a wash bottle containing DI water, gloves, sleeve, pen and marker pen,
* pH/DO/temperature meters (if not measured by the plant),
* 500-mL or 1-L glass bottles (2~3, if some properties are not available from the plant),
* a bucket and pipette (clean, 2~4 L bucket, 50~100 mL pipette, if have to take water sample from influent/effluent mixed liquor of the aeration tank).

(2) Preparation for centrifuge and storage:
1.5 mL and 15 mL tubes (sterile, for 1.5 mL and 9 mL sample),
Centrifuge, Freezer (-80°C), pipetter and tips
* Parafilm and/or sealed plastic bags as secondary container (if will send pellet samples).

(3) * Other materials for possible physic-chemical analyses: as required by methods (table 1).

2. Sampling

(4) Fill the Sampling Data Sheet A (Appendix IIA) at the first time and Sheet B (Appendix IIB) weekly, as fully as possible.

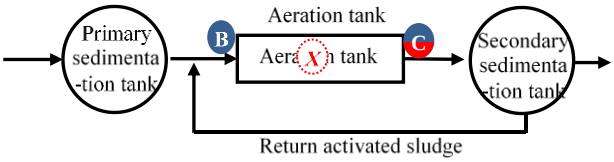
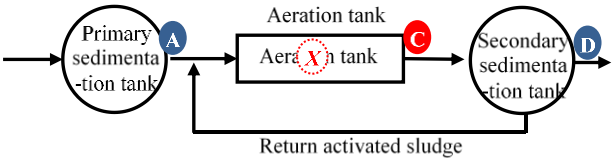
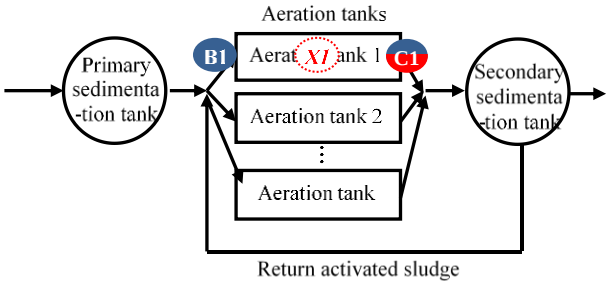
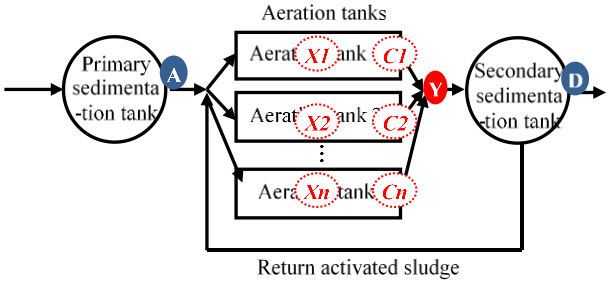
Table 1 List of parameters in weekly sampling data sheet

Type	Parameters
Microbial sample (i.e. mixed liquor)	MLSS/MLVSS (biomass), pH, DO, liquor temperature.
Influent and effluent samples	Liquor temperature, pH, DO, BOD and/or COD, ammonium, nitrate and nitrite, TN, TP. (optional: heavy metal)
Operational parameters	Influent flow rate, return sludge flow rate (or recycle ratio), SRT, liquor volume in tank
Weather data	Air temperature, precipitation

(5) Figure out sampling positions (see Table 2 for typical cases and options):
Take the mixed liquor sample for micobiome analyses (so-called microbial sample) from the outlet or middle of the aerobic zone in an aeration tank.
It is acceptable to use the data from the plant lab. Considering a plant lab usually take samples after primary and secondary sedimentation tanks (position A and D in Table 2), we suggest alternative options in Table 2. So you do not need to do any physic-chemical analyses if the plant lab can provide.
* Explanation: in this study, the performance and environment conditions (water properties) of the sampled activated sludge are important to link community and functioning. If we target one tank in a multiple-tank system (case 2), water properties at

position B and C are exactly what we want, and position A and D can be unacceptable when the tank has different performance from others (it happens). So if we want to use water properties at position A and D from the plant lab to reduce our work loading, a combined sampling (case 2 alternative option) should be considered.

Table 2 Sampling positions in typical activated sludge WWTP

Cases and Options	Microbial sample	Influent sample	Effluent sample
Case 1: one-aeration-tank system **Prior**: perhaps more lab work, but better for research 	Position C (or X)	Position B	Position C
Alternative: perhaps less lab work and acceptable. 	Position C (or X)	Position A	Position D
Case 2: multiple-aeration-tank system **Prior**: perhaps more lab work, but better for research 	Position C1 (or X1)	Position B1	Position C1
Alternative: perhaps less lab work and acceptable, but maybe a bit more field work 	Position Y (alternatively, combination* of C1~Cn; or combination* of X1~Xn)	Position A	Position D
Red, microbial sample; blue, influent/effluent sample; unfilled circles, alternative sampling positions.	Required	Unnecessary if plant lab analyzed	

* Combine the mixed liquor samples by the proportions related to flow rates in different tanks.

** Additional suggestion (recommend to do so if applicable):
Take a microbial sample from the secondary influent weekly to get what immigrate into activated sludge.

Protocol:

(a) Take 150 mL (you may use three 50-mL tubes) water samples from the influent of the aeration tank (i.e. secondary influent or primary clarifier effluent, before combined with return sludge).
(b) Centrifuge the water sample at 15000g for 10 min, and discard the supernatant.
(c) Resuspend the pellets by 3 mL water, mix well and divide into 3 aliquots in 1.5-mL tubes, centrifuge at 15000g for 10 min to get pellets, and store the pellets at -80C.

(6) Take out tubes and bottles, make labels on the top and side by a marker pen.
(7) Rinse the sampler by mixed liquor at the position.
(8) For mixed liquor sample (microbial sample): take about 1 liter mixed liquor, shake or stir this 1-L mixed liquor and then transfer 15 mL to the 15-mL tube. Cap the tube tightly and chill on the ice.

* If MLSS/MLVSS is not available from the plant lab, please full fill a 500-mL glass bottle with mixed liquor. Cap the bottle tightly and chill on the ice.

** For combined sampling (as Case 2 alternative option in Table 2, if position Y is not applicable), take 15 mL mixed liquor from each of the parallel running tanks in the same chain and combined them in lab by proportion related to the flow rates in these tanks (often equally).

*** If necessary, take influent/effluent samples as showed in Table 2. If there is activated sludge at this position (e.g. position B and C in Table 2), take about 2~4 liter sample into a bucket, let the sludge settled for 15 min (or more if needed), then transfer supernatant to a 500-mL (or 1-L as needed) bottle. If there is no activated sludge (e.g. position A and D in Table 2), directly take water sample into a bottle. Fill the bottle to overflow, cap the bottle tightly and chill on the ice.

(9) Wash the sampler by clean water thoroughly after use.
(10) Measure DO/pH/temperature of the mixed liquor, influent and effluent at the positions.
(11) Filled sampling information in Sampling Data Sheet (Appendix IIB). Attention to sample labels.
(12) Keep samples on ice and transport to lab ASAP (within 12 hours).

3. Mixed liquor sample centrifuge and storage in lab

(13) Shake up the sample in 15-mL tube, divide into 4x 1.5mL and 1x 9mL (or 4x 1.5 mL + 2x 2 mL) and discard the rest. Make labels.

* Keep the tubes on ice to keep liquor samples cold. Attention to sample labels.

(14) 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.

(15) If you will ship pellets, you may use Parafilm to seal the cap of each tube, or put tubes of each sample into a plastic bag to avoid unexpected spillage and cross contamination when shipping. Then preserve the pellets at -80°C (-20°C is NOT acceptable).

4. (If necessary) Physic-chemical analyses in lab:

(16) If nitrite and/or nitrate 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.

(17) For other chemical analyses, the water sample should be acidified by H_2SO_4 to pH<2 and preserved at 2~4°C dark place if you cannot measure right after sampling.

(18) 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.

Property	Preservation	Standard method	Alternative method
DO	Measure on site	ISO 5814:2012 (Electrochemical probe)	Reliable DO meter
pH	Measure on site	ISO 10523:2008 (pH meter)	Reliable pH meter
Liquor temperature	Measure on site	Standard thermometer	
COD_{Cr}	pH<2, 4°C, <7 days or <28 days	ISO 6060:1989 (Dichromate method)	ISO 15705:2002 (Sealed tube method) HACH Method 8000
NH4-N	pH<2, 4°C, ASAP, <24 hours	ISO 7150-1:1984 (Salicylate method)	HACH Method 10205
NO_3-N and NO_2-N	0.2 μm filter, 4°C, ASAP, <24 hours	ISO 10304-1:2007 (IC method)	
TN	pH<2, 4°C, <1 week	ISO 11905-1:1997 (Peroxodisulfate method)	HACH Method 10208
TP	pH<2, 4°C, <28 days	ISO 6878:2004 (Ammonium molybdate method)	HACH Method 8190
TSS	4°C, <7 days	ESS Method 340.2 (Dried at 103-105°C)	US EPA method 1684
VSS	4°C, <7 days	ESS Method 340.2 (Ignited at 550°C)	US EPA method 1684
(Optional) Heavy metals	0.2 μm filter, pH<2 (by HNO_3),	US EPA method 6010C (ICP-AES for Al, Sb, As, Ba,	US EPA method 6020A (ICP-MS)

Property	Preservation	Standard method	Alternative method
(except Cr^{VI})	< 6 months (Hg <28 days)	Be, Cd, Cr, Co, Cu, Pb, Mn, Ni, Ag, Tl, Zn, etc.)	

* Nitrate and nitrite: better if measured separately, but acceptable if measured as one composite parameter.

5. **After one-year sampling, extract DNA according to Appendix IV unless we send out a new protocol.**

6. **Packing and shipping samples after one-year sampling**

For international shipping, please read Appendix V before shipping samples to USA.

For shipping within USA---

(19) Contact Joy Van Nostrand (joy.vannostrand@ou.edu) and Daliang Ning (ningdaliang@ou.edu) before you prepare.
** Oklahoma university may be closed for a holiday or special weather :)*

(20) Prepare tools: Carton box, foam box as cooler, some frozen ice packs, wadded paper, other files required by courier.
* If shipping sludge pellet samples, you need to use dry ice. Please consult a courier for notes, proper documentation and labeling requirements when using dry ice.

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

(22) 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.

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

(24) Send as soon as possible after packing. Let Joy or Daliang know once you send.

Appendix IIA Sampling Data Sheet A (yearly)

A1- Plant basic information						
Name of WWTP						
WWTP ID						
Plant contact			Fax			
Office Phone			Email			
Address						
Longitude			Latitude			
Air temperature	annual mean		max		min	
Age of Plant		Designed Capacity (m^3/d)				
Actual Influent Flow Rate (m^3/d)						
Influent of the whole plant (yearly average): BOD_____(COD_____) NH_4-N_____ TN_____ TP_____		Effluent of the whole plant (yearly average): BOD_____(COD_____) NH_4-N_____ TN_____ TP_____				
Industrial wastewater in influent:		□ No □ Yes, percentage_____% □ unknown				
Storm water in influent		□ Never □ Sometimes □ Always if rain □ unknown				
Sludge Age (SRT) (Days)						
HRT in the whole plant						
A2- Tank basic information						
Age of this tank		Designed liquor volume				
Shape and Size						
When did the current activated sludge system set up?						
Anaerobic zone or anaerobic tank ahead/behind? Yes or No Where:_____						
Anoxic zone or anoxic tank ahead/behind? Yes or No Where:_____						
Nitrification? Yes or No	Denitrification? Yes or No	Phosphorus removal? Yes or No				
Activated Sludge Process Type[1]						
Nitrification Process Type[2]						
Denitrification Process Type[3]						
Corresponding investigator		Email				

Additional Notes:

Appendix II Sampling Data Sheet B (weekly)

Sampling Date (MM-DD-YY)	____-____-_____	Investigator	

B1- Operational parameters

Parameters	When sampling	Average in last 7 days
Influent flow rate **of the tank** (m^3/d)		
Recycling Ratio(return sludge flow/influent flow)		
Mixed liquor volume in the tank (m^3)		
Waste sludge flow rate (m^3/d)		

If the tank is SBR:	Discharge Volume			Volume exchange ratio		
	Cycle time	Fill time	React time	Settle time	Draw time	Idle time

Shock loading or other abnormal events (e.g. operation stops, excessive use of chemicals etc.) related to this tank in last 7 days	

B2- On-site data

Air temperature (°C)		Weather	

Positions	Sampling time	Sample ID	Liquor temp.	DO	pH
[inf]					
[eff]					
[ML]					

B3- In-lab data

MLSS		MLVSS		SVI		SRT[4]	

Positions	CBOD or COD		NH_4-N	NO_3-N + NO_2-N	TN	TP
[inf]						
[eff]						

Metals										
[inf]										
[eff]										

B4-Weather data in last 7 days

Parameter	-6 d	-5 d	-4 d	-3 d	-2 d	-1 d	Today
Temp. mean							
Temp. max							
Temp. min							
Precipitation							

Cells with grey shading are optional. See Note [4] for how to get SRT. Waste sludge flow rate is not necessary if SRT is already available. Abbr.: [inf], influent sample; [eff], effluent sample; [ML], mixed liquor sample for microbial analyses (microbial sample). Temp, temperature. SBR, sequencing batch reactor.

Welcome additional notes and documents to clarify the answers.

Appendix IIC Notes about the Sampling Data Sheet

[1] List of Active Sludge Process Types:

□ 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 Types:

□ 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 Types:

□ 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.)

Welcome additional notes and documents to clarify the answers.

[4]

How to get SRT

SRT (sludge retention time) could be one of the most important parameters controlling active sludge system. Here are some ways to figure out SRT.

(1) Ask the WWTP for accurate SRT (the best way)

Most plants must have SRT values. In some references, "Sludge age" means exactly the same thing as SRT, but in other cases, "sludge age" is quite different from SRT as the following equations. Please clarify it when talking with people in the plant.

$$\text{SRT} = \frac{MLSS \times V_{ae}}{TSS_{WAS} \times Q_{WAS}}$$

$$\text{Sludge age} = \frac{MLSS \times V_{ae}}{TSS_{inf} \times Q_{inf}}$$

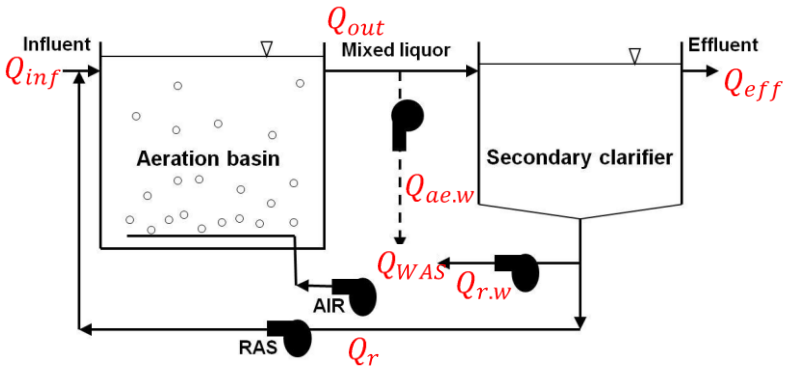
MLSS	mixed liquor suspended solid (mg/L);
V_{ae}	liquor volume in aeration tank (m^3 or gallon);
TSS_{WAS}	total suspended solid in waste activated sludge (mg/L);
Q_{WAS}	flow rate of waste sludge (m^3/d or gallon/d);
TSS_{inf}	total suspended solid in waste activated sludge (mg/L);
Q_{inf}	influent flow rate (m^3/d or MGD).

(2) Calculate from MLSS, V_{ae}, TSS_{WAS} and Q_{WAS} by the first equation. (the most accurate)

$$\text{SRT} = \frac{MLSS \times V_{ae}}{TSS_{WAS} \times Q_{WAS}}$$

(3) Estimate from V_{ae}, waste sludge flow rate(s) and sludge recycle ratio. (easier and reasonable)

If you cannot get the values of SRT and TSS in waste sludge from the plant but get an average flow rate of waste sludge, you may calculate SRT in waste sludge as follow.



Welcome additional notes and documents to clarify the answers.

- If waste sludge is all from return sludge (Q_{WAS}=$Q_{r.w}$):

$$TSS_{WAS} = TSS_{RAS} = \frac{MLSS \times (Q_{inf} + Q_r) + \Delta M - TSS_{eff} \times Q_{eff}}{Q_r + Q_{WAS}}$$

$$\approx \frac{MLSS \times (Q_{inf} + Q_r)}{Q_r + Q_{WAS}} = \frac{1+r}{r+r_w} \times MLSS$$

$$\text{SRT} \approx \frac{MLSS \times V_{ae}}{\frac{1+r}{r+r_w} \times MLSS \times Q_{WAS}} = \frac{(r+r_w) \times V_{ae}}{(1+r) \times r_w \times Q_{inf}} = \frac{r+r_w}{r \times r_w + r_w} HRT$$

- If waste sludge is all from aeration tank (Q_{WAS}=$Q_{ae.w}$):

$$\text{SRT} = \frac{MLSS \times V_{ae}}{MLSS \times Q_{WAS}} = \frac{V_{ae}}{Q_{WAS}} = \frac{HRT}{r_w}$$

- If waste sludge is from return sludge and aeration tank (Q_{WAS}=$Q_{ae.w}$+ $Q_{r.w}$):

$$TSS_{WAS} \times Q_{WAS} = MLSS \times Q_{ae.w} + TSS_{RAS} \times Q_{r.w} \approx \left[Q_{ae.w} + \frac{1+r-r_{wa}}{r+r_{wr}} Q_{r.w}\right] \times MLSS$$

$$\text{SRT} \approx \frac{(r+r_{wr}) \times V_{ae}}{(r+r_{wr}) \times Q_{ae.w} + (1+r-r_{wa}) \times Q_{r.w}} = \frac{r+r_{wr}}{r \times r_w + r_{wr}} HRT$$

TSS_{RAS} total suspended solid in return activated sludge (mg/L);
Q_{inf} influent flow rate (m^3/d or MGD);
Q_{eff} effluent flow rate (m^3/d or MGD);
Q_r return activated sludge flow rate (m^3/d or MGD);
Q_{WAS} waste sludge flow rate (m^3/d or gallon/d);
$Q_{ae.w}$ the flow rate of waste sludge from aeration tank (m^3/d or gallon/d);
$Q_{r.w}$ the flow rate of waste sludge from return activated sludge (m^3/d or gallon/d);
$r = Q_r / Q_{inf}$ sludge recycle ratio;
$r_w = Q_{WAS} / Q_{inf}$ sludge waste ratio;
$r_{wa} = Q_{ae.w} / Q_{inf}$ aeration tank sludge waste ratio;
$r_{wr} = Q_{r.w} / Q_{inf}$ return sludge waste ratio;
ΔM the sludge growth rate in sedimentation tank (kg/d);
TSS_{eff} TSS in effluent of sedimentation tank (mg/L).

(4) Estimate from HRT, MLVSS, BOD and TKN (the easiest)

If you cannot get SRT, TSS in waste sludge and Flow rate of waste sludge, you may estimate SRT from HRT, MLVSS, BOD and TKN as below.

$$\text{MLVSS} = \frac{SRT}{HRT}\left[X^0_{inert} + Y_{het}\frac{1+(1-f_d)b_{het}SRT}{1+b_{het}SRT}\Delta BOD + Y_{nit}\frac{1+(1-f_d)b_{nit}SRT}{1+b_{nit}SRT}\Delta TKN\right]$$

MLVSS mixed liquor volatile suspended solid (mg/L);

$HRT = V_{ae}/Q_{inf}$ hydraulic retention time (d or h);

X^0_{inert} influent biomass and can be set as $0.2 \times BOD_5^{in}$

BOD_5^{in} 5-day biological oxygen demand in the influent before combining with return sludge;

Y_{het} true-yield coefficient for heterotrophs (Y_{het} =0.45 kg VSS/kg BOD_L)

BOD_L the ultimate BOD;

f_d the fraction of newly synthesized biomass that is degradable by endogenous decay ($f_d = 0.8$);

b_{het} endogenous decay coefficient for heterotrophs (b_{het}=0.1/day);

ΔBOD removal of BOD_L across the aeration tank

$$\Delta BOD = 2.44 \times \Delta BOD_5 = 2.44 \times \left(BOD_5^{in} - BOD_5^{out}\right)$$

BOD_5^{out} 5-day biological oxygen demand in the effluent of this tank;

Y_{nit} true-yield coefficient for all nitrifiers (Y_{nit} =0.45 kg VSS/kg N for all nitrifiers);

b_{nit} endogenous decay coefficient for all nitrifiers (b_{het}=0.15/day);

ΔTKN removal of Total Kjehldahl Nitrogen across the tank;

$$\Delta TKN = TKN_{in} - TKN_{out}$$
$$\approx \Delta TN + \Delta NO_3N + \Delta NO_2N$$
$$= (TN_{in} - TN_{out}) + (NO_3N_{out} - NO_3N_{in}) + (NO_2N_{out} - NO_2N_{in})$$

TN_{in} and TN_{out} total nitrogen in the influent and effluent of the tank;

NO_3N_{in} and NO_3N_{out} nitrate nitrogen in the influent and effluent of the tank;

NO_2N_{in} and NO_2N_{out} nitrite nitrogen in the influent and effluent of the tank.

[Ref] Rittmann BE, Laspidou CS, Flax J, Stahl DA, Urbain V, Harduin H et al. (1999). Molecular and modeling analyses of the structure and function of nitrifying activated sludge. Water Science and Technology 39: 51-59.

So if you do not know the exact values of Y_{het}, Y_{nit}, b_{het}, b_{nit}, and f_d in the plant, you may firstly use the above values to estimate SRT as below.

$$\text{MLVSS} = \frac{SRT}{HRT}\left[0.2 \times BOD_5^{in} + \frac{1.098 + 0.02196 \times SRT}{1 + 0.1 \times SRT}\left(BOD_5^{in} - BOD_5^{out}\right) + \frac{0.45 + 0.0135 \times SRT}{1 + 0.15 \times SRT}(TKN_{in} - TKN_{out})\right]$$

Actually, Y_{het}, Y_{nit}, b_{het}, b_{nit}, and f_d can be different from one plant to another, and affected by various factors such as temperature. Therefore, the estimated SRT may not be so accurate, however, it has been much better than nothing. If you have SRT values at several time points, you can use these SRT values to calibrate all the coefficients in the equation and estimate the SRT values you missed. In this case, this method is very helpful and relatively reliable.

Appendix III DNA Extraction Protocol

In 2014, we all used Powersoil® DNA Isolation Kit to extract DNA from activated sludge. This method would continue to be used unless a new protocol was sent out.

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) Generally, use pellets from 2x 1.5 mL mixed liquor to extract DNA by one prep of the kit.

* You may use 4 x 1.5 ml if the sample contains too much sands (if the WWTP received storm) or the MLSS is too low (<2000 mg/L).

(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).

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. 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 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