Sampling and DNA extraction from **Anaerobic Digester Sludge**

Standard Protocol

Version 1.0
Skill Prerequisites: DNA handling

Introduction - Sampling

This protocol describes sampling and storage of sludge from anaerobic digesters at wastewater treatment plants prior to performing microbial analysis. It is important to follow standardised procedures to get optimal and reproducible results from the microbial analysis.

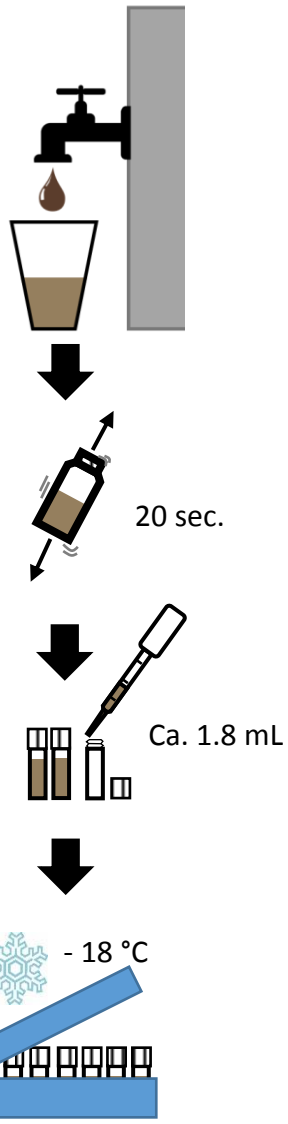
Materials - Sampling

We recommend the following equipment for optimal sampling.

Sampling and storage

- A bucket
- Plastic bottle with lid, 1 L (DELTALAB S.L., 444613)
- Pasteur pipettes with wide opening, 3 mL (DELTALAB S.L., 200006B)
- Sample tubes ”cryo tubes”, 2.0 mL (DELTALAB S.L., 409002.1)
- Storage box for cryo tubes (DELTALAB S.L., M-510)
- Permanent freeze resistant marker pen (Sharpie fine-point)

Sampling and storage



Preparation

Label 3 sample tubes [2.0 mL cryo tubes]. Information to put on the tube label: Date, location, reactor name/number and replicate number (1, 2, 3).

Sampling

The sample should be drawn from the digester during mixing to ensure that the sludge is sufficiently homogenous.

1. Open the valve and allow any old sludge in dead volumes (pipes and hoses) to be discarded to ensure that the sample does not consist of old sludge which does not reflect the reactor conditions. Pour at least 1 L of fresh sludge into the bucket.
2. Transfer approximately 0.5 L of the sludge to the 1 L container with lid.
3. Shake the container vigorously for 20 seconds to ensure that the samples is well mixed.
4. Transfer ~1.8 mL sludge to each of the labelled sample tubes [2.0 mL Cryotubes] using a Pasteur pipette.

Storage (”Biobank”)

The samples should be frozen immediately and stored at –18 °C or colder in a storage box. They can be stored for years and act as a ”Biobank” for the treatment plant.

Clean the equipment

Wash the 1 L container and the bucket with some soap and water. Wash subsequently with lots of water to remove leftover soap and dry the equipment.

Introduction – DNA extraction

This protocol explains DNA extraction from digester sludge from wastewater treatment plants. The protocol is based on the FastDNA® spin kit for soil protocol (MP biomedicals) with some modifications, mainly streamlining and longer bead beating.

The key in sampling and DNA extraction is consistency and hence this protocol should be followed to the letter. If you choose to deviate from the protocol do it consistently throughout your experiment.

Materials – DNA extraction

DNA Extraction and QC

- FastDNA® Spin kit for soil (MP Biomedicals)
- FastPrep-24 (MP Biomedicals)
- Spintubes (DNAse free), 1.5 mL
- Greiner tubes, 15 mL
- Ice
- Pipettes (Range 1 µL to 1000 µL)
- Nanodrop1000 (Thermo Scientific)
- Electrophoresis chamber and power source
- Gel-Doc
- DNAse free tips (10 µL, 300 µL and 1000 µL)
- Gelred (Biotium)
- 1kb Generuler
- TAE-buffer 50x
- Nuclease Free H_2O (Qiagen)
- Permanent marker (Sharpie)
- Personal protective equipment

Method

Preparation for DNA extraction

When preparing many samples it is recommended that all tubes are aligned according to the processing and marked with sample numbers prior to starting the DNA extraction. Beadbeating tubes should be marked in more than one location as the beating process can erase the text.

1. Sample input
    a. Target volume: 50 µL
2. Prepare tubes for the whole workflow (pr. sample):
    a. 1 x Lysing Matrix E tube (from kit)
    b. 1 x SPIN filter (from kit)
    c. 1 x Catch tube (from kit)
    d. 3 x 1.5 mL DNAse free tubes
    e. 1 x 15 mL Greiner tube
3. Thaw sample aliquot at room temperature and store on ice until use.

DNA Extraction

Bead beating

1. Mix sample before use i.e. with vortexing.
2. Transfer **980** µL Sodium Phosphate Buffer (PBS) to a Lysing Matrix E tube.
3. Transfer and 120 µL MT Buffer to the Lysing Matrix E tube.
4. Transfer **50 µL** digester sludge to a Lysing Matrix E tube using a wide tip (or cut tips with a pair of scissors, to allow small chunks to enter and exit the tip).
5. Perform bead-beating in the FastPrep-24 instrument
    a. Time: 4 x 40s
    b. Speed: 6 m/s
    c. Adaptor: Custom
    d. Remember to balance samples like in centrifuges.
    e. Between each 40s interval the samples should be kept on ice for 2 min to cool down.

Protein Precipitation and binding of DNA to matrix

1. Spin down samples at >10,000 g for 10 min, preferably at 4°C.
2. While samples are spinning add 250 µL PPS (Protein Precipitation Solution) to fresh 1.5 mL spintubes.
3. After centrifugation, transfer supernatant to 1.5 mL spintubes with PPS and then shake the tubes 10 times by hand (keep the tubes on ice until all samples are processed).
4. Centrifuge the tubes at 14,000 g for 5 minutes to pellet the precipitate. While the centrifuge is running, re-suspend the Binding Matrix and add 1.0 mL to a 15 mL Greiner tube.
5. Transfer the supernatant to the 15 mL tube with Binding Matrix suspension.
6. Invert by hand for 2 minutes to allow binding of DNA to the matrix.
7. Place the tube in a rack for 3-5 minutes (or until the liquid appears clear) to allow settling of the silica matrix.
8. Remove and discard up to 2x750 µL of supernatant being careful to avoid settled Binding Matrix.
9. Re-suspend Binding Matrix in the remaining amount of supernatant.

DNA washing and elution

1. Transfer approximately 750 µL of the mixture to a SPIN™ Filter and centrifuge at 14,000 g for 1 minute.[1]
2. Empty the catch tube.
3. Ensure that ethanol has been added to the Concentrated SEWS-M.
4. Add 500 µL prepared SEWS-M and gently re-suspend the pellet using the force of the liquid from the pipet tip - or by stirring with a pipet tip.
5. Centrifuge at 14,000 g for 1 minute.
6. Empty the catch tube and use it again.
7. Centrifuge at 14,000 g for 2 minutes to “dry” the matrix of residual wash solution.
8. Discard the catch tube and replace it with a new tube.[2]
9. Allow the SPIN™ Filter to dry for 5 minutes at room temperature with open lid.

[1] If you have more sample than 750 µL, you should repeat this step.

[2] The new tube is the tube that the sample is to be stored in so be sure to label it properly.

10. Gently re-suspend Binding Matrix (above the SPIN filter) in 60 µL of DES. - Use a pipet tip to stir the matrix until it gets liquid. Make sure not to disrupt the filter.
11. Centrifuge at 14,000 g for 1 minute to bring the eluted DNA into the clean catch tube. Discard the SPIN filter.
12. Store DNA at -20°C for short-term storage and -80°C for long-term storage.

DNA QC (optional)

1. Quality and Concentration Check
   a. Nanodrop DNA measurement. Use guidelines provided by the vendor. A260/280 should be 1.8 to 2.0. A260/230 2.0 to 2.2. Also compare the UV-vis absorbance curve to that of pure DNA[3].

2. Run gel electrophoresis
   1. Prepare a 1% agarose gel
      1. 1 g agarose, 100 mL 1xTAE-buffer, heat in microwave and gently swirl to completely dissolve agarose. After slight cooling add 2 µL gelred and cast gel.
   2. Prepare samples by mixing 1 µL 6x loading buffer, 2 µL sample (20-50 ng) and 3 µL nuclease free water. Prepare ladder according to vendor recommendations.
   3. Run electrophoresis approximately 80 min at 120V (6 V/cm).
   4. Capture gel image on gel-doc, save and analyze. The majority of DNA should be in the range 3-10+ kb. If it is below the DNA is heavily degraded and it might affect the downstream uses.

[3] DNA extracted with 'FastDNA spin kit for soil' is known to contain contamination producing a peak around 230 nm. The contaminant can lead to overestimation of DNA concentration. The contamination does not effect PCR, but might impact ligation/transposome based sequencing library protocols.

Literature

Instruction Manual, FastDNA® SPIN Kit for Soil, Revision # 6560-200-07DEC, MP Biomedicals