Chapter 4
Preparation of the Microcosm N-System

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Abstract In this chapter, the preparation of the microcosm test, including the apparatus, the dissolved oxygen (DO) meters (electrode DO meter and fluorometric DO meter), the calibration of the DO meter, and the oxygen dissolution rate are described. Additionally, the treatment of the microcosm N-system from successive subculturing is also explained.

4.1 Apparatus

- 300 mL Erlenmeyer flask
- Silicon plug (Matsuura Machinery, Catalog No. 6-343-16, Type No. C-40)
- Incubator (25 °C, L/D cycle = 12 hr/12 hr, illuminance = 2400 lux (photosynthesis photon flux density = 36 μmol/m^2/s))
- Dissolved oxygen (DO) meters
- Black rubber tube (only needed when light leaks from DO meter)
- Stirrer (unnecessary if the DO meter is able to perform measurements in standing water)
- Stirring rotor bar (unnecessary if the DO meter is able to perform measurements in standing water)

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- Perforated cap for DO electrode (unnecessary if the DO meter is able to perform measurements in standing water)
- Vinyl tube (unnecessary if the DO meter is able to perform measurements in standing water)
- Circular glass cover (unnecessary if the DO meter is able to perform measurements in standing water)
- Insulating material (e.g., silicon sponge sheet) and blank paper or a white acrylic plate (unnecessary if the DO meter is able to perform measurements in standing water)

4.2 Dissolved Oxygen (DO) Meter Protocols

4.2.1 Electrode DO Meter

1. Wash the tip (gold cathode, silver anode) of the DO meter, a cap, an O-ring, and a rotor well in pure water (demineralized water), and ensure that they are dry before proceeding.
2. Remove any color change from the cathode by polishing its surface. Polish horizontally with a super fine-grit sandpaper (greater than a #1000) to clean the gold cathode. If the electrode is not smoothed, it will cause the membrane to tear during attachment.
3. When refuse collects in the ditch around the gold cathode, remove it using the tip of a toothpick. Additionally, this prevents touching of the cathode, as the triangular part of the electrode is very fragile.
4. Affix an electrode and a plastic bottle to the meter as shown in Fig. 4.1, and wind plastic tape around it so that it will not leak from around the adjoining part.
5. Leave undisturbed for 2–3 min until there remains 14% of the ammonium solution or 1–2 h until there remains 3% of the ammonium solution, with the

Fig. 4.1 Wash of electrode

Fig. 4.2 Setup of DO electrode

tip of the DO meter completely submerged. If a terminal end has black dirt on it and does not become whitish, leave undisturbed for additional time.

6. Wash away the ammonium solution in pure water. If it does not wash well, dip it into pure water for a while.
7. Remove shavings by washing well with pure water, and then rinse it out three times in a potassium chloride solution (KCl).
8. Fill the tip with KCl and attach a membrane. Confirm that the film is not wrinkled and that air bubbles are not present inside of the membrane. When air bubbles are present, reattach the membrane. Remove it by pulling the edge of the membrane with an O-ring when a wrinkle is observed. Cut away any extra membrane afterward.
9. Place the glass cover and a stirring rotor bar into the cap, and attach it to the tip of the electrode. Prevent the silver electrode from touching the cap.

When using electrodes that require water flow to measure the DO (e.g., polarographic oxygen electrodes), use a perforated cap with a stirring rotor bar in the sensor region, and stir only near the sensor to measure DO. A stirring rotor bar can be moved smoothly by placing a circular glass cover at the bottom of the perforated cap and placing the rotor on it. A method of attaching this stirring system to the YSI-5750 is shown in Fig. 4.2. Insert the electrode (within which the stirring system has been emplaced) until it is in contact with the bottom of the 300 mL Erlenmeyer flask, and affix it using a rubber or silicon gasket or clip. Do not block the mouth of the flask. Attach an insulating material and blank paper to the stirrer so that the heat of the stirrer will not reach the flask. A white acrylic board (which has been processed to a thickness of 5 mm and has a gap of 5 mm formed below it) may be substituted for the insulating material and blank paper. Carry the flask equipped with an electrode on this board, stir it, and then measure the DO.

4.2.2 Fluorometric DO Meter

Because light leaks out from the side of the electrode, a fluorescence-type DO meter, such as the ProODO (YSI, Japan), is used to interrupt light in a black rubber tube (Fig. 4.3). Affix this electrode using a rubber or silicon gasket or a clip from the

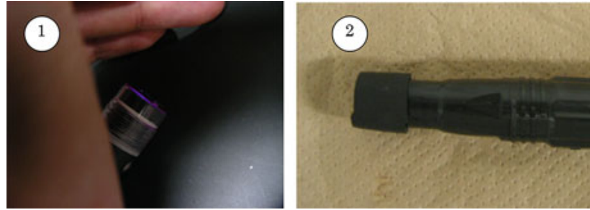

Fig. 4.3 Interruption of light in a black rubber tube

bottom of the 300 mL Erlenmeyer flask to a depth of ~1 cm. Do not block the mouth of the flask. Stirring of the flask is not necessary, as spatial inhomogeneity is considered an ecological characteristic.

Additionally, it is appropriate to use the fluorescence-type (optical) DO meter in the microcosm test due to its ease of maintenance and stable performance.

4.2.3 Calibration of the DO Meter

Pour 210 mL of distilled water or Milli-Q water into a 300 mL Erlenmeyer flask, attach a DO electrode in the manner prescribed in Sects. 4.2.1 and 4.2.2, and measure DO continuously overnight every 30 min. Review the DO concentration to confirm that it is stable, and then calibrate the concentration at that time as 100%. Additionally, finish calibrating atmospheric pressure before the calibration of DO when there is an automatic correction function based on the atmospheric pressure.

4.2.4 Oxygen Dissolution Rate

The rate (δD) at which oxygen moves between the atmosphere and the water is influenced by the state of the electrode, the incubator, the stirrer, the opening of the mouth of the flask, and other variables. Therefore, every experimental replicate (i.e., the flask, electrode, stirrer, etc.) should be tested, and the oxygen dissolution rate should be determined individually or in bulk by calculating the average. If possible, it is desirable to measure δD twice, immediately before and immediately after an experiment, and to obtain the mean DO concentration; δD should be measured according to the protocol described below.

4.2.4.1 Experiment

Pour 210 mL of distilled or Milli-Q water into a 300 mL Erlenmeyer flask, and aerate with oxygen or nitrogen. Measure the DO concentration of the liquid phase so that it is higher than the equilibrium level in the atmosphere (in the case of oxygen) or so

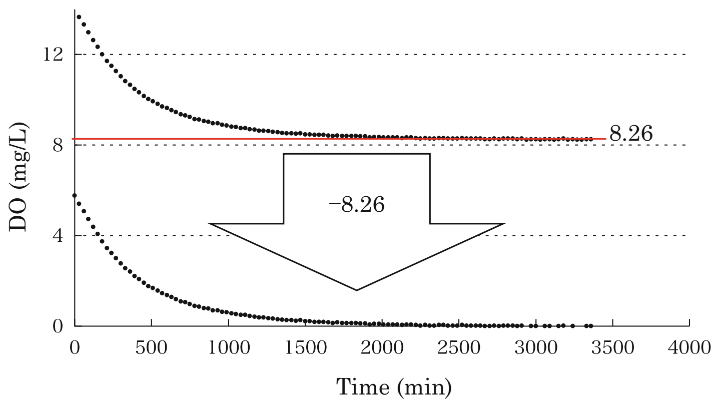

Fig. 4.4 Reduction of 8.26 from measured value

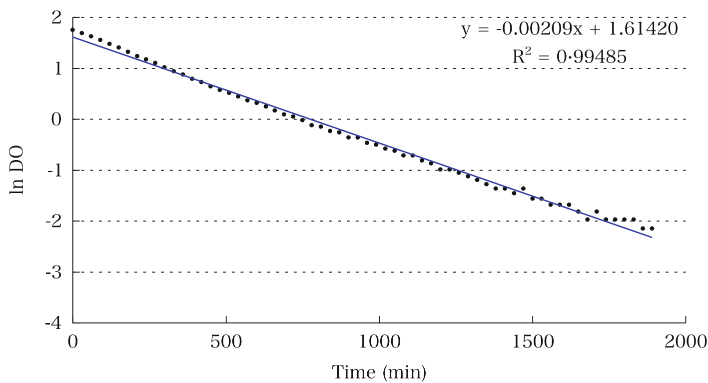

Fig. 4.5 Linear approximation by logarithm with deduction of 8.26

that it is lower (in the case of nitrogen). Leave the flask undisturbed for ~60 min without closing the cap. Then, replace the oxygen or nitrogen of the vapor phase in the flask with air, and measure the DO for 24–48 h, as described in Sect. 4.2.3.

The method of calculating the δDO with data when aerated with oxygen is shown in Fig. 4.4 as an example. The black point in the upper section of Fig. 4.4 represents the DO data when aerated with oxygen. Dissolved oxygen decreases exponentially and reaches equilibrium at 8.26 mg/L. When 8.26 mg/L is subtracted from the original DO data, it results in the graph shown in the bottom panel of Fig. 4.4.

The linear approximation of the natural logarithm ($\ln x = \log_e x$) for the data (>0) subtracted from 8.26 mg/L is shown in Fig. 4.5. However, large errors arise as the line approaches the equilibrium concentration, and, after approximately 700 min, it cannot be approximated well because of the amount of noise.

The slope of the approximately straight line shows the constant rate of oxygen transfer. Assuming that the constant rate of oxygen transfer is a and the equilibrium

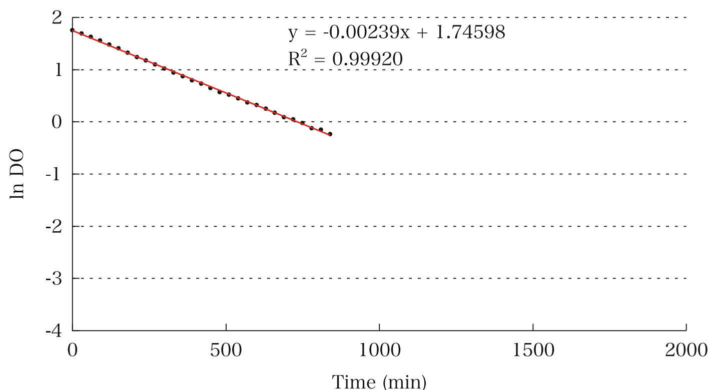

Fig. 4.6 Linear approximation with removal of measurement error

concentration of DO is b, the oxygen transfer rate (δDO) at a certain DO concentration x can be calculated according to the following formula:

$$\delta \mathrm{DO} = a(x - b).$$

Since $b = 8.26$ mg/L (Fig. 4.4) and $a = -0.00239$ (Fig. 4.6), δDO (mg/L/min) in this example is

$$\delta \mathrm{DO} = -0.00239\ (x - 8.26).$$

This oxygen transfer rate (δDO) is used in Sect. 6.2, "Calculation of Respiration and Production Quantities in the Microcosm System," of this book.

4.3 Microcosm N-System

The maintenance culture of the microcosm N-system must remain aseptic. The culture used for experimentation adhered to a culture maintenance method. The inoculum dose of the N-system seed is 10 mL. A TP (Taub + peptone) medium (shown in Table 3.1) is employed for cultivation. After adjusting the TP medium, dispense it into a 300 mL Erlenmeyer flask by 200 mL volume, cap it with a silicon plug, and then autoclave it at 121 °C for 15 min to sterilize it. After allowing it to cool to room temperature, add 10 mL of the N-system, which was cultured for 2–8 weeks, as a seed, and culture it in an incubator with an L/D cycle = 12 hr/12 hr and an illuminance = 2400 lux (photosynthesis photon flux density = 36 μmol/m^2/s), at a temperature of 25 °C. Stir it lightly once a day until the culture begins 1–3 days later, then leave it at rest, and culture it afterward. Subculturing is performed once every 2 months. Use a National (i.e., Panasonic) FL20SS-W/18 as

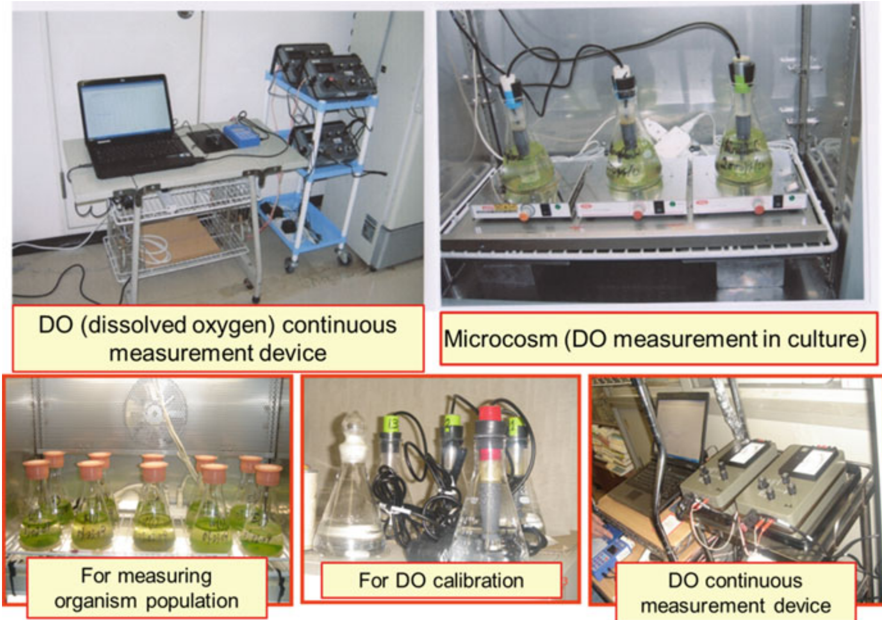

Fig. 4.7 Microcosm N-system apparatus

a source of light, replace all fluorescent bulbs every 3 months or measure the illuminance or photosynthesis photon flux density every month, and replace them as needed. Various devices associated with culturing of the microcosm are shown in Fig. 4.7. The DO electrode that was attached to the microcosm was connected to a DO meter outside of the incubator, and the wave patterns of the DO were continuously monitored using a data logger on a personal computer at the same time as they were recorded. When an abnormality is observed within a wave pattern, immediately reset the DO sensor. Preparation of the N-system used for biomass and chemical analyses and the one used for measuring DO should be performed separately. Additionally, both microcosms should be cultured equally until an electrode for DO measurement is attached.

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