Chapter 2
Standardization of the Microcosm N-System

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Abstract In this chapter, the perspective needed for examining graded impact statements is described. Such an evaluation is comprised of the examinations of various culture conditions for standardizing the microcosm N-system, the two-species cultures, the microcosm, and the mesocosm.

2.1 Standardization of the Microcosm N-System

In order to use the microcosm for environmental impact assessments, it is necessary to standardize the microcosm culture conditions. To this end, important parameters of the culture conditions for microbial cultivation were investigated.

2.1.1 Species Composition

Seventeen species, consisting of six species of protozoans and metazoans as predators, seven species of algae as producers, and four species of bacteria as decomposers, were used to investigate the effect of species composition on the stability and reproducibility of the microcosm system, with the aim of developing a standard, aquatic, flask-sized microcosm system. Three species each of protozoans and metazoans were used as predators (consumer). The protozoans (Ciliata) were *Cyclidium glaucoma*, *Tetrahymena pyriformis*, and *Colpidium campylum*. The

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Y. Inamori (ed.), *Microcosm Manual for Environmental Impact Risk Assessment*,
https://doi.org/10.1007/978-981-13-6798-4_2

metazoans were of two types, namely, rotifers and aquatic oligochaetes. The former included *Philodina erythrophthalma* and *Lecane* sp., and the latter was *Aeolosoma hemprichi*. These protozoans and metazoans frequently appear in both natural ecosystems, such as lakes, marshes, rivers, and seas, and in artificial ecosystems, such as biofilms and activated sludge, and they are easily cultured and accurately counted. These strains were isolated from water treatment facilities and a polluted lake.

Three species of algae belonging to Chlorophyta, *Chlorella* sp., *Scenedesmus quadricauda*, and *Chlamydomonas monticola* and four species of blue-green algae, *Tolypothrix* sp., *Oscillatoria agardhii*, *Anabaena flos-aquae*, and *Microcystis viridis*, were used as producers. The strains were sourced from the Microbial Culture Collection Center of NIES in Tsukuba, Japan. These algal species are often observed in lakes and ordinal ponds. Four species of bacteria were used as decomposers, namely, *Pseudomonas putida* (gram-negative aerobic rod bacteria), *Bacillus cereus* (gram-positive aerobic endospore-forming rod bacteria), *Acinetobacter* sp. (gram-negative rod bacteria), and a coryneform bacterium (gram-positive rod bacteria). Mixed cultures of these bacteria were utilized.

Taub's basal medium (200 mL) containing 100 mg/L of polypeptone was placed in a cotton-plugged 300 mL volume Erlenmeyer flask and sterilized by an autoclave at 1 atm and 121 °C for approximately 20 min. A stock culture of each microorganism was added, and the flask was placed in a growth chamber at 25 °C under cool white fluorescent lighting for 12 h (2500 lux) and in the dark for 12 h, sequentially. Cultivation was conducted under static conditions.

Certain species of protozoan predators were able to be established within the system, but others were not. All species of metazoan predator were able to coexist, and filter feeders, such as rotifers, and detritus feeders, such as oligochaete, were also able to coexist. All species of blue-green algae (as producers) were able to establish themselves, notwithstanding any other microorganisms. All species of chlorophytes were able to be established, and the combination of and interaction between chlorophytes and protozoans appeared to be of importance. All species of bacterial decomposers were able to establish themselves, with none disappearing in any of the microcosms. It was apparent that ecosystem stability did not always increase as a function of species richness. From these results, the species composition of a standardized microcosm was determined as the combination of one species of protozoa, *Cyclidium glaucoma* (Ciliata); three metazoan species, including two rotifers, *Lecane* sp. and *Philodina erythrophthalma*, and one oligochaete, *Aeolosoma hemprichi*, as predators (consumers); two species of chlorophytes, *Chlorella* sp. and *Scenedesmus quadricauda*, and one species of blue-green algae, *Tolypothrix* sp., as producers; and four species of bacteria, *Pseudomonas putida*, *Bacillus cereus*, *Acinetobacter* sp., and a coryneform bacterium.

2.1.2 Culture Vessel

Constructing optimum culture conditions is necessary for generating a standardized microcosm. Specifically, the following must be optimized: (i) the movement of the experimental water body, (ii) the wall effects, (iii) species composition and abundance, and (iv) light and temperature conditions. To ensure an optimum microcosm cultivation volume, an 18 mL test tube and 100 mL, 300 mL, 500 mL, and 1000 mL Erlenmeyer flasks were used for investigation.

In culture vessels of any volume, all microorganisms in the microcosm transited the same growth curve, and no difference was observed among any of the culture vessels. The numbers (N) of each microorganism in the steady state were approximately as follows: 100 N/mL of *Cyclidium glaucoma*, 30 N/mL of *Lecane* sp., 40 N/mL of *Philodina erythrophthalma*, and 10 N/mL of *Aeolosoma hemprichi*. This indicates that the volume of the microcosm, ranging from 18 mL to 1000 mL, does not affect the succession of microbiota for the purposes of conducting the microcosm test. Although there is no influence on the succession of microbiota, a 300 mL Erlenmeyer flask is appropriate from the standpoints of handling, cultivation space, and sampling of the microorganisms.

2.1.3 Stirring

The effect of stirring on the stability of the microcosm was investigated. The microbiota in the microcosm was divided into two groups, influenced species and uninfluenced species, in the succession period. Specifically, the blue-green alga, *Tolypothrix* sp., grew in a fragmentary manner when stirred. However, there was no difference in the microbiota between the stirred microcosm and static microcosm by the 16th day after cultivation began. This indicates that stirring, as an outside factor, disturbs the stability of the microcosm during the succession period but has no affect in the stable period. In the microcosm, which is used as a model of natural ecosystems, spatial inhomogeneity is important as an ecological characteristic. Although some differences were observed in the growth patterns of certain microbiota, a stable system could be obtained under both stirring and static conditions.

2.1.4 Temperature

Culture temperatures were set to 10, 20, 25, and 30 degrees Celsius (°C), and the growth patterns of microorganisms in the microcosm were observed. The effect of temperature on microorganism growth was conspicuous, and the speed of succession

was faster under higher-temperature culture conditions. Namely, growth was delayed, the peak abundance of *Cyclidium glaucoma* appeared 20 days after cultivation began, and a high population density was maintained at 10 °C. However, the population sizes of other microanimals were lower than 10 N/mL, and the dominant species of bacteria was represented by only three individuals. At 20 °C, the peak abundances of bacteria and *Cyclidium glaucoma* appeared 7 days after cultivation began, and the population of *Cyclidium glaucoma* stabilized at ~150 N/mL. Other microorganisms also had more than 1 N/mL after 21 days, but the dominant species of bacteria was represented by only one individual. At 25 °C, all microorganisms exhibited concentrations of more than 1 N/mL by the 7th day, and a steady state was maintained after the 14th day. In the steady state, microanimals coexisted as ~100 N/mL of *Cyclidium glaucoma*, 30 N/mL of *Lecane* sp., 40 N/mL of *Philodina erythrophthalma*, and 10 N/mL of *Aeolosoma hemprichi*. At 30 °C, the succession pattern was fairly similar to that at 25 °C, but a decrease in the abundance of *Cyclidium glaucoma* by the 7th day was conspicuous. The microcosm culture allowing for high stability and serial transferring on a monthly cycle was determined to be 25 °C.

2.1.5 Illuminance

Material circulation in ecosystems begins with photosynthesis by plants and microalgae, such as chlorophytes and blue-green algae, which filled this niche in the experimental microcosm. To establish a standard condition for illuminance, the light/dark (L/D) cycle was investigated. An L/D cycle, with 12 hr each, was considered appropriate because the succession of microbiota was smooth. Even under a 16 hr/8 hr cycle of light/dark illuminance, the microcosm reached a steady state; however, the lifespan of the fluorescent lamp was short. Under both 24 hr/0 hr (total light) and 0 hr/24 hr (total darkness) conditions of illuminance, the succession of microbiota was unstable.

2.1.6 Substrate Concentration

While the medium is important for cultivating the microcosm, the substrate concentration is of greater importance. To obtain a stable microcosm, the effect of the substrate concentration was investigated; 25 mg/L, 50 mg/L, and 100 mg/L of polypeptone were added to Taub's basal medium, and the microcosm was cultured under these conditions. The microcosm was able to reach a steady state under all of these conditions, but there was an observed tendency toward population decline and extinction of some species of microorganisms under the 25 mg/L of polypeptone condition. From the results obtained in these experiments, it was determined that a 100 mg/L concentration of polypeptone was safe.

2.1.7 *Standard Cultivation Conditions of the Microcosm N-System*

From the experiments described in Sects. 2.1.1, 2.1.2, 2.1.3, 2.1.4, 2.1.5, and 2.1.6 of this chapter, the standard culture conditions of the flask-sized microcosm were determined as follows: (i) species composition involves the combination of one species of ciliate protozoan, *Cyclidium glaucoma*; three species of metazoan predators, two rotifers, *Lecan*e sp. and *Philodina erythrophthalma*, and one oligochaete, *Aeolosoma hemprichi*; two species of chlorophytes, *Chlorella* sp. and *Scenedesmus quadricauda*; and one species of blue-green algae, *Tolypothrix* sp., as producers, and four species of bacteria, *Pseudomonas putida*, *Bacillus cereus*, *Acinetobacter* sp., and a coryneform bacterium; (ii) the culture vessel size is that of a 300 mL Erlenmeyer flask; (iii) there is no stirring; (iv) culturing occurs at 25 °C and under 2400 lux, with a 12 hr/12 hr L/D cycle; (v) Taub's basal medium is supplied with 100 mg/L of polypeptone as the substrate. A basic pattern that was observed was an increase in the abundance of microorganisms in the microcosm under the standard culture conditions (Fig. 2.1). A similar increase was observed when a new nutrient medium was provided to seed the microcosm N-system, continuing the transition toward the stationary phase. In other words, the microcosm N-system has very high plasticity and stability, and the system is thus a superior tool for repeated experimentation.

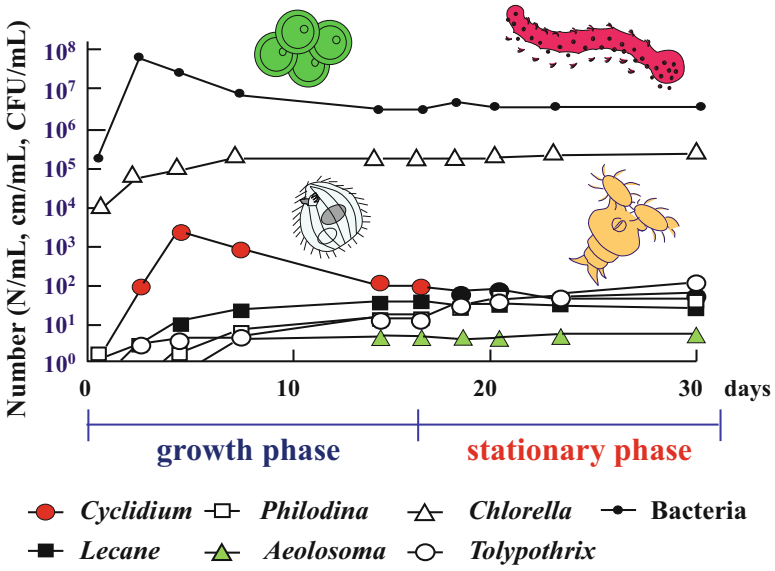

Fig. 2.1 Growth pattern of microorganisms in microcosm N-system

2.2 Environmental Assessment Using a Model Ecosystem

To estimate the effect of chemical substances on the microcosm, the step-by-step test method, consisting of three tests, was considered to be essential (Japan Sewage Works Association 2012). A conceptual diagram of the examination of the graded ecosystem impact is shown in Fig. 2.2. A two-species culture test was used to investigate prey-predator interactions between bacteria and microanimal predators. Microanimal predators were selected from among the protozoans and metazoans, taking note of the frequency of their appearances in both natural ecosystems, such as rivers, lakes, marshes, and seas, and in artificial aquatic ecosystems, such as biofilms and activated sludge, as well as the ease and accuracy with which they may be cultured and counted. The microanimals used were *Cyclidium glaucoma*, *Tetrahymena pyriformis*, *Colpidium campylum*, *Philodina erythrophthalma*, and *Aeolosoma hemprichi*. They have been cultured and serially transferred in a lettuce and egg (LE) medium for several years.

The flask microcosm described in this book consisted of four species of bacterial decomposers, one species of protozoa, three species of metazoan predators (consumers), and three species of microalgae as producers. This system displayed very high reproducibility and a robust reflection of a natural ecosystem. It is therefore suggested that this small-scale, repeatable microcosm can be used as a tool for screening tests at the ecosystem level.

A natural lake model ecosystem (i.e., a mesocosm) constitutes natural lake water in which naturally occurring microbiota exist (Graney et al. 1994; Harris 2013). It contains several species of predatory microanimals (consumers), microalgae as producers, and bacterial decomposers. This system can serve as an intermediate

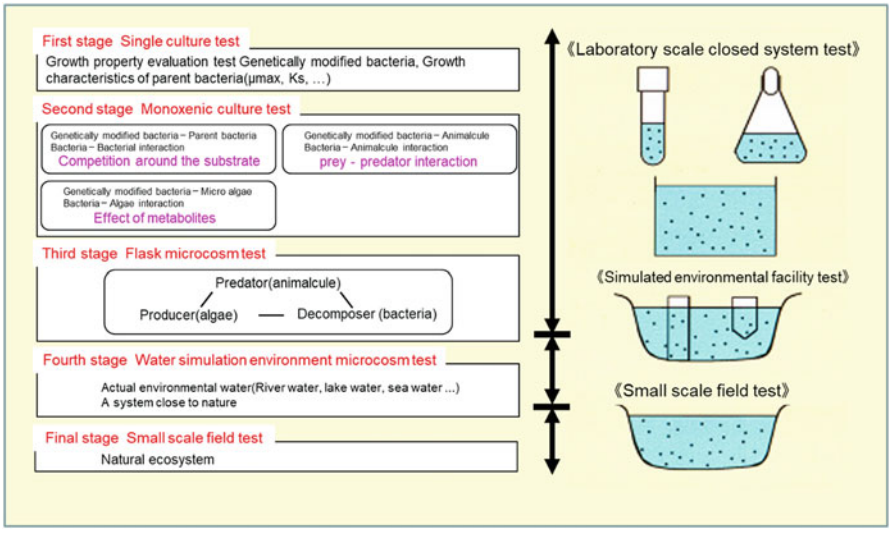

Fig. 2.2 Conceptual diagram of stepwise ecological impact assessment test (tier test)

between a flask-sized microcosm and a natural aquatic ecosystem. As described above, using a step-by-step assessment method (i.e., a tier test), the safety of target chemicals, genetically engineered microorganisms, and microbial pesticides can be more accurately evaluated.

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