ANAEROBIC DIGESTION FEASIBILITY PROTOCOL FOR FRUIT AND VEGETABLE PROCESSING WASTE

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Introduction

This protocol is intended to serve fruit and vegetable processors by analyzing a waste stream (substrate) to determine the applicability of anaerobic digestion as a treatment method and/or energy source. Food processors and area farmers pursuing a regional digester may also test wastewater-manure blends. An individual processor may be interested in installing a digester for the plant's wastewater treatment or a group of processors and/or farmers may be organizing a regional digester. The protocol includes methods to assess a waste stream's pretreatment and operating requirements, estimate biogas production using an anaerobic respirometer and analyze the subsequent methane fraction with gas chromatography. It provides recommendations for suitable methane applications. Processor objectives, wastewater parameters and respirometer results are considered to determine if further investigation such as a pilot-scale digester is appropriate. To facilitate the collection of pertinent information from the processor, assessment worksheets were developed based on literature and site-specific experience (see Appendix I). Conclusions for an individual processor may include a recommendation to consider a regional digester.

Step 1. Processor's Goals and Objectives

Obtain the following information from the food processor and compare these goals and expectations with reasonable deliverables. See Appendix A for assessment worksheets.

1. Determine the processor's interest in anaerobic digestion.

    a. If a regional digester or co-digestion is being pursued, the protocol must be carried out for all contributors, and a representative substrate must be tested. Check all governing regulations, as some locales prohibit the mixing of certain waste streams.

    b. There should be no difference in assessment for individual treatment vs. energy objectives.

        i. An energy study may conclude 'only adequate for treatment'.
        ii. A treatment study may also conclude 'adequate for energy recovery.'

c. Methane potential can only be an estimate; there are several determining factors in full-scale operation such as mixing effects which cannot be accounted for in lab-scale testing.

2. Determine the desired biogas applications. There are many indirect considerations for electric generation which should be addressed upfront. (This is outside the scope of this protocol. Consult an appropriate professional). For example, if the electric power will be sold back to a common grid, the utility company may assess significant hook-up fees.

3. Identify the discharge requirements: the COD (mg/L) and/or BOD (mg/L) as per regulations for applicable effluent discharge. BOD is the conventional measurement of wastewater quality, however COD is easier to measure and more representative of anaerobic treatment potential than BOD.

   a. It is assumed that fruit and vegetable processing waste streams contain below the regulated limits for nitrogen and phosphorus (and other nutrients and metals). If this is not the case, these nutrients must also be factored in at this step.

   b. The required COD or BOD discharge concentrations will be compared with waste stream concentrations to determine the degree of treatment required. If adequate treatment cannot be achieved by anaerobic digestion alone, an alternate treatment must be considered in conjunction or instead. See Section 4.2 to estimate treatment potential.

4. Assess the relative economic status of the project.

   a. Some operating costs and methane cost savings are discussed throughout the protocol. Digestion of any given waste may require regular addition of an alkalinity source, nitrogen, phosphorus, or metals, and thus an added operating cost. This may be a significant or negligible cost depending on the nutrient and level of deficiency.

   b. These may be used to supplement a full cost-benefit analysis. (This is beyond the scope of this protocol).

5. Determine the processor's desired timeline for operational treatment or compliance.

a. This protocol may require several months for sampling and testing. Further pilot-scale testing may be recommended, which would require several months additional.

b. Full-scale system design and permit applications may take over a year.

c. Construction, start-up and stabilization may require over a year additional.

Step 2. Plant Profile

Obtain the following information from the food processor. See Appendix A for plant assessment worksheets.

1. Determine the plant's average monthly energy usage (natural gas in thm/month, and/or electricity in kWh/month). This can be calculated from billing costs.

2. Identify the plant's wastewater streams (by commodity or location in the plant). Characterize the fluctuation in flow and COD loading throughout the year and average temperature of each stream.

   a. Convert COD (or BOD) to kg/day and flow to m^3/day.

   b. Identify waste streams that are most appropriate for anaerobic digestion, with high soluble organic loading.

      i. Determine if streams currently may be combined to equilibrate COD and volume loadings, or may be readily combined by installing piping. Alternately, consider if streams with low organic loading could be readily separated and diverted, such as storm water.

      ii. A high peak in volume or COD may require a large equalization tank, adding to construction costs.

3. Identify the different commodities processed and the fluctuation in volume processed throughout the year. See Appendix A for assessment worksheets.

   a. Correlate this with the data from section 2.2 above.

   b. If data for section 2.2 is not available, the plant's commodity data and water use may be used to approximate ranges for BOD loading and waste stream flow using published data for each commodity (ex: gal/day per ton processed). Weight these accordingly for each

commodity processing rate per year (tons/month * # of months/year).

4. List any known cleaners, sanitizers or chemical additives used in processing that may be present in the different waste streams. If certain compounds are only used in specific areas (waste streams) specify which.

   a. Compounds may include (but are not limited to):

      Oxidizing sanitizers
      - Hyperchlorites, chlorine, chloramines
      - Organic bromine
      - Iodine, alcohol-iodine, iodophors
      - Hydrogen peroxide, peroxy acids

      Biocides and non-oxidizing sanitizers
      - Organic acids (e.g. acetic acid, propionic acid, formic acid, carboxylic acids)
      - Acid anionic sanitizers
      - Acid-quat sanitizers
      - Quaternary ammonium compounds

   b. Any compounds present may serve as potential sources of toxicity that may interfere with anaerobic digestion. It may be possible for a full scale digester may acclimate and adapt to such toxicity.

   c. If specific concentrations can be determined, compare the present levels to a known IC_{50} (inhibitive concentration) if available. See Blum and Speece (1991).

   d. For concentrations near the IC_{50} level, anaerobic treatment and methane production may be severely retarded. An alternate wastewater treatment should be pursued if the chemical(s) of concern cannot be replaced with one(s) less toxic.

Step 3. COD Conversion to Heat and Electric Potential

1. If historical plant data is unavailable (section 3.3), analyze the processing plant's average daily wastewater COD concentration and daily water volume usage. Characterize COD levels for as many commodities as possible. If time does not permit testing of all commodities, consult published COD estimates to identify 'high' and 'low' COD level commodities in order to ensure that representative commodities are tested at the very least. Take samples during a transition period to include at

least two commodities. Note: Using results from only the 'worst' commodity (i.e. that with the highest COD) may provide overestimated results in methane potential.

a. Test COD using Hach Method 8000.

   i. Collect field duplicates.
   ii. Verify testing methods using lab duplicates. The percent difference should be less than 20%.

b. Compare test results against published commodity ranges. (See Wastewater Characteristics and Quantities Associated with Fruit and Vegetable Processing in Michigan).

2. Consider the average and peak COD loading ($kg/m^3/d$) and assume an appropriate reactor type based on Table 1 below, with a given COD removal treatment efficiency (e.g. 80 to 90%):

Table 1 Types of Anaerobic Digesters

Reactor Type	**COD loading***	**COD removal rates***
Contact (ANCP)**	1-5 $kg/m^3/d$	70-95%
Hybrids	5-15 $kg/ m^3/d$	70-95%
UASB**	5-20 $kg/ m^3/d$	80-95%
Filters	5-20 $kg/ m^3/d$	70-90%
EFB**	10-40 $kg/ m^3/d$	60-85%

*Totzke (2006)
** ANCP = Anaerobic Contact Process
UASB = Up-flow Anaerobic Sludge Blanket
EFB = Expanded/ fluidized bed

3. Calculate a COD removal rate (kg/d) based on reactor treatment efficiency:

COD removal rate (kg/d) = minimum %COD removal (%/ 100) x average COD influent (kg/m^3) x flow rate (m^3/d) [1]

4. Based on COD removal, calculate the heating and electric potentials.

a. For estimated heating potential (Speece, 1996):

Btu/d = $12x10^6$ Btu / 1000 kg COD removed x COD removal rate (kg/d) [2]

b. For estimated electric wattage potential (Speece, 1996):

$$MWhr = (1\ MW/10^7\ Btu) \times (12x10^6/\ 1000\ kg\ COD\ removed) \times COD\ removal\ rate\ (kg/d) \times 24\ hr/hr \quad [3]$$

5. Compare these results to the processor's energy needs (section 4.2). If the results are appropriate for the processor's intended anaerobic applications, continue on to section 5.

Step 4. Substrate Characterization Tests

The COD conversions to Btu and MW estimates do not account for variability in substrate biodegradability. To properly assess the anaerobic digestibility of a given waste stream, several other parameters must be analyzed. Historical data should be available (collected monthly and annually per MDEQ regulations).

1. Retrieve historical wastewater effluent data for the past 1 to 2 years.

2. Collect wastewater effluent samples, throughout the season if possible. Samples must be representative of both the dominant commodity(s) and those with the highest COD loading (which may be more seasonal, e.g. pumpkins).

3. Test the samples for the parameters listed in Table 2.

Table 2 Substrate Characterization Analyses

Analysis	Method	Suggested Range	Source
pH	pH meter	6.5 to 8.2	Speece, 1996
Alkalinity	Hach 8203	2000 to 3000 mg/L $CaCO_3$	Speece, 1996
COD	Hach 8000 (EPA approved)	> 1000 mg/L COD	Speece, 1996
Total Nitrogen Series	EPA-350.1, 353.2, 351.3, 350.2/351.3	3 to 5 mg/L N per 100 mg/L COD	Bouallagui et al., 2004
Ammonia	EPA-350.1	> 40 to 70 mg/L NH_4^+ < 1500 mg/L NH_4^+	Speece, 1996 Calli, et al. 2005
Total Phosphorus	Hach 8190	0.5 to 1 mg/L P per 100 mg/L COD	Bouallagui et al., 2004
Total Solids (TS)	Hach 8271 (EPA approved)	< 10% for batch tests	Carucci et al., 2005
Volatile Suspended Solids (VSS)	Hach 8158, 8164	Calculate VSS:TS. The higher, the better.	
Sulfide	EPA-376.2	> 2 mg/L S, <200 mg/L S	Isa et al., 1986
Sulfate	EPA-375.4	< 5000 mg/L SO_4 for treatment < 300 mg/L SO_4 for biogas applications	Isa et al., 1986

4. If processed commodities include foods high in polyphenols or long chain fatty acids (> 100 mg/L), then pretreatment may be required (see Literature Review), which would raise capital and/or operating costs depending on the chosen treatment method.

   a. High-phenol foods include (www.kirkmanlabs.com):

      - Apricots
      - Berries
      - Cherries
      - Dill
      - Licorice (Anise)
      - Mint
      - Olives
      - Oranges
      - Pineapple
      - Peppers
      - Red grapes
      - Tomatoes
      -

5. Analyze the test results and assess the implications for the processor's goals as described below.

   a. If pH is too low, then acetate production does not occur and methane will not be produced. If pH is too high, then less ammonia (NH_3) ionizes to ammonium (NH_4^+) and becomes toxic.

   b. Estimate the alkalinity amendment required.

      Added alkalinity (mg/L /day) = 10%(inf. COD mg/L) – Inf. alkalinity (mg/L) [4]

      Note: This is only a rough estimate, and alkalinity production will not be accurately represented at a lab scale.

      i. Low-protein waste will generate little to no alkalinity during digestion.

      ii. Consider the cost of alkalinity treatment per year and include as an operating cost. Cost can vary widely with alkalinity source and potentially be significant.
      See Speece (1996) for treatment options and cost calculations.

iii. Alkalinity should be tested and determined later during full-scale reactor operation in proportion VFA levels, and residual alkalinity calculated.

c. COD/N/P should be approximately 100/4/1 (Bouallagui et al., 2004). Low N or P levels will ultimately limit digestion, but may take more than 20 to 40 days to manifest in a large scale system.

i. Co-digestion with a nutrient-rich substrate may help. Otherwise, nutrients must be added.
Note: Manure and yogurt are high in phosphorus. Manure and beans are high in nitrogen.

ii. Estimate the cost of nutrient addition per year. The cost will depend on which compound(s) are chosen as a nutrient source. These costs account for operating costs.

Cost of N addition ($/yr) = [Required N (kg N/1000 kg COD) x COD loading (kg/day) - Available N (mg/L) x 1 kg/1000 mg x wastewater loading (L/day)] x cost of N ($/kg as N) x days of operation/yr [5]

Cost of P addition ($/yr) = [1/4 x Required N (kg N/1000 kg COD) x COD loading (kg/day) – Available P (mg/L) x 1kg/1000 mg x wastewater loading (L/day)] x cost of P ($/kg as P) x days of operation/yr [6]

d. Total solids (TS) >10% can inhibit digestion, particularly in batch tests.

e. Volatile solids (VS) is the organic fraction, which is available for biodegradation.

f. High sulfide concentrations will produce H_2S gas, and sulfate concentrations will promote sulfate reducing bacteria which produce H_2S gas. The biogas may need to be pretreated.

i. Tennessee air permits required < 1800 ppm H_2S, so an industrial digester installed a biogas scrubber (Rosdil, 2006).

ii. Some internal combustion engines can utilize biogas if the hydrogen sulfide is less than 60 ppm.

iii. Hydrogen sulfide at 800 to 1000 ppm can be fatal to humans.

g. High salt concentrations may inhibit digestion. Over time, a digester may be able to acclimate to a higher degree.

h. High phenol or LCFA concentrations may require pretreatments (see Literature Review), which would raise capital and/or operating costs, depending on the chosen treatment method.

6. If initial test concentrations are within the acceptable ranges, continue to Section 6. If some of the concentrations are outside the appropriate ranges, estimate the costs to rectify these issues. If the required nutrient addition or toxicity treatment is economically prohibitive, the waste-stream is inappropriate for anaerobic treatment.

Step 5. Anaerobic Respirometry

The respirometer is used to conduct the biodegradability tests (similar to serum bottles). Microbial seed, nutrient media and wastewater substrate are measured out in multiple reaction flasks. Flasks are stirred and incubated at a constant temperature via a water-bath. The respirometer measures individual real-time biogas production for each flask. Computer software calculates progressive gas production rates and cumulative gas production. Gas samples of the headspace will be collected by syringe and analyzed by gas chromatograph to determine the percent methane production.

1. Collect an appropriate feed stock sample from the processing plant. Note: Synthesized feed solution CANNOT be used. The full array of processing waste constituents cannot be replicated and may include disinfectants or toxic compounds that could disrupt anaerobic digestion, the affects of which must be properly analyzed. Any solid waste in the sample should be removed or processed to a liquid consistency and diluted to a total solids content of 1 to 5%. Note: To test the biodegradability of intact solid constituents consider implementing a bench-scale reactor instead.

2. Identify an anaerobic seed source. Possible resources include a local anaerobic digester, a lagoon at the food processing plant showing evidence of anaerobic activity, manure (rumen) slurry, or a lab culture.

a. To maximize biological activity, collect fresh seed just before setting up the respirometer. Color can serve as an indicator of anaerobe quality. Active seed will exhibit a dark black color; inactive or unviable seed may be grey or light olive green.

b. Refrigerate the seed sample if necessary. Anaerobes will remain dormant yet viable at low temperatures. Note: A longer refrigeration period will result in a longer start-up time; in this case such a delay is not the result of toxicity or acclimation.

3. Set up the respirometer. See Appendix B for the respirometer set-up procedure, and the Challenge System respirometer manual.

4. Collect gas samples from the head space of each reaction flask. Analyze the samples via gas chromatography and record the CH_4 and CO_2 fractions throughout normal reactor operation.

   a. At a minimum, sample the flasks following peak gas production and at the end of the run.

   b. Designate one gas-collection syringe for all sampling to ensure the same operation and sample volume for each flask.

   c. Between samplings flush the syringe with ambient air several times (rather than headspace gas), to minimize the volume drawn from the flasks and reduce negative pressure in the bubble counters.

   d. Use gas cylinders of standardized composition (CH_4 and CO_2) to establish a calibration curve. Verify calibration with each sampling event.

6. If methane production remains low or unstable 15 days after startup, consider these amendments:

   a. Add a greater volume of seed to each flask.

   b. Add more micronutrients (S, Fe, Ni, Co)

   c. If solid waste is being tested, be sure to dilute to < 5% TS.

7. End the respirometer test once all flasks cease gas production. Test the pH and COD of each flask.

8. Analyze the respirometer results.

   a. Graph the cumulative biogas production (mL) vs. time (days).

   b. Graph the biogas production rate (mL/hr) vs. time (days)

c. Calculate the approximate percent theoretical methane production.

i. Subtract out methane production from the seed control.

ii. The theoretical (maximum possible) methane production is 395 mL CH_4 per 1000 mg COD at 35°C.

d. Compare methane potential to current energy usage. Use the results from sections 5.3.2 or 5.4.1 (COD) and section 6.8 (respirometer) to reassess the economic benefit of an anaerobic system (first evaluated in section 4.5). One thm can be provided by 96.7 ft^3 natural gas.

9. If the overall respirometer results are poor (after allowing 30 for seed acclimation), then conduct a toxicity test (section 7) to determine if substrate components are a source of methanogen inhibition. Poor results are constituted by:

a. < 60% CH_4 fraction in biogas

b. < 60% theoretical CH_4 produced.

10. If respirometer methane production is good, then move ahead to section 8 to interpret the overall results.

Step 6. Toxicity Test

If overall respirometer tests are poor, conduct a toxicity test.

1. See Speece (1996) and/or Owen et al. (1979) for toxicity test methods. If the methane production rate decreases as the substrate level added increases, this is an indication of toxicity.

2. If toxicity is confirmed, conclude 'no further study' is necessary and report anaerobic digestion to be an inappropriate treatment technique for this substrate.

3. If toxicity is not an issue, essential nutrients may be lacking or the seed-to-substrate ratio in the reaction flasks may be too large to distinguish gas production from the substrate. Consider a different respirometer set-up and run the test again with fresh seed.

Step 7. Interpretation

1. Compare the predicted COD destruction and methane production to the intended treatment goals and biogas applications. Compare methane results to theoretical methane production and energy potential.

2. Consider implications of various results, including economic and environmental consequences.

    a. If nutrients are lacking and respirometer results are poor, co-digestion may be worth considering.
    b. Re-evaluate the protocol for the blended substrate conditions.

3. If conclusions support anaerobic digestion, the processor should then conduct a pilot scale reactor study.

    a. Small batch tests such as the respirometer provide are not representative of large-scale mixing effects or sustained alkalinity needs. Pilot scale reactors must be tested before full-scale projects are pursued.

References

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APPENDIX A

PROCESSING PLANT ASSESSMENT WORKSHEETS

Worksheet 1 of 3

Plant Name: _____ Contact Info: _____

Assessment Date: _____ Completed By:_____

1. Have you taken any measures to reduce water use or waste production?

   _____ Yes _____ No

Please explain: _____

_____

2. Have you considered anaerobic digestion?

   _____ Yes _____ No

Please explain: _____

_____

3. Anaerobic digestion can potentially produce methane (natural gas).

   Does the plant use natural gas?

   _____ No

   _____ Yes, approximately _____ thms per month

4. a. What is the current wastewater treatment method? _____

   b. Are there discharge requirements?

   BOD (mg/L) _____

   Other _____

5. Please provide the plant's current process-flow diagram(s).

Please note: All identifying information will be kept in strict confidence.

6. Please list in the table below any cleaners, sanitizers or chemical additives used in processing. If certain compounds are only used in specific processing lines (and waste streams) please specify which.

Such compounds may include (but are not limited to):

Oxidizing sanitizers

- Hyperchlorites, chlorine, chloramines
- Organic bromine
- Iodine, alcohol-iodine, iodophors
- Hydrogen peroxide, peroxy acids

Biocides and non-oxidizing sanitizers

- Organic acids (e.g. acetic acid, propionic acid, formic acid, carboxylic acids)
- Acid anionic sanitizers
- Acid-quat sanitizers
- Quaternary ammonium compounds

Chemical Used	**Processing Line/ Waste stream ID**	**Application Frequency (ex: metered in, or weekly)**

Please note: All identifying information will be kept in strict confidence.

Worksheet 2 of 3

Plant Name:	Contact Info:
Assessment Date:	Completed by:

Please fill out one worksheet per wastestream, entering as much information as possible

Wastestream Location /ID:		**Avg. Temp:**		**Data from Year** _____
Commodities Processed on this line:				

	Volume (gal/d)	COD (g/L)	BOD (g/L)	pH	T Phos (mg/L)	NH4 (mg/L)	Potassium (mg/L)	Magnesium (mg/L)	Iron (mg/L)	TSS (mg/L)
Jan										
Feb										
Mar										
Apr										
May										
Jun										
Jul										
Aug										
Sep										
Oct										
Nov										
Dec										

Worksheet 3 of 3

Plant Name:	Contact Info:
Assessment Date:	Completed by:

Commodity:	_____	_____	_____	_____	_____	_____	NOTES
Data from Year _____	Avg. Volume Processed (ton/d)	Avg. Volume Processed (ton/d)	Avg. Volume Processed (ton/d)	Avg. Volume Processed (ton/d)	Avg. Volume Processed (ton/d)	Avg. Volume Processed (ton/d)	
Jan							
Feb							
Mar							
Apr							
May							
Jun							
Jul							
Aug							
Sep							
Oct							
Nov							
Dec							

APPENDIX B

RESPIROMETER SET-UP PROCEDURE

1. Flask Allocation

Determine the desired flask assignments including which will serve as controls and which as duplicates. Then determine the appropriate volume allocation of substrate, seed and nutrient solution for each flask, using the following guidelines.

First, allocate the substrate. Include between 150 and 250 mg COD. More than 150 mg should insure a significant net biogas volume; less than 250 mg should insure that the trial finishes in less than 45 days, though this will also depend on the seed activity. This COD amount will correspond to a given volume of substrate based on its COD concentration (mg/L). Subtract this substrate volume from the working flask volume, where the working volume is equal to the total volume less 10% for the headspace. This remaining volume consists of the seed and nutrient solution:

$$V_{Seed + Nutrients} = 0.9\ V_{Total} - V_{Substrate} \quad [1]$$
$$\text{where } V_{Substrate} = 200 \text{ mg COD} / X \text{ mg/L } COD_{Substrate}$$

The seed and nutrient volume is subdivided at a ratio of 1 part seed to 4 parts nutrient solution. Resazurin, an oxygen indicating dye, should be added to each flask for a final concentration of 1 mg/L. For 600 mL flask volumes, add approximately 0.5 mg. As resazurin is a fine powder, it is easiest to first mix a concentrated solution (1000 mg/L) dissolving 10 mg in 10 mL of water and then pipetting 0.5 mL into each flask.

Most flasks will not include all three components. For these cases, replace any component (or partial volume, e.g. ½ nutrients or ½ [wastewater, seed, nutrients]) with de-ionized water. This insures the different flasks have comparable dilutions.

2. Nutrient and Metal Solutions

The nutrient solution is composed of mineral and metal solutions. This is such because the trace metals are at such a low concentration in the nutrient solution that it is necessary to mix a more concentrated metal solution first. The nutrient solution is taken from Shelton and Tiedje (1984).

Measure out one liter of de-ionized water into an Erlenmeyer flask with a stir bar on a stir plate. Add to the flask the following measured amounts.

- 0.500 g $MnCl_2 \cdot 4H_2O$
- 0.050 g H_3BO_3
- 0.050 g $ZnCl_2$
- 0.030 g $CuCl_2$
- 0.010 g $Na_2MoO_4 \cdot 2H_2O$ [1]
- 0.500 g $CoCl_2 \cdot 6H_2O$
- 0.050 g $NiCl_2 \cdot 6H_2O$
- 0.050 g Na_2SeO_3

[1] In the original text, this is denoted 'Na_2Mo_4', and assumed to be an error.

This constitutes the metal solution. Only a small fraction will be used for the nutrient solution; the remaining can be stored for later testing.

New mineral solution should be made for each respirometer trial. For the mineral solution, measure out enough de-ionized water to satisfy the flask allocation requirements of nutrient solution, and pour into an Erlenmeyer flask with a stir bar on a stir plate. Add to the flask the following amounts per liter of water.

- 0.270 g KH_2PO_4
- 0.350 g K_2HPO_4
- 0.530 g NH_4Cl
- 0.075 g $CaCl_2 \cdot 2H_2O$
- 0.100 g $MgCl_2 \cdot 6H_2O$ [2]
- 0.020 g $FeCl_2 \cdot 4H_2O$

[2] In the original text, this is denoted '$MgCl \cdot 6H_2O$', and assumed to be an error.

Once all is dissolved, pipette 1 mL of metal solution into the mineral solution. Transfer this mixed solution into glass jars with autoclavable caps. Measure out the volume of de-ionized water required for the flask allocations into similar glass containers. Sparge the headspace of each container with nitrogen gas for at least ten seconds and cap tightly. Autoclave the containers for ten minutes. Once they have cooled to room temperature (typically the following day), carefully transfer to a large Erlenmeyer flask, taking care not to aerate the solution. Continuously sparge the flask headspace with nitrogen gas while stirring gently with a stir bar and stir plate. Add 1.20 g $NaHCO_3$ per liter of solution, and cover the flask with parafilm.

3. Flask Set-up

Remove the wastewater sample(s) from storage to warm up. Collect the seed sample at this time (if possible) and leave it at ambient temperature (or remove from storage and warm to ambient temperature. Add a stir bar to each clean reaction flask and then measure out the flask constituents, doing one at a time for all flasks. Consider the following order: de-ionized water, nutrient solution, resazurin dye, wastewater and seed. Cap the flasks tightly, with a new septum in each cap. Take care not to over tighten the caps and break them.

The color of the resazurin in the flasks should turn from blue to pink as the seed (and sometimes the wastewater) is added, and again from pink to 'colorless' as the flasks are placed in the water bath and stirred. The color change is indicative of reducing conditions and microbial activity.

As soon as possible, fill the water bath and place in the flasks, and then start the water bath heater. It is best to bring up the flask temperatures gradually with the

water bath, rather than place the flasks in a pre-heated bath and potentially shock the system. Vent the flasks at this time and hook them up to the respective gas counters. Set up the software program and start data collection immediately. The first eight to twelve hours, the gas pressure will equilibrate as the temperature rises. Thus, the reported gas rates will not be representative of true seed activity or actual gas production during this equilibration period. After this initial start up period, check that the seeded flasks have returned to septic conditions and are no longer tinted pink; any pink flasks at this point may indicate a loose cap.