Chapter 7
Example Assessments of the Microcosm N-System

Kazuhito Murakami, Kunihiko Kakazu, Kakeru Ruike, and Ken-ichi Shibata

Abstract In the example assessment, not only chemicals but also metals, radiation, and nonindigenous microorganisms were used as test materials. Estimations of the impacts of 106 examples of test materials (6 of surfactants, 3 of germicides, 14 of herbicides, 12 of pesticides, 2 of endocrine-disrupting chemicals, 1 of an antibiotic, 1 of an algal toxin, 6 of organic matter and solvents, 2 of nutrients, 15 of metals, 2 of radiation, 6 of microbial pesticides, 8 of genetically modified bacteria, 13 incorporating biomanipulation, and 2 involving climate change) are described in this chapter. It was made clear by the results of these tests that it is sufficient to analyze the P/R ratio with a branching-type ANOVA in the microcosm test. However, the characteristics of the constituent organisms were also evaluated, and the analyses are explained here.

7.1 Surfactants

The stability of aquatic environments in Japan has been improved, but many problems still remain in need of urgent resolution. One such problem is that great quantities of surfactants flow directly into aquatic environments with gray water, deteriorating the aquatic ecosystem, even though there is little problem in areas with waste management facilities because sewage is effectively treated. To assess the environmental effects of surfactants at the ecosystem level, it is necessary to analyze the mechanisms of community stability under the natural conditions present in aquatic environments because natural ecosystems are extremely complex and are

K. Murakami (✉)
Chiba Institute of Technology, Narashino, Chiba, Japan
e-mail: kazuhito.murakami@p.chibakoudai.jp

K. Kakazu · K. Ruike
University of Tsukuba/Foundation for Advancement of International Science, Tsukuba, Ibaraki, Japan
e-mail: kakazu@fais.or.jp; ruike@fais.or.jp

K. Shibata
Yokohama National University, Yokohama, Kanagawa, Japan
e-mail: shibata-kenichi-ym@ynu.jp

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exposed to unpredictable environmental factors. Instead of working with a natural ecosystem, it is convenient to use microcosms consisting of biotic and abiotic factors that originated from a natural ecosystem because they allow for both biological simplicity and replication (Beyers 1963; Cook 1967; Margalef 1969; Kawabata et al. 1978). In this section, the effects of surfactants on the aquatic ecosystem and the biodegradability of surfactants are described.

7.1.1 Linear Alkylbenzene Sulfonate (LAS)

A microcosm was used to assess the effects of the anionic surfactant, linear alkylbenzene sulfonate (LAS), on an aquatic ecosystem. The addition concentration of LAS was adjusted to 1, 5, and 10 mg/L and was added on the 16th day of the stable state into the microcosm. Linear alkylbenzene sulfonate supplied to this experiment was composed of a sodium linear-dodecyl benzene sulfonate standard (C12) and LAS at a purity of more than 99%.

The endpoints were the population density (structural parameter) and DO (functional parameter), and the population was measured by an optical microscope and counted from the beginning of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of B16–30 (days 16–30), which was the ratio of the abundance and population density (N_{30}) on the 30th day. The DO was measured continuously from the 16th day onward, and the P/R ratio was calculated from the amounts of production (P) and respiration (R). Using the structural parameter, the impact of LAS was evaluated in comparison with the control system according to the behaviors of the microorganisms in the microcosm on each of the 14 days after the addition of LAS for which measurements were taken. The population that did not change relative to the control system was maintained at a 1 mg/L surfactant load and recovered to almost the same population density, while there was a decrease in the density of microanimals in the microcosm with the 5 mg/L load. In the microcosm with a 10 mg/L load of LAS, *Cyclidium glaucoma* and *Philodina erythrophthalma* disappeared, and the populations of *Lecane* sp., *Aeolosoma hemprichi*, and *Tolypothrix* sp. were stable at low densities, and the microcosm ecosystem did not collapse.

As a result of the observed changes in the structural parameter, the m-NOEC (microcosm maximum effect-free concentration) of LAS was estimated as 1 mg/L. While the NOEC of LAS for *Daphnia magna* in a single-species examination was 1180–3250 μg/L and the NOEC for algae was 400–18,000 μg/L, it was thought that the m-NOEC exhibited slight unevenness. It was shown that the evaluations of the microcosm test using the structural parameter (i.e., population density) and the functional parameter (i.e., the P/R ratio) were consistent. The microcosm test is an effective tool for assessing the effects of surfactants on ecosystems because it allows for the evaluation of the effects of surfactants from the viewpoint of the interactions among microorganisms, material cycling, and energy flow. Based on these characteristics, the microcosm test is a useful method of performing environmental assessments that can reflect the behavior of natural aquatic ecosystems.

Nearly the same effects and degradation were observed when LAS was added to the scaled-up microcosm, which included the same microbiota as those in the culture medium of the flask-sized microcosm. This microcosm was scaled up to a 10 L glass jar (depth, 30 cm; surface area, 490 cm^2) containing 7 L (depth, 16 cm) of TP medium. In this experiment, concentrations of LAS, adenosine triphosphate (ATP), nutrients, DO, pH, oxidation reduction potential (ORP), chemical oxygen demands (COD), dissolved organic carbon (DOC), suspended solid (SS), and chlorophyll *a* (Chl.*a*) were also analyzed. When the initial concentration of LAS was 1.5 mg/L, changes in the abundance of all microorganisms were the same as those in the control system. At 2.5 mg/L, the abundance of *Cyclidium glaucoma* decreased for 2 days after the addition of LAS, but it slowly increased again. At 5.0 mg/L, *Cyclidium glaucoma* and *Tolypothrix* sp. were eliminated from the system. The abundance of *Philodina erythrophthalma* and *Aeolosoma hemprichi* decreased for 2 days after the addition of LAS, but it slowly increased again until equilibrating with that of the control system. The abundance of bacteria was ten times higher than that of the control on day 2 after the LAS addition. At 10 mg/L, the abundance of *Philodina erythrophthalma* and *Lecane* sp. decreased for 2 days after LAS addition and then slowly increased. *Aeolosoma hemprichi* was eliminated from the system. The abundance of bacteria was 100 times higher than in the control on days 2–7 after LAS addition. The effect of LAS on *Chlorella* sp. was not recognized in the LAS concentrations added in this experiment. The NOEC of LAS on the population density was less than 1.5 mg/L.

When the initial concentration of LAS was less than 2.5 mg/L, the concentration of ATP remained the same as in the control system (Fig. 7.1). At LAS concentrations of 5.0 mg/L and 10.0 mg/L, the concentration of ATP decreased, which corresponded to a decrease in the abundance of microorganisms, such as *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Tolypothrix* sp. It was found that ATP reflected the decrease in the population densities of the microcosm system. Evaluation on the basis of ATP showed that the NOEC of LAS was less than 2.5 mg/L. At initial LAS concentrations of less than 2.5 mg/L, the concentration of DO and the P/R ratio remained the same as those in the control system. At an LAS concentration of 5.0 mg/L, the concentration of DO and the P/R ratio decreased for 4 days after the addition of LAS, but, toward the end of the experiment, DO recovered to the same level as in the control system. At an LAS concentration of 10.0 mg/L, the concentration of DO decreased and converged to 3.6 mg/L; the P/R ratio also decreased for 4 days from 1.1 to −5.1, but, toward the end of the experiment, it recovered to the same level as in the control system. The NOEC of LAS was less than 2.5 mg/L when evaluated according to the concentration of DO and the P/R ratio. No effect of LAS on other measured parameters, such as pH, ORP, and nutrients, was observed at these initial LAS concentrations. These results demonstrate that the effect of LAS on microcosm population densities was simultaneously reflected by the concentrations of ATP and DO. Moreover, it was suggested that ATP and DO were sensitive to the changes in population densities caused by the addition of LAS and, thus, that using these parameters can allow for precise environmental assessments to be performed.

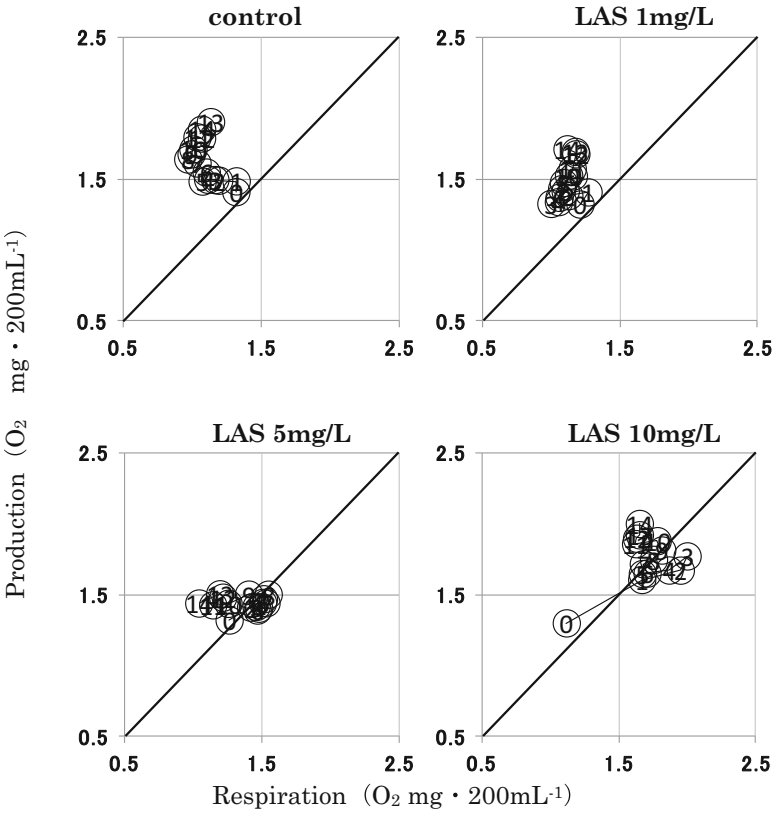

Fig. 7.1 Time course of P/R ratio in LAS-added microcosm N-system

7.1.2 Alcohol Ethoxylate (AE)

The addition concentration of alcohol ethoxylate (AE) was adjusted to 2, 10, 25, 50, and 100 mg/L and added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the DO concentration (functional parameter), and the population was measured using an optical microscope and counted the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of the abundance and the population density (N_{30}) on the 30th day. The concentration of DO was measured continuously from the 16th day onward, and the P/R ratio was calculated from the amount of production (P) and respiration (R). Using the structural parameter, a constant population was maintained for all microorganisms in the control system through the experimental period, and a constant population was likewise maintained at a 2 mg/L AE load. A decrease in microanimals was confirmed in both the 10 mg/L and 25 mg/L AE load systems until the 2nd and 4th days, respectively, but their populations had recovered to nearly the same as that in the

control system after the 7th day. The populations of *Cyclidium glaucoma*, *Aeolosoma hemprichi*, *Philodina erythrophthalma*, and *Lecane* sp. were affected, and the fluctuations grew large with increasing addition concentrations of AE.

The functional parameter (i.e., the P/R ratio) remained constant as production and respiration remained the same in the control system throughout the measurement period; approximately the same behavior was observed even at an AE concentration of 2 mg/L (Fig. 7.2). Production and respiration volumes increased just after the addition of 10 mg/L of AE, and a change was observed in comparison with the control system. However, the behavior of the 10 mg/L AE load system was similar to that of the control system after the 2nd day (i.e., the system was only disturbed temporarily, and it recovered afterward). Production and respiration volumes suddenly increased just after the addition of 25 mg/L of AE, and a remarkable change was observed; the P/R ratio remained less than 1 for 7 days after the addition of AE. This continuous state of a P/R < 1 meant the reduction of food resources by overconsumption, and it was thought that a load of 25 mg/L of AE heightened the risk of ecosystem collapse in the microcosm because a risk for future ecosystem collapse was expressed by the constituent microorganisms. A system with a 25 mg/L load of AE exhibited strong disturbance and risked collapse, but its behavior was similar to that of the control system after the 12th day. There was also an observed difference in the influence of the culture period, and it was suggested that the pattern of influence was similar to that of the 10 mg/L AE load. The importance to strengthen including degree and periods from influence by the chemical substance addition to recovery was suggested.

The results of the P/R ratio and the behavior of the population were consistent. However, a change might occur in the behavior of the P/R ratio even if the population is maintained at the same level as in the control system, and it is thought that the behavior of the P/R ratio broadly reflects changes in the population. The utility of the risk evaluation using the P/R ratio as an evaluation index was also demonstrated. No influence was observed in either the P/R ratio or the population densities at an AE concentration of 2 mg/L, which was estimated as the m-NOEC as a result of this experiment. However, it was thought that there was a need for endpoint setting that considered resistance to change, the analysis of patterns of resiliency, and relaxation of the influence of an autoregulatory function for ecosystem disturbance in natural environments modeled by the microcosm.

7.1.3 Sodium Dodecyl Sulfate (SDS)

The addition concentration of sodium dodecyl sulfate (SDS) was adjusted to 4, 8, and 16 mg/L and was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30. It was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and the population density (N_{30}) on the 30th day.

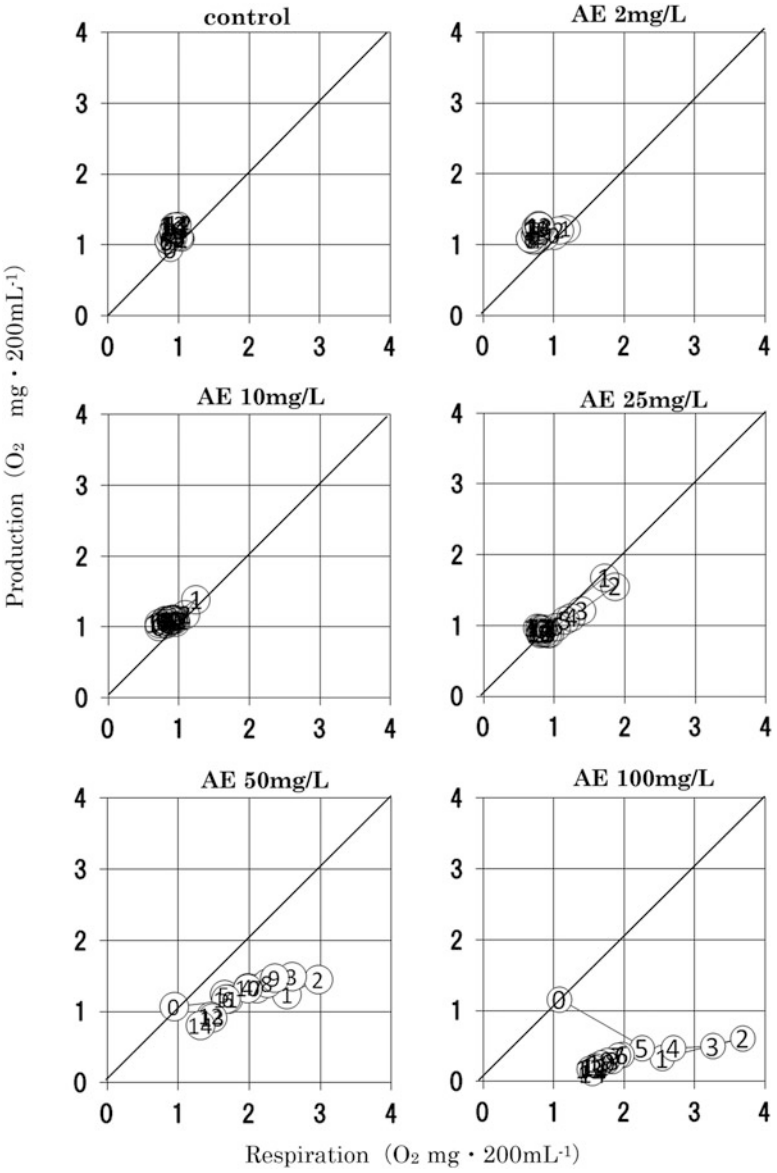

Fig. 7.2 Time course of P/R ratio in AE-added microcosm N-system

The concentration of DO was measured continuously from the 16th day onward, and the P/R ratio was calculated from the amount of production (P) and respiration (R). Using the structural parameter, the influence of SDS was evaluated in comparison with the control system based on the behaviors of microorganisms in the microcosm observed for each of the 14 days after the addition of SDS for each SDS load system. Increases in the abundance of *Lecane* sp. at the 4 mg/L and 8 mg/L SDS loads, a

decrease in and recovery of *Tolypothrix* sp. at the 8 mg/L SDS load, and a decrease in the abundance of *Tolypothrix* sp., a decrease and recovery of *Cyclidium glaucoma*, and an increase in the abundance of *Lecane* sp. at the 16 mg/L SDS load were observed.

The influence of SDS was evaluated using the functional parameter (i.e., the P/R ratio) by statistically comparing the production and respiration volumes 14 days after the addition of the SDS load. Of the system after SDS was understood for life in a system as an example of a more complimentary analysis, and the toxicity became extinct, there was no difference between the addition and control systems, and the m-NOEC for SDS was estimated as 4 mg/L (Figs. 7.3 and 7.4). The respiration volume in the microcosm deviated from that of the control system after the 3rd day of SDS addition and was later restored in the 8 mg/L SDS load system. A change in the respiration volume was recognized on the next day following the addition of SDS and recovered afterward. However, a difference in the production volume between the control system and the experimental microcosm was produced at the end for the evaluation period, with the production volume being adversely affected in the 16 mg/L SDS load system.

A tendency toward an increase in the abundance of biota was observed, but it was able to judge it from 4 mg/L of SDS addition system (the m-NOEC) because there were no changes in the ecological balance of production and respiration. It was found that there was a negative influence on the population at an SDS concentration of 8 mg/L, but the density recovered to the control system level, whereas it could not recover in the 16 mg/L SDS load system. These results were similar to the predicted values from the mesocosm test of SDS according to the high correlation between the mean of the natural ecosystem (mesocosm test) (mean NOEC) and the m-NOEC. From these results, it can be estimated that the m-NOEC of SDS is 4 mg/L.

7.1.4 Trimethyldodecyl Ammonium Chloride (TMAC)

In the addition system with 10 mg/L of trimethyldodecyl ammonium chloride (TMAC), an increase in the abundance of the cyanophycean, *Tolypothrix* sp., and the ciliate, *Cyclidium glaucoma*, was observed, and the reverse was noted for the populations of the rotifer, *Philodina erythrophthalma*, and the oligochaete, *Aeolosoma hemprichi*. With the increase in the abundance of *Tolypothrix* sp. in particular, the color of the microcosm changed from green to brown. This is the reverse of the pattern reported for the decrease in the abundance of *Tolypothrix* sp. observed in the 16 mg/L SDS addition system.

A statistically significant difference between the addition and control systems was not recognized, and the m-NOEC was estimated as equal to 1 mg/L of TMAC (Fig. 7.5). There was a significant difference at the TMAC concentration of 10 mg/L. An impact assessment was performed by concentration setting of common ratio 2 in TMAC to calculate the m-NOEC. As significant differences emerged at the 0.2 mg/L TMAC load, the amount of production and respiration changed remarkably as the

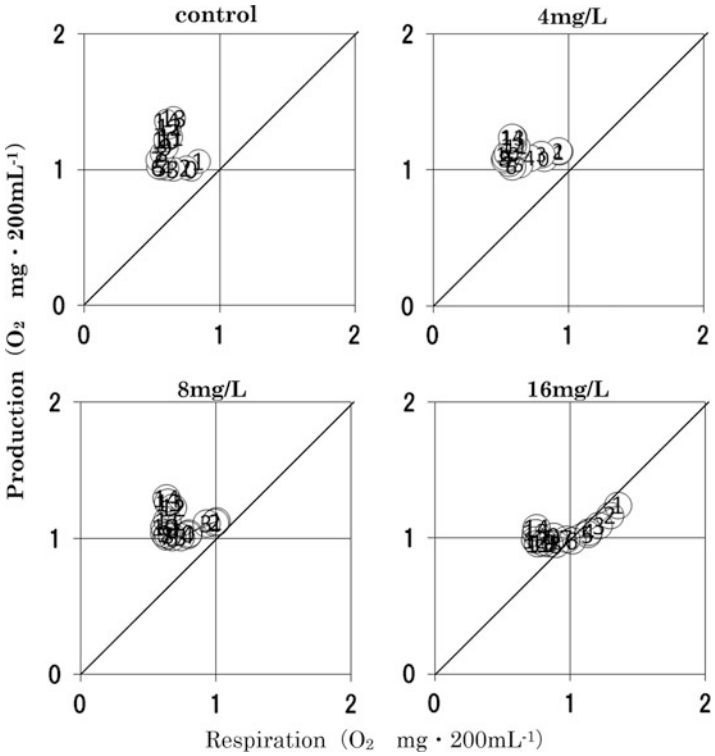

Fig. 7.3 Time course of P/R ratio in SDS-added microcosm N-system

concentration increased. From these results, it can be estimated that the m-NOEC of TMAC is 1 mg/L.

7.1.5 Soap

The addition concentration of soap was adjusted to 10, 30, 50, 100, 150, and 200 mg/L, and it was added on the 16th day of the stable state into the microcosm. Soap supplied to this experiment was composed of sodium fatty acids (C8–C18). The endpoint was the population density (structural parameter) measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. Using the structural parameter, the same density of microorganisms was maintained in the 10 mg/L and 30 mg/L soap addition systems as in the control system. *Cyclidium glaucoma* disappeared at concentrations of up to 50 mg/L 2 days after the addition of soap, but its population slowly recovered 4 days after the addition of soap and was

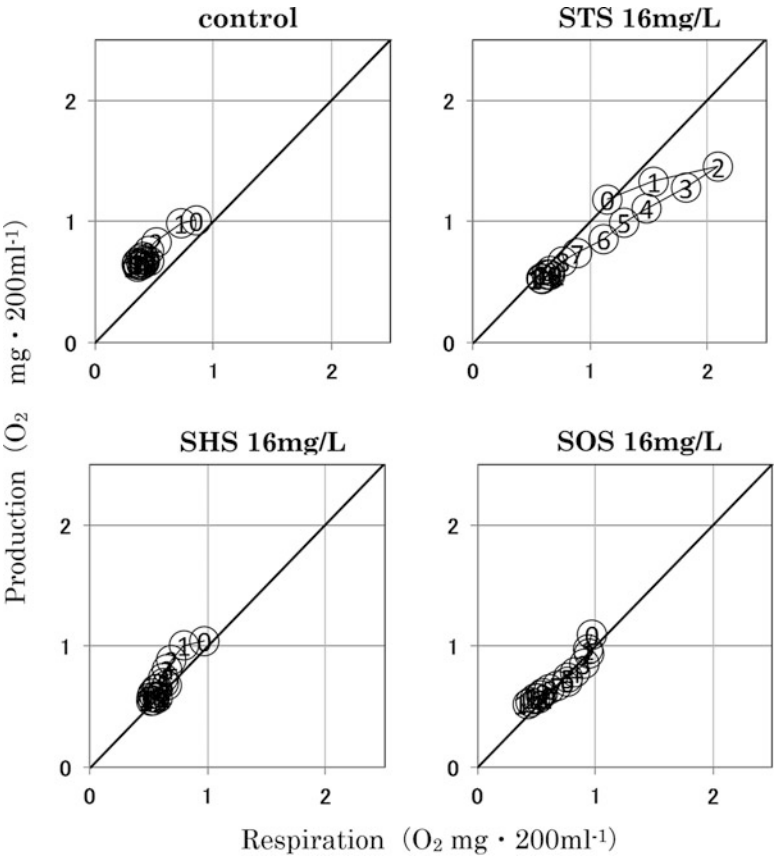

Fig. 7.4 Time course of P/R ratio in STS-, SHS-, and SOS-added microcosm N-system

maintained at a density of 1.0×10^2 N/mL. With the addition of 200 mg/L of soap, *Cyclidium glaucoma* disappeared from the system. It seems that the recovery of *Cyclidium glaucoma* was due to the degradation of complexed soap by bacteria. The density of *Tolypothrix* sp. slowly decreased to approximately 2.0×10^1 cm/mL 11 days after the addition of soap concentrations of up to 50 mg/L. The slow decrease in the density of *Tolypothrix* sp. was considered to have been driven by the light inhibition of soap scum. The densities of *Lecane* sp. and *Philodina erythrophthalma* slowly recovered in concentrations of up to 50 mg/L, ranging from 6.0×10^1 N/mL to 1.1×10^2 N/mL. The reason for the slow recoveries of *Lecane* sp. and *Philodina erythrophthalma* was assumed to be that they fed on bacteria, which increased in density due to the organic nature of complexed soap. With the addition of 200 mg/L of soap, the population of *Aeolosoma hemprichi* slowly decreased and finally disappeared from the system. Only *Chlorella* sp. was independent of the complexed soap at all concentrations. It follows from these

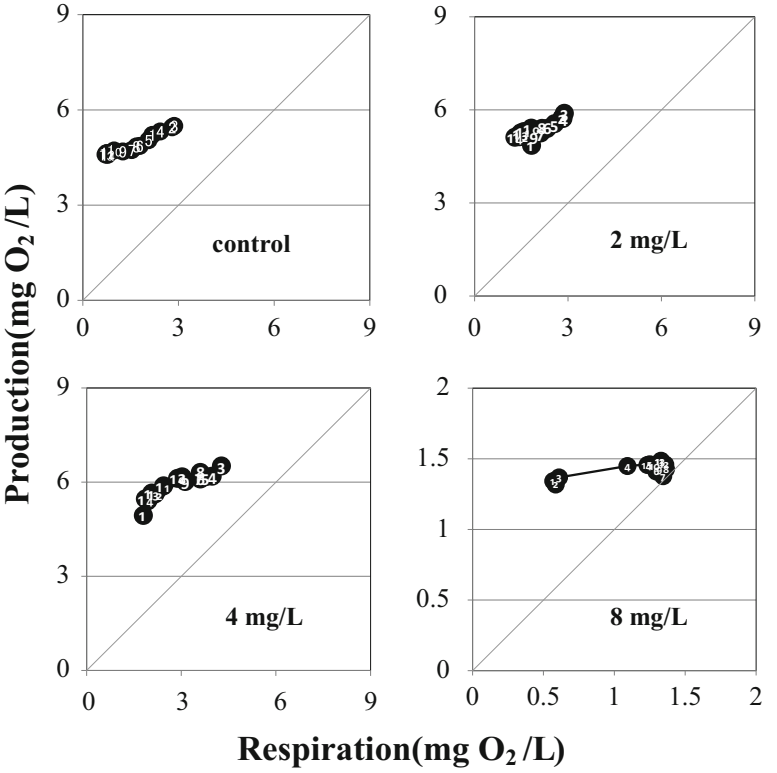

Fig. 7.5 Time course of P/R ratio in TMAC-added microcosm N-system

results that the populations of microorganisms in the system were not affected by complexed soap concentrations below 30 mg/L. As a result, it can be estimated that the m-NOEC of soap is less than 30 mg/L.

7.1.6 Complexed Soap

The addition concentration of complexed soap was adjusted to 10, 30, 50, 100, 150, and 200 mg/L, and it was added on the 16th day of the stable state into the microcosm. Complexed soap used in this experiment was composed of 58% soap and a lime soap-dispersing agent (LSDA). The endpoint was the population density (structural parameter) measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. Using the structural parameter, the abundance of microorganisms maintained with the addition of 10 mg/L and 30 mg/L of complexed soap was the same as in the control system. *Cyclidium*

glaucoma disappeared within 2 days of the addition of complexed soap concentrations of up to 50 mg/L, but slowly recovered in the 4 days after the addition of complexed soap and maintained a density of 1.2×10^2 N/mL. With the addition of 200 mg/L of complexed soap, *Cyclidium glaucoma* disappeared from the system. It seems that the recovery of *Cyclidium glaucoma* was due to the degradation of complexed soap by bacteria. The population density of *Tolypothrix* sp. slowly decreased to approximately 3.0×10^1 cm/mL with 15 days after the addition of complexed soap concentrations of up to 50 mg/L. The reason for the slow decrease in the population of *Tolypothrix* sp. was considered to be light inhibition by soap scum. The populations of *Lecane* sp. and *Philodina erythrophthalma* also slowly recovered at complexed soap concentrations of up to 50 mg/L, ranging from 3.0×10^1 N/mL to 8.9×10^1 N/mL. The reason for the slow recoveries of *Lecane* sp. and *Philodina erythrophthalm*a is considered to be their predation upon bacteria, which increased in abundance due to the organic nature of complexed soap. With the addition of 200 mg/L of complexed soap, the population of *Aeolosoma hemprichi* slowly decreased and finally disappeared from the system. Only *Chlorella* sp. was independent of complexed soap at all concentrations. It follows from these results that the population of microorganisms in the system was not affected by complexed soap concentrations below 30 mg/L. As a result, it can be estimated that the m-NOEC of complexed soap is less than 30 mg/L.

7.2 Germicides

Germicides (antiseptics) are antimicrobial substances, and they are applied to living tissues/skin to reduce the possibility of infection, sepsis, or putrefaction. Blast disease has been known as one of the serious blights on rice in Japan, and a preventative method of sterilizing seed rice with formalin was developed in the 1930s. Organomercuric agents were used widely after World War II, but due to another type of organic mercury-causing Minamata disease, of which the risk was noted in the 1960s, organomercuric agents were prohibited from use. For treating rice blast disease, blasticidin and kasugamycin were discovered and developed for agricultural use. Furthermore, some effective chemical agents for blast disease, such as dithiocarbamate in the 1960s and azole and benzimidazole in the 1970s, were developed. New chemical agents, including quinone outside inhibitors (QoI), have even been developed recently.

7.2.1 2,3,4,6-Tetrachlorophenol

2,3,4,6-Tetrachlorophenol (TCP) is used as an insecticide, sterilizer, wood preserver, and formicide (ant-specific insecticide). The organism that is the most susceptible to its effects is the water flea, and its ECOSAR class is that of phenols. The amount of

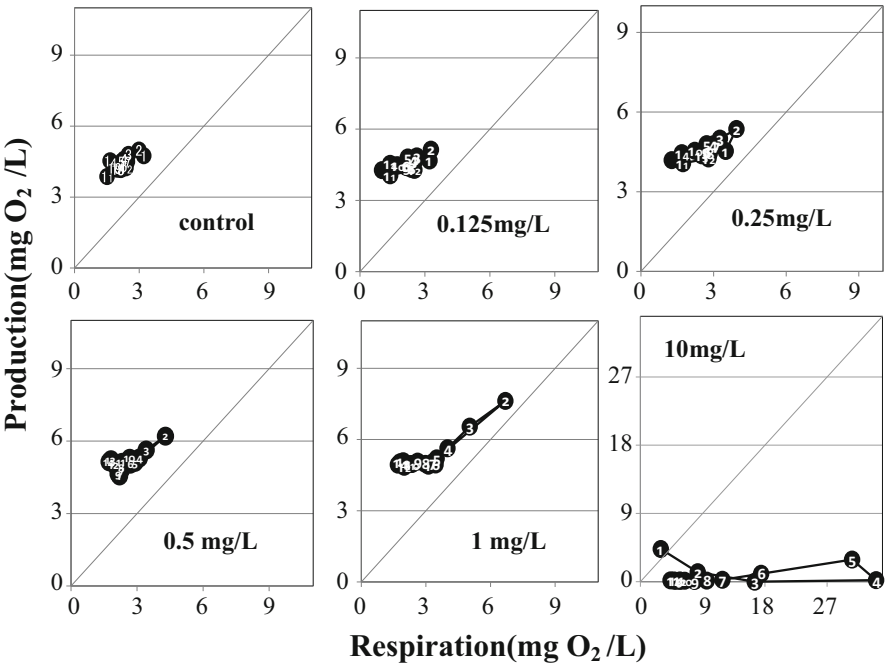

Fig. 7.6 Time course of P/R ratio in 2,3,4,6-TCP-added microcosm N-system

production and respiration decreased in the microcosm with even a 1 mg/L addition, and effects were observed. The influence on the amount of production decreasing to 0 occurred at an addition concentration of 10 mg/L, and it was clear that TCP more strongly influences production than respiration (Fig. 7.6). As a result of holding P and R constant, a test of the significant difference in the P/R ratio was performed using a branching-type ANOVA. Having been subjected to impact assessment, MCP was found, and the m-NOEC was determined to be 1 mg/L. Additionally, as a result of having compared the mesocosm and microcosm test results, it was clear that they were closely correlated.

7.2.2 Mancozeb

Mancozeb was added to the microcosm N-system at concentrations of 0.3 and 3.0 mg/L 16 days after cultivation began (i.e., during the stationary phase). It was determined that there was a statistically significant difference in both the 0.3 and 3 mg/L loads of Mancozeb. However, the lowest effective concentration was determined because it maintained almost stable production and respiration volumes at 0.3 mg/L of added Mancozeb (Fig. 7.7).

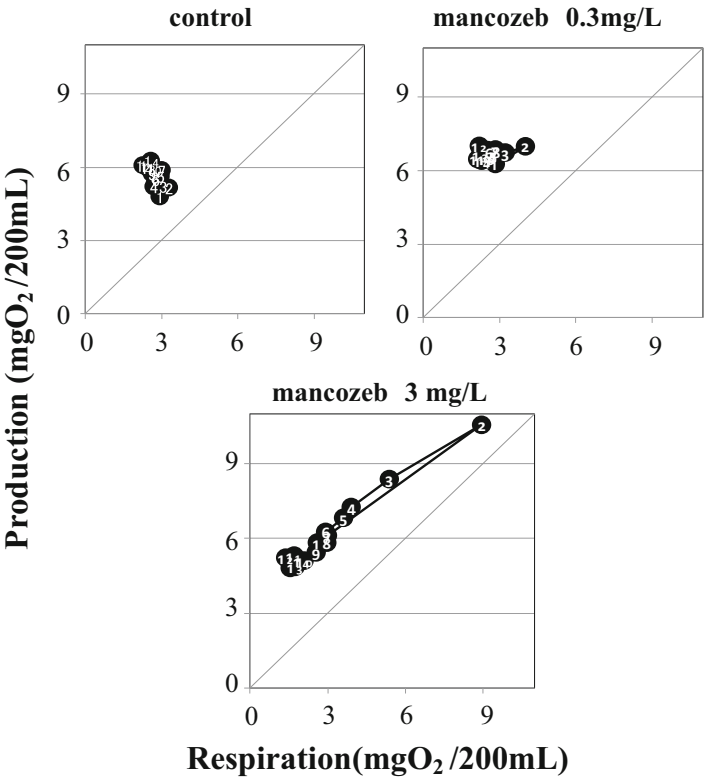

Fig. 7.7 Time course of P/R ratio in Mancozeb-added microcosm N-system

7.2.3 Thiurum

Thiurum has been used as a dithiocarbamate-based sterilizer (pesticide) or a repellent against birds. The vulcanized accelerants in the latex are cited as the main use of this chemical other than as a sterilizer. The thiurum concentration added in the microcosm tests were set to 0.25, 0.5, 1, 2, 4, and 8 mg/L, and the methanol concentration as the solvent was adjusted to 0.32%. Experiments involving the addition of thiurum to the microcosm were conducted 16 days after the microcosm culture began (i.e., during the stationary phase).

As a result of exposure to thiurum, bacteria, *Lecane* sp., *Philodina erythrophthalma*, and *Aeolosoma hemprichi* tended to multiply, which allowed bacteria to uptake as a nutrient source the methanol from the organic substances added as a solvent, and microanimals predated them. Microalgae, such as *Chlorella* sp., were not affected by the addition of thiurum. Furthermore, the addition of 0.32% of a methanol solvent did not affect the microcosm. *Cyclidium glaucoma* and *Aeolosoma hemprichi* decreased in their populations with the addition of 1.0 mg/L of thiurum. From the single-species culture test, it was made clear that *Aeolosoma*

hemprichi was strongly affected by thiurum. However, at the 1.0 mg/L addition concentration, the population of *Aeolosoma hemprichi* decreased before then increasing. The populations of *Philodina erythrophthalma* and *Lecane* sp. increased 2 days after the addition of thiurum. At the thiurum addition concentrations of 4.0 mg/L and 8.0 md/L, the populations of *Philodina erythrophthalma* and *Lecane* sp. first decreased and then increased remarkably. This was because of the monopoly of *Lecane* sp. and *Philodina erythrophthalma* on the food source that occurred due to the extinction of *Cyclidium glaucoma* and *Aeolosoma hemprichi*. Additionally, the increase in the bacterial population after the addition of thiurum was related to the decrease in predation pressure by microanimals, which decreased due to the addition of thiurum. Furthermore, this was because the survival of microanimals recovered in the thiurum-added microcosm. Photolysis and biological degradation were both possible with the addition of thiurum, and it was therefore assumed that microanimals were able to multiply remarkably again.

After analyzing the residual properties of thiurum through high-performance liquid chromatography (HPLC), the thiurum concentration exhibited little change in the control system where there were no microorganisms, and it was shown that photolysis did not occur under the microcosm culture conditions. In the 1.0 mg/L thiurum addition system, the thiurum concentration in supernatant was reduced to half 1 day after addition and decreased to below the detection limit by 2 days later. From this, it was thought that thiurum was biodegraded by the microorganisms in the microcosm within a short period of time after its addition. After analyzing thiurum concentrations in the microorganisms sampled by filtration, the characteristics of thiurum accumulation were not accepted.

From the evaluation of thiurum toxicity in the single-species culture test, the EC_{50} of *Philodina erythrophthalma* and *Aeolosoma hemprichi* were estimated as 6.0 mg/L and 1.5 mg/L, respectively. However, the concentration at which an increased influence was observed in comparison with the control system was determined as the influence concentration in the microcosm test. The concentration at which the influence of thiurum increased in the single-species culture test was also determined to compare both test methods. *Philodina erythrophthalma* was affected at a concentration of 4.0 mg/L of added thiurum in the single-species culture test, the same as in the microcosm test. *Aeolosoma hemprichi* was affected at a concentration of 1.0 mg/L of added thiurum in the single-species culture test, which was also the same as in the microcosm test. Generally, the influence of thiurum was more difficult to observe in the single-species culture test, but it was readily observed in the microcosm test. These findings are thought to have resulted from the differences in the basal media of the microorganism populations between the two methods.

7.3 Herbicides

Selective herbicides control specific weed species while leaving the desired crop relatively unharmed. Meanwhile, nonselective herbicides (sometimes called total weed killers in commercial products) can be used to clear plants from the ground,

industrial and construction sites, and railways and railway embankments, as they kill all plant material with which they come into contact. Apart from selective/nonselective herbicides, other important distinctions among these chemicals include their persistence (i.e., their residual action or how long the product stays in place and remains active), means of uptake (whether it is absorbed by aboveground foliage only, through roots, or by other means), and the mechanism of action. Historically, products such as common salt and other metal salts were used as herbicides; however, these have gradually fallen out of favor, and in some countries, a number of these are banned due to their persistence in soils and toxicity and due to concerns regarding groundwater contamination. Herbicides have also been used in warfare and armed conflicts. Modern herbicides are often synthetic mimics of natural plant hormones, which interfere with the growth of target plants. The term "organic herbicide" has come to mean herbicides intended for organic farming. Some plants also produce their own natural herbicides, such as the genus *Juglans* (walnuts) or the tree of heaven; the actions of natural herbicides and other related chemical interactions are termed allelopathy. Due to herbicide resistance, a major concern in agriculture, a number of products combine herbicides with different means of action. Integrated pest management may use herbicides alongside other pest control methods.

Though herbicides are known to exhibit high toxicity in the aquatic ecosystems that contain biological interactions, material circulation, and energy flow, the environmental risk associated with the use of herbicides remains poorly understood. The microcosm, which is a model microbial ecosystem consisting of a producer, consumers, and decomposers, is useful for evaluating the environmental risk to an ecosystem, and positioning of this model ecosystem as the standard docimasy is thus important.

7.3.1 Linuron

The addition concentration of Linuron was adjusted to 0.01, 0.05, 0.1, 1.0, and 3.0 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the DO concentration (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day onward, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

The m-NOEC of Linuron as a herbicide was determined to be 0.1 mg/L using the functional parameter (Fig. 7.8), but no effect was observed in the time series of biotic succession. It was also determined that the m-NOEC of Linuron as herbicide was in the range of 1–10 mg/L by the structural parameter. The activity of the microcosm increased with the addition of 1 mg/L and decreased with the addition of 10 mg/L of Linuron. The strength of Linuron loading (i.e., the influence concentration) was

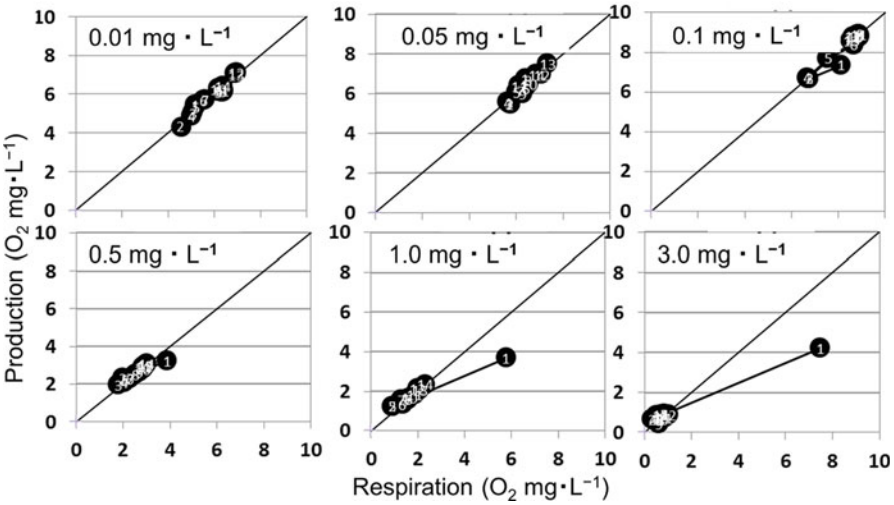

Fig. 7.8 Time course of P/R ratio in Linuron-added microcosm N-system

greater for production than for consumption. Although it is difficult to analyze the functions of production and consumption in the microcosm based on the abundance of each microorganism and measuring of the DO concentration, the above information can be obtained using the integrated Environmental Impact Risk Assessment System (iEIRAS).

7.3.2 Fomesafen

Fomesafen partially inhibits the production of chlorophyll, and it is used for broad leaf weeds, with the obstruction of a photosynthesis mainly serving to damage the cell membrane. Additionally, pesticide registration in Japan is not yet complete. Acetone was used as a solvent and was regulated to concentrations of 0.24% after addition to the TP medium. The addition concentration of Fomesafen was adjusted to 0.5, 0.6, 0.7, 1.0, 2.0, 5.0, 10, 20, 30, and 50 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the result of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The concentration of DO was measured continuously from the 16th day onward, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

A change in the abundance of microorganisms was observed according to the time series of the structural parameter. All organisms living in all of the experimental

systems entered an acute period after the addition of Fomesafen. The abundance of *Lecane* sp. decreased at addition concentrations of more than 20 mg/L of fomesafen, the number of *Aeolosoma hemprichi* decreased significantly at concentrations of more than 0.6 mg/L, *Cyclidium glaucoma* perished, and the population of *Philodina erythrophthalma* increased and then decreased from herbicide addition in the sub-acute period. The abundance of *Lecane* sp. decreased at addition concentrations of 5 mg/L or more during the chronic period, *Aeolosoma hemprichi* significantly decreased at concentrations of more than 0.6 mg/L, *Cyclidium glaucoma* perished at concentrations of more than 2 mg/L, and *Philodina erythrophthalma* increased and decreased in abundance through the addition of fomesafen. As for the phyto-plankton, *Chlorella* sp. decreased to about half of its original population size in all microcosms, and there were few changes. The population of *Scenedesmus quadricauda* tended to increase incrementally, but it exhibited chronicity even when there was a tendency toward increasing and then decreasing at concentrations of more than 30 mg/L. *Tolypothrix* sp. increased in abundance but decreased with the addition of more than 20 mg/L of Fomesafen.

From the viewpoint of $B_{16–30}$ (days 16–30 of the experiment), there was no significant decrease in the abundance of any organisms at an addition concentration of 0.5 mg/L, but the population of zooplankton decreased by more than half with concentrations of greater than 0.6 mg/L. From this, it was estimated that the m-NOEC was 0.5 mg/L for the structural parameter. Additionally, a strong influence on zooplankton was observed with the addition of Fomesafen, and a decrease in the abundance of *Chlorella* sp. was observed, while an increase was recognized for the populations of both *Scenedesmus quadricauda* and *Tolypothrix* sp. This depended on the difference in the resistance of the microbial species in the microcosm, and it is thought that the presence or absence of the influence differed and might be produced by different choices of herbicide, resulting in different effects on a given plant.

There was no observed difference in the functional parameter (DO concentration) at addition concentrations of 0.6 mg/L or 10 mg/L, but activity decreased at 30 mg/L, and the DO concentration decreased with the addition of 50 mg/L of fomesafen (Fig. 7.9). The P/R ratio was stable at ~1 for addition concentrations of 0.6 mg/L and 10 mg/L, but some variation was observed at a concentration of 30 mg/L, and the value greatly declined with the addition of 50 mg/L of fomesafen (Fig. 7.9). From these results, it can be estimated that the m-NOEC was approximately 30 mg/L for the functional parameter.

7.3.3 Simazine

Simazine only affected the growth of *Tolypothrix* sp., which was measured for 15 days after the addition of the herbicide, and it decreased to 1/40th of its original value with the addition of 0.08 mg/L or more of Simazine. The influence of this chemical was mitigated by the diversity of the constituent species in the microcosm. Simazine remained very stable in the microcosm. It was found that the toxicity—the

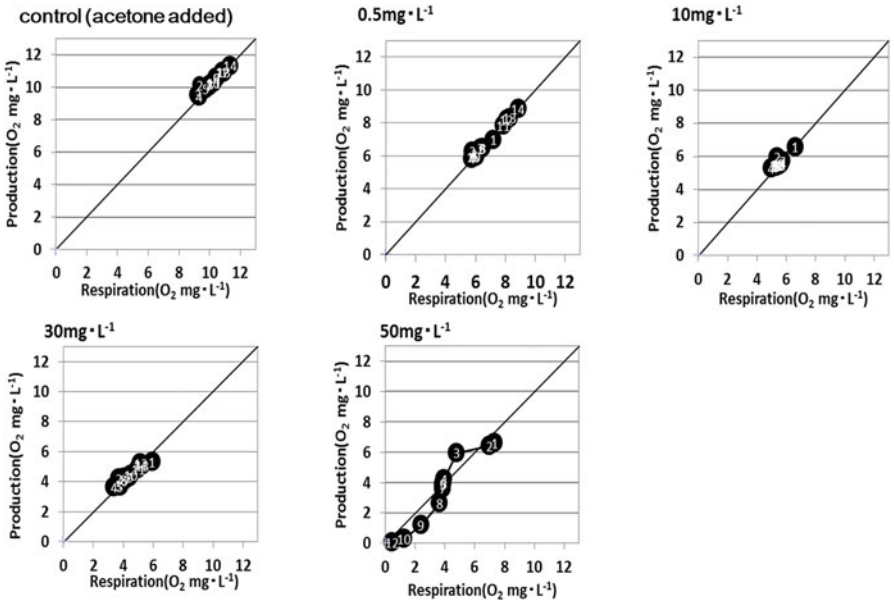

Fig. 7.9 Time course of P/R ratio in Fomesafen-added microcosm N-system

influence and stability of pesticides in the aquatic model ecosystem—could be evaluated from the behavior of the constituent microorganisms and pesticides. Addition concentrations of simazine were set to 0.08, 0.16, 0.32, and 0.64 mg/L. In the control system, only methanol was added to the system, and both methanol-addition and no-addition systems were constructed to compare the influence of methanol as a pesticide solvent.

Using the structural parameter, there was no observed effect on the population of the methanol-addition system in comparison with the no-addition system, which was used as a control. There was no effect on the populations of constituent microorganism with the addition of 0.16, 0.32, or 0.64 mg/L of Simazine, except for a decrease in the bacterial population 2 days after the addition of 0.64 mg/L of simazine. Changes were not observed in either the amount of respiration (R) or in the amount of production (P) in the control system (methanol no-addition), but, in the control system with methanol added, both the amounts of respiration and production increased. In addition systems with low simazine concentrations, changes similar to those observed in the methanol-addition control system were recognized, but a decrease in the amount of production was observed as the concentration of simazine increased. Although there was no change in the P/R ratio, which ranged between 1.6 and 1.8, in either of the control systems (i.e., both with and without methanol), the P/R ratio decreased to 0.8 after the addition of 0.64 mg/L of Simazine (Fig. 7.10).

In the multivariation analysis of the amounts of respiration and production after the addition of simazine, a meaningful decrease in the amount of production was recognized between the 0.64 mg/L simazine load and the control with added

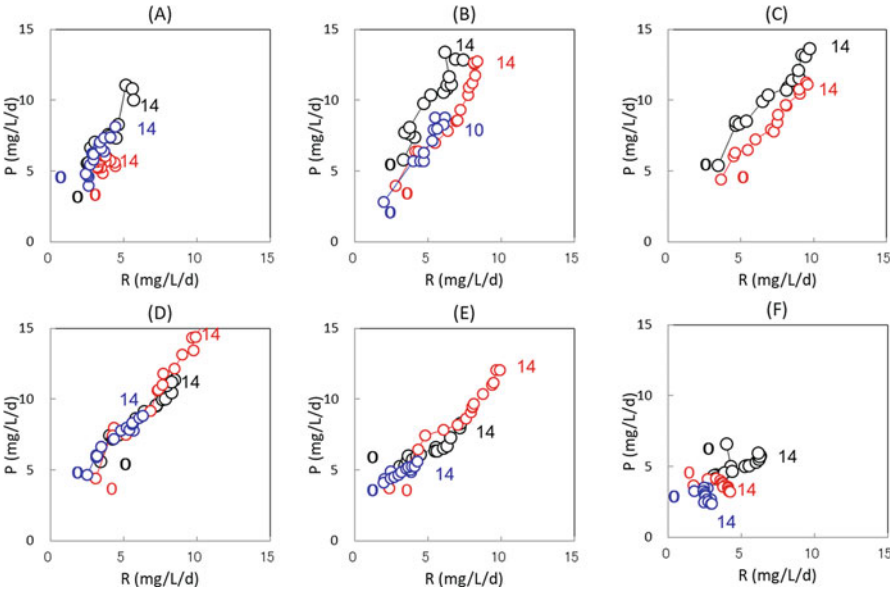

Fig. 7.10 Time course of P/R ratio in Simazine-added microcosm N-system. (**a**) Control. (**b**) Control + methanol. (**c**) 0.08 mg/L. (**d**) 0.16 mg/L. (**e**) 0.32 mg/L. (**e**) 0.64 mg/L

methanol from the 4th day after addition onward, and both the low Simazine load (0.64 mg/L) and high load (16 mg/L) systems were defined from the 1st day after the addition of the herbicide. In these cases, the amount of production did not decrease meaningfully, whereas the P/R ratio decreased in the high load system. The toxicity of Simazine was not recognized as having an influence on the populations of microorganisms at the concentration ranges employed in this experiment. Thus, the amount of production appears to have significantly decreased as a direct effect of simazine alone, and there was no influence on the populations of microorganisms, and the P/R ratio tended to decrease. It is necessary to use the measured amounts of production and respiration together to investigate more than simply the changes in the populations of microorganisms in an ecosystem when performing an ecosystem impact assessment of pesticides because there are differences in ecosystem responses depending upon the kind of pesticide used and the methods of assessment.

7.3.4 Benthiocarb

Benthiocarb affected the growth of *Cyclidium glaucoma* from a concentration of 1.0 mg/L in the two-species cultivation test and from a concentration of 2.0 mg/L in the microcosm test. The influence was mitigated by the diversity of constituent

species in the microcosm. *Aeolosoma hemprichi* was eliminated, and the number of *Chlorella* sp. decreased to one-third its original value at Benthiocarb concentrations above 0.5 mg/L. In the microcosm, the concentration of benthiocarb decreased to 30% for 15 days after the addition of 1.0 mg/L. It was found that the toxicity of Benthiocarb could be evaluated based on the behaviors of the constituent microorganisms and pesticides. Addition concentrations of benthiocarb were set to 1.0, 2.0, 4.0, and 8.0 mg/L. As the controls, only methanol-addition and methanol no-addition systems were constructed to compare the influence of methanol as a pesticide solvent.

Using the structural parameter, there was no difference observed in the populations of microorganisms between the methanol-addition and no-addition control systems. In the microcosms where 1.0–4.0 mg/L of Benthiocarb was added, there was also no significant influence on the population; however, both *Aeolosoma hemprichi* and *Philodina erythrophthalma* perished with the addition of 8.0 mg/L of Benthiocarb. The functional parameter and the amounts of respiration (R) and production (P) increased with the addition of methanol, but not only was there no observed influence on the ecosystems of the methanol-addition system and in the case of Benthiocarb loading, but there was also no difference recognized at the high load of 8.0 mg/L of benthiocarb. The P/R ratio did not change with the addition of Benthiocarb (Fig. 7.11). Differences in the amounts of production and respiration on each measurement day after the addition of Benthiocarb were not recognized as statistically significant in comparison with either of the methanol control systems according to the results of ANOVA. No significant difference in the P/R ratio was recognized between the methanol-addition system and Benthiocarb-load systems, either. With respect to the toxicity of Benthiocarb to animals, it was reported that the LD_{50} values for carp and *Daphnia* are 3.6 mg/L and 1.7 mg/L, respectively, but the concentration responsible for the mortality of microanimals in the microcosm was approximately 8.0 mg/L. However, no influence from Benthiocarb itself was observed on the amounts of production or respiration at the concentration in which microanimal populations became extinct.

7.3.5 Propyzamide

Propyzamide is a benzamide herbicide used for the weeding of a particularly gramineous plant and works by obstructing the microtubular composition that is necessary for cell division. Acetone was used as a solvent and regulated to a concentration of 0.24% after addition to the TP medium. The addition concentration of Propyzamide was adjusted to 0.1, 0.2, 1, 3, and 5 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population

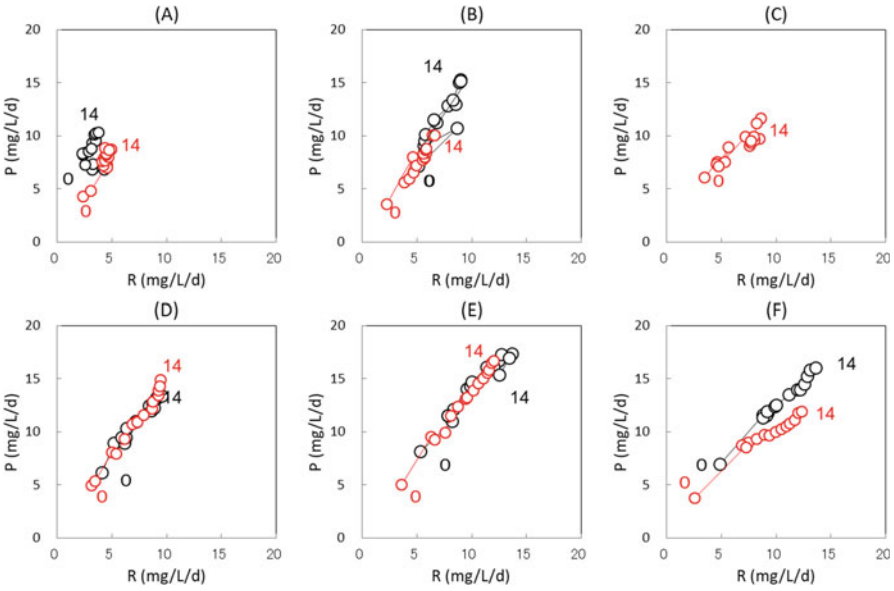

Fig. 7.11 Time course of P/R ratio in Benthiocarb-added microcosm N-system. (**a**) Control. (**b**) Control + methanol. (**c**) 1.0 mg/L. (**d**) 2.0 mg/L. (**e**) 4.0 mg/L. (**f**) 8.0 mg/L

density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

Based on examination of the population density (N_{30}) in each herbicide addition system, no extinction of zooplankton was observed, but a tendency to decrease was recognized for all microbiota at a Propyzamide concentration of 5 mg/L. Based on the microorganismic abundance ($B_{16–30}$) at each concentration, *Cyclidium glaucoma* and *Lecane* sp. decreased at high addition concentrations. This is because bacteria, the prey of both *Cyclidium glaucoma* and *Lecane* sp., decreased in abundance with the addition of Propyzamide. Additionally, the phytoplankton were hardly affected, except for *Scenedesmus quadricauda*. The selectivity of Propyzamide is that it works on only rice seeds, but a future examination of the differing influence of this chemical on phytoplankton in the microcosm is necessary.

Significant changes in the concentration of DO were not observed with the addition of 1 mg/L of Propyzamide, but production and respiration amounts increased at addition concentrations of more than 3 mg/L (Fig. 7.12). Additionally, it may be said that the activity *per* cell of both microanimals and microalgae increased at concentrations of more than 1 mg/L because the abundance decreased. However, the P/R ratio was stable, so it was estimated that there was no effect on the microcosm ecosystem. Furthermore, the NOEC value is greater than 2.2 mg/L with respect to reproduction (NOECREP) for 21 days in *Daphnia magna* and 0.32 mg/L for 3 days in the chlorophycean, *Pseudokirchneriella subcapitata*. It is thought that

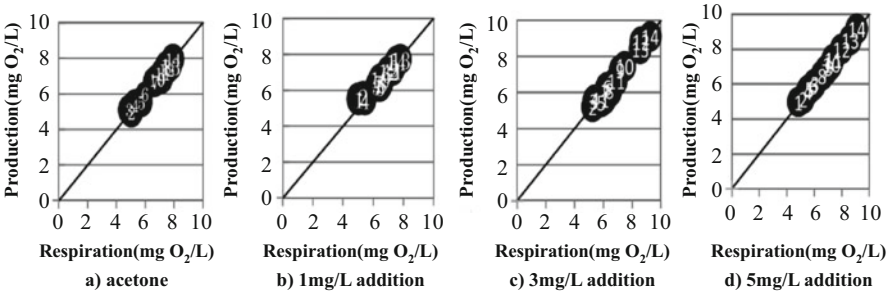

Fig. 7.12 Time course of P/R ratio in Propyzamide-added microcosm N-system. (**a**) Acetone. (**b**) 1 mg/L addition. (**c**) 3 mg/L addition. (**d**) 5 mg/L addition

the influence on the ecosystem was reduced because the interaction among the organisms in the microcosm functioned where one ecosystem is constructed.

From these outcomes, it was estimated that the m-NOEC of the structural parameter was greater than 5 mg/L because, while mainly the zooplankton were affected, extinction was not observed. Similarly, the m-NOEC of the functional parameter was also estimated as being greater than 5 mg/L, and there was an observed tendency toward increased production and respiration in all addition systems. Populations of phytoplankton and zooplankton decreased with the increasing addition concentration of propyzamide, but the activity *per* individual rose simultaneously, and it was thought that equilibrium was maintained. Influence on ecosystem was relaxed than a single creature by the microbial interaction in microcosm, and it was made clear that a difference was observed in comparison with the effects of Propyzamide in the single-species test.

7.3.6 2-Methyl-4-Chlorophenoxyacetic Acid (MCPA)

2-Methyl-4-chlorophenoxyacetic acid (MCPA), a chemical substance similar to auxin, disturbs hormonal balance and is therefore a hormonal drug. The addition concentration of MCPA was adjusted to 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were population density (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

Extinction of the constituent microorganism was not confirmed from the evaluation of the population density, nor was ecological collapse observed in the

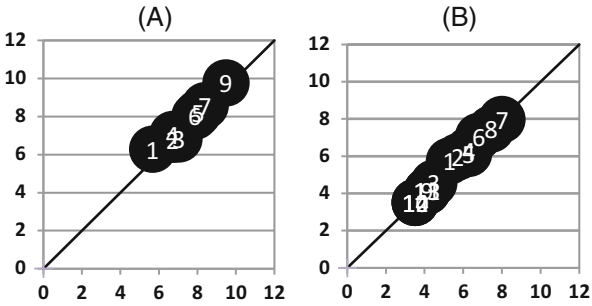

Fig. 7.13 Time course of P/R ratio in MCPA-added microcosm N-system. (**a**) 0.3 mg/L addition. (**b**) 0.4 mg/L addition

microcosm. The populations of *Aeolosoma hemprichi* and *Cyclidium glaucoma* decreased remarkably by the 30th day. The influence of MCPA was recognized as a tendency to decrease population sizes at concentrations ranging from 0.3 to 0.4 mg/L. Most species were observed to increase in population size with the addition of 0.2 mg/L of MCPA because a system is opposed to this for weed killer MCPA, and it may be said that populations increased because they were active. Therefore, the m-NOEC of the structural parameter was estimated to be 0.4 mg/L. There was no observed difference in the functional parameters of the control and addition systems until an addition concentration of 0.3 mg/L was reached. However, activity gradually decreased at an addition concentration of 0.4 mg/L, and the results were similar at concentrations of more than 0.5 mg/L. While the P/R ratio of the control system was ~1 and stable, the activity tended to increase at a concentration of 0.3 mg/L and decrease at a concentration of 0.4 mg/L loading (Fig. 7.13). Thus, it can be estimated that the m-NOEC of the functional parameter is 0.4 mg/L.

7.3.7 Alachlor

Alachlor is an acid amide herbicide, and it is considered to wither plants by interrupting normal cell division at the position of new growth by synthesizing inhibitors of very-long-chain fatty acids. On the 16th day after the start of the microcosm culture, Alachlor was adjusted to concentrations of 0.1, 1, and 4 mg/L and added. Evaluation of production (P) and respiration (R) in the microcosm showed that the m-NOEC of Alachlor was present in the range of 1–4 mg/L (Fig. 7.14).

7.3.8 3-(3,4-Dichlorophenyl)-1,1-Dimethylurea (DCMU)

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) is a typical, urea-based, photosynthesis-inhibiting herbicide. Its herbicidal action is mild, and it is used as a

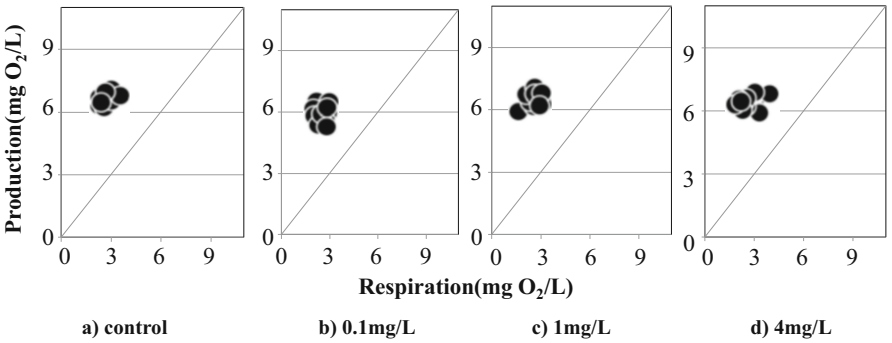

Fig. 7.14 Time course of P/R ratio in Alachlor-added microcosm N-system. (**a**) Control. (**b**) 0.1 mg/L. (**c**) 1 mg/L. (**d**) 4 mg/L

common soil conditioner. The m-NOEC in the microcosm test was evaluated to be 0.05 mg/L using the structural parameter and to be 0.1 mg/L based on the functional parameter.

7.3.9 Pentachlorophenol

The effect of pentachlorophenol (PCP) on microcosms was investigated by Dr. Sugiura. PCP was added at the start of the microcosm culture (day 0), and the abundance of organisms and amounts of respiration and production were tracked. With the addition of PCP, both the respiration volume and production volume became larger than in the control system. The m-NOEC of PCP was estimated to be 0.07 mg/L (Sugiura 1992).

7.3.10 Atrazine

The effect of atrazine was investigated by Dr. Sugiura. Atrazine was added and adjusted to concentrations of 0.07, 0.1, and 0.15 mg/L at the start of the microcosm culture (day 0). The locus of DO differed depending upon the amount of atrazine added. With the addition of atrazine, both the respiration volume and production volume became larger than in the control system. The m-NOEC of atrazine was estimated to be 0.03 mg/L (Sugiura 1992).

7.3.11 3,4-Dichloroaniline

The effect of 3,4-dichloroaniline (3,4-DCA) was investigated by Dr. Sugiura. 3,4-Dichloroaniline was added at the start of the microcosm culture (day 0), and the number of organisms (abundance) and amounts of respiration and production were tracked. With the addition of 3,4-DCA, both the respiration volume and production volume became larger than in the control system. The m-NOEC of 3,4-DCA was estimated to be 1 mg/L (Sugiura 1992).

7.3.12 Paraquat

The effect of paraquat was investigated by Dr. Sugiura. Paraquat was added at the start of the microcosm culture (day 0), and the number of organisms and amount of respiration and production were tracked. The m-NOEC was estimated to be 0.01 mg/L (Sugiura 1992).

7.3.13 2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T)

The effect of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was investigated by Dr. Sugiura. 2,4,5- Trichlorophenoxyacetic acid was added at concentrations of 0.1, 1.0, 10, and 100 mg/L at the start of the microcosm culture (day 0). The change over time in the population density and community metabolism with the addition of 10 mg/L did not differ from those of the control system. In the 100 mg/L addition system, *Philodina erythrophthalma*, *Aeolosoma hemprichi*, *Scenedesmus quadricauda*, and *Tolypothrix* sp. perished, and the change over time in the community metabolism also differed greatly from the control system. From these results, the addition of 10–100 mg/L of 2,4,5-T was estimated to have affected this system (Sugiura 1992).

7.3.14 Cafenstrole

On the 16th day after the start of the microcosm culture (stationary phase), cafenstrole was added at concentrations of 0, 0.5, 1.0, 2.5, and 5.0 mg/L. The influence exerted by this chemical on the microorganisms constituting the microcosm depended upon the concentration added. At an addition concentration of 0.5 mg/L, *Cyclidium glaucoma*, *Lecane* sp., and *Aeolosoma hemprichi* began to be affected, with *Aeolosoma hemprichi* being affected at 1.0 mg/L, and both *Aeolosoma hemprichi* and *Cyclidium glaucoma* disappeared within 2 days after the addition of

2.5 mg/L of cafenstrole. With the addition of 5.0 mg/L of cafenstrole, *Aeolosoma hemprichi* disappeared within 2 days after addition, and both *Lecane* sp. and *Cyclidium glaucoma* disappeared from the system on the 7th day following chemical addition. In this way, predatory microanimals that contribute to the material circulation and energy flow in the microcosm were deemed susceptible to cafenstrole.

7.4 Pesticides

Pesticide has been widely used and it improved crop yields dramatically, and our life has become rich. On the other hand, pesticide is recognized that it influences lives including humans very seriously such as defoliant which has been sprayed in the Vietnam War. Biological toxicity of pesticide is regulated by various bioassay tests, but its ecosystem toxicity is not enough cleared.

7.4.1 Chlorpyrifos

Dursban 4E, an emulsification pesticide, was prepared as Chlorpyrifos. This is because Leeuwangh et al. (1994) and Daam and van den Brink (2007) used Dursban 4E, which is an emulsification pesticide product as Chlorpyrifos, Daam et al. (2008) used Dursban 40EC, and López-Mancisidor et al. (2008a, b) used Chas 48EC, an emulsification pesticide product, in their mesocosm experiments. Chlorpyrifos was added at the start of microcosm cultivation, and the microcosm test was conducted with concentrations of 125, 250, 500, and 1000 μg/L, with the common ratio set to 2.

At any concentration loaded, the P/R ratio was around 1, but the production and respiration amounts were significantly lower at a Chlorpyrifos loading of 1000 μg/L (Fig. 7.15). The NOEC of Chlorpyrifos was estimated to be 500 μg/L. Even if an organism perished with the addition of 10,000 μg/L of Chlorpyrifos, there was no observed impact on the production or respiration at all. Chlorpyrifos is a neurotoxin and reduces the activity of animals and causes a drop in the respiration amount *per* individual. It was thought that the amount of respiration in the entire microanimal population in the microcosm decreased because the population decreased with a rise in death rates. However, it was thought that no influence appeared in the amounts of production and respiration when chlorpyrifos was added during the stationary period because the respiration amount had been supplemented by decomposition of the microanimals and the increase in the abundance of many organisms with the decrease in predation. On the other hand, the community structure of the system changed when chlorpyrifos was added when the culture began because Chlorpyrifos drove the succession of microanimals in the microcosm, and it is thought that, as a result, an influence on production and respiration appeared.

The NOEC of Chlorpyrifos in the mesocosm test was reported as 0.06 μg/L. However, NOEC of the microcosm test was 500 μg/L, which is much higher.

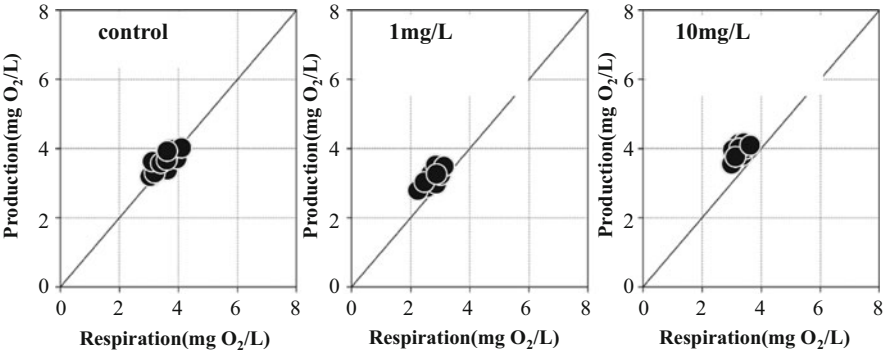

Fig. 7.15 Time course of P/R ratio in Chlorpyrifos-added microcosm N-system

Hydrolysis is high in Chlorpyrifos, and the quality of soil adsorption characteristics is also high, and it is thought that it is stabilized while adsorbed in the soil. Therefore, it is thought that Chlorpyrifos remains behind within a system for a long term by soil adsorption and stabilization. Chlorpyrifos is adsorbed to the bottom sediment when Chlorpyrifos density decreases by hydrolysis detaching by chemical equilibrium, and even one revelation being supplied to the mesocosm test where there is bottom sediment underwater. Because there was no soil in the microcosm, the residual property of Chlorpyrifos was low, and it was thought that it was less toxic than in the mesocosm test in which there was soil. It is necessary to examine the analytical methods that noted the quality of soil and bottom sediment adsorption characteristics in the future.

7.4.2 Fenitrothion

Fenitrothion was adjusted so that acetone levels became 0.05% after addition to the culture fluid in diluted form. The concentrations of Fenitrothion were set to 0.01, 0.10, 1.00, and 10.00 mg/L. The Fenitrothion was added 16 days after microcosm culturing began. As a control, an acetone-only addition system and a no-addition system were constructed to compare the influence of acetone as a solvent for pesticides.

Based on the structural parameter, there was no influence of acetone itself (i.e., in the acetone-only system) on the populations of microorganisms, in comparison with the no-addition system, both of which served as control systems. There was no remarkable change observed in the populations of each species, in comparison with these control systems, at concentrations of 0.01 mg/L of Fenitrothion, but the population size of *Aeolosoma hemprichi* decreased with the addition of 0.10 mg/L and 1.00 mg/L. There was no observed influence on the other microorganisms at these concentrations of Fenitrothion. However, *Aeolosoma hemprichi*, *Philodina erythrophthalma*, and *Lecane* sp. perished, and the population of *Cyclidium*

glaucoma decreased remarkably and then recovered with the addition of 10 mg/L of fenitrothion. Thus, there was a recognized influence from this pesticide on microanimals in the microcosm. In contrast, the microalgae, *Chlorella* sp., *Scenedesmus quadricauda*, and *Tolypothrix* sp., were not influenced by the addition of less than 10 mg/L of Fenitrothion. The functional parameter, the quantity of production (P) and respiration (R), increased in comparison with the no-addition control system. There was no difference between the P/R ratios of systems with Fenitrothion concentrations ranging from 0.01 to 10 mg/L and the acetone-only control system. The P/R ratio did not change with the addition of Fenitrothion (Fig. 7.16).

To understand the effects of Fenitrothion, an ANOVA was performed between the acetone-only control system and each Fenitrothion addition system, but no meaningful difference was recognized in the amounts of production and respiration or in the P/R ratios of each microcosm; the influence of the Fenitrothion concentration remained the same. The NOEC of Fenitrothion varied widely by species; it is reported as 0.009 mg/L for *Daphnia magna*, a very small amount, whereas it was ~1.0 mg/L for rotifers and microalgae. To focus on the constituent animals of the microcosm, because the abundance of *Aeolosoma hemprichi* decreased with the addition of 0.1 mg/L of Fenitrothion and *Aeolosoma hemprichi* and two species of rotifer, *Philodina erythrophthalma* and *Lecane* sp., perished with the addition of 10 mg/L of Fenitrothion, it may be said that influence at the same level as the aquatic animal that tolerance is relatively strong. However, even at the concentration at which constituent animals in the microcosm perished, there was no observed influence by Fenitrothion alone on the amounts of production and respiration.

7.4.3 Lindane

Lindane was added to the microcosm N-system on the 16th day after cultivation began (stationary phase) at concentrations of 0.003, 0.03, and 0.12 mg/L. There was no statistically significant difference between the addition and control systems, and stable production and respiration amounts were maintained that were equal to those in the control system, at Lindane concentrations of 0.003, 0.03, and 0.12 mg/L (Fig. 7.17). It was estimated that the m-NOEC of Lindane is greater than 0.12 mg/L.

7.4.4 Carbendazim

A benrate hydration agent was supplied for investigation. A benrate hydration agent is a main product of Carbendazim, composed primarily of approximately 50% of benomyl, and the benomyl is converted into Carbendazim underwater, having a half-life of 2 h. The addition concentration of Carbendazim was adjusted to 0.1, 1.0, 3, 5, and 10 mg/L, and it was added on the 16th day of the stable state into the microcosm.

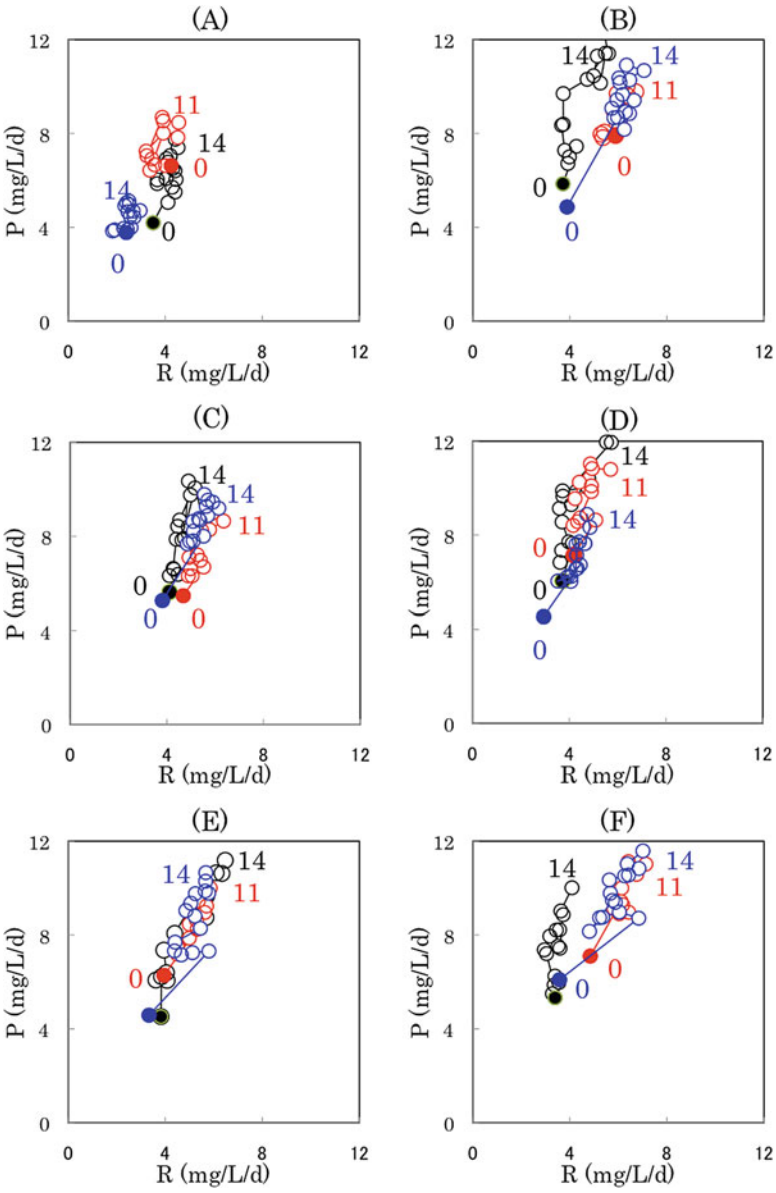

Fig. 7.16 Time course of P/R ratio in Fenitrothion-added microcosm N-system. (**a**) Control. (**b**) Control + aceton. (**c**) 0.01 mg/L. (**d**) 0.10 mg/L. (**e**) 1.00 mg/L. (**f**) 10.00 mg/L

The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was

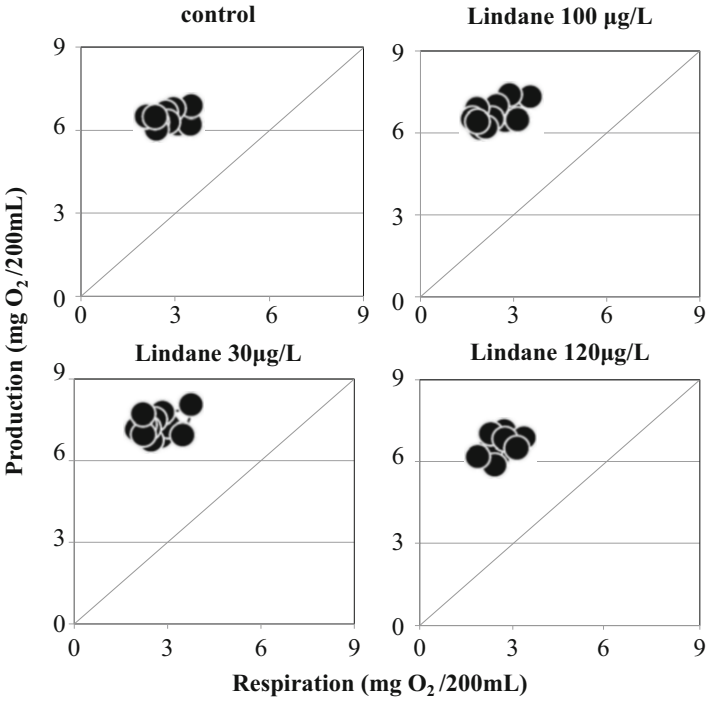

Fig. 7.17 Time course of P/R ratio in Lindane-added microcosm N-system

the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

The structural parameter was evaluated according to the categories of acute influence $B_{16–20}$ (0–4 days), subacute influence $B_{20–23}$ (5–7 days), chronic influence $B_{23–30}$ (8–14 days), and abundance of microorganisms (N_{30}) on the 30th day after culturing began. As a result, no large decreases or extinctions of species were seen in the periods of subacute or acute influence, but a large decrease in the abundance of *Cyclidium glaucoma* and *Aeolosoma hemprichi* was observed in the addition system with more than 1 mg/L of Carbendazim during the period of chronic influence. Additionally, phytoplankton did not exhibit any remarkable increase or decrease, and no influence upon them was recognized. Because Carbendazim is a sterilizer, at first this influenced the bacteria which act as decomposers in the microcosm, and it influenced zooplankton predation on them afterward. It is thought that there was little impact on the phytoplankton producers. Decreases in the abundance and extinction of zooplankton were observed for N_{30} as concentrations increased, but an increase in the abundance of *Philodina erythrophthalma* was also recognized in the 10 mg/L addition system. This is considered to be due to the extinction of

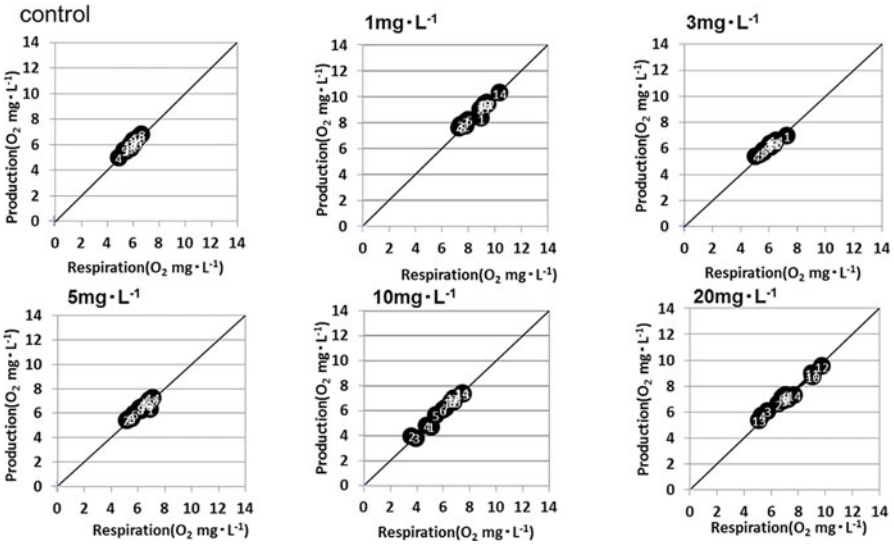

Fig. 7.18 Time course of P/R ratio in Carbendazim-added microcosm N-system

Cyclidium glaucoma and *Aeolosoma hemprichi*, and *Philodina erythrophthalma* supplemented the consumers and increased to maintain the system. As a functional parameter, the amplitude of the DO damping as the concentration increased, along with the activity of the system, indicated a tendency to decline slightly. However, all systems were stable, with P/R ratios of ~1. The m-NOEC of Carbendazim, as a fungicide, was detected within the range of 0.1–1 mg/L in the structural parameter and as more than 10 mg/L in the functional parameter (Fig. 7.18).

7.4.5 Dimethoate

Dimethoate is organophosphorus pesticide that inhibits a nerve transduction system (acetylcholinesterase (AChE) activity) necessary for transmitting information for the life support of the pest and is mainly used for exterminating pests of fruit trees. The addition concentration of Dimethoate was adjusted to 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

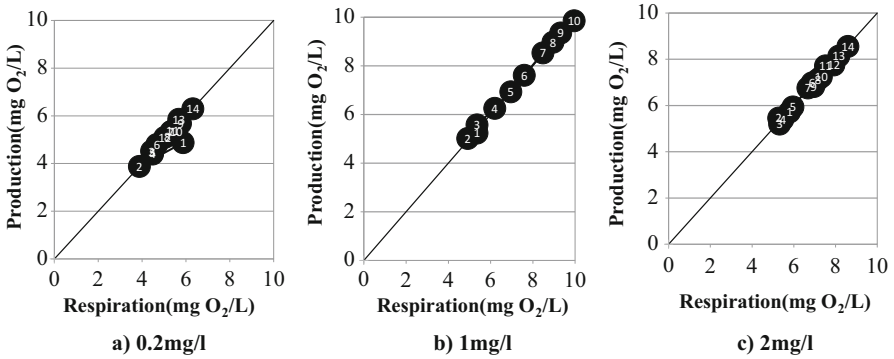

Fig. 7.19 Time course of P/R ratio in Dimethoate-added microcosm N-system. (**a**) 0.2 mg/L. (**b**) 1 mg/L. (**c**) 2 mg/L

The population (structural parameter) of phytoplankton decreased over time after the addition of dimethoate, and only *Tolypothrix* sp. maintained its population at all addition concentrations. The abundance of *Cyclidium glaucoma* decreased sharply at concentrations of 0.5 mg/L or more during the subacute period, and extinction was observed at a concentration of 1 mg/L during the acute period. A remarkable difference was observed in the abundance of phytoplankton and zooplankton from $B_{16–30}$ (days 16–30). As for the phytoplankton, *Scenedesmus quadricauda* and *Tolypothrix* sp. tended to increase in abundance, relative to the control system, at concentrations of more than 1 mg/L, but *Chlorella* sp. did not. All species of zooplankton decreased in abundance, as compared with the control system, at concentrations of more than 0.2 mg/L. It is thought that differences might occur among the influences on species due to the mechanisms of the insecticide used for pest extermination. As a functional parameter, a difference in the DO concentration was not recognized between dimethoate addition systems with concentrations between 0.05 mg/L and 0.2 mg/L and the control system, but the activity rose with 0.5 mg/L of added dimethoate. Additionally, activity became uniform at concentrations ranging from 0.5 mg/L to 1 mg/L, and an active decline was observed at a concentration of 2 mg/L. However, it is necessary to examine systems with concentrations of more than 2 mg/L because there is not a system to decrease than control system on DO level. As for P/R ratio, activity also rose using test additions ranging from 0.2 mg/L to 1.0 mg/L of dimethoate, but it began to decrease when more than 1.0 mg/L and up to 2.0 mg/L was used (Fig. 7.19).

The m-NOEC of the structural parameter was estimated to be less than 1 mg/L because of the extinction of *Cyclidium glaucoma*, and m-NOEC of the functional parameter was in the range of 1 mg/L to 2 mg/L. Because the abundance of phytoplankton and the amount of production decreased over time, it is thought that there is no influence on the function *per* individual phytoplankton. On the other hand, it is thought that the function *per* individual decreased in zooplankton

because one species perished and other species maintained their populations with decreased consumption.

7.4.6 Dinotefuran

In recent years, the neonicotinoid pesticide, Dinotefuran, has been widely used as one of the essential pesticides, but information regarding its influence on the ecosystem hydrosphere through drainage and river water remains lacking. Here, an environmental impact assessment of Dinotefuran, a neonicotinoid pesticide, on microbial community function and structure, was conducted using the experimental flask-sized microcosm. To assess its influence, Dinotefuran was added at several concentrations on the 16th day after microcosm cultivation began. Plankton observation using an optical microscope was conducted on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30 after cultivation began, and the change observed in the populations in the flask at each addition concentration was used as the structural parameter. Additionally, the DO concentration was measured consecutively from the 16th day of the culture period onward to evaluate ecosystem function, using the P/R ratio as the functional parameter.

The results based on the structural parameter revealed that the extinction of species was not observed in all systems. A species less than 0.5 for $B_{16–30}$ (the population ratio in the period ranging from the 16th day to the 30th day of the experiment) was recognized at Dinotefuran concentrations of 0.25 and 1.0 mg/L. A decrease in the population of zooplankton was observed in all addition systems from N_{30} (the abundance on the 30th day). Additionally, the population ratio of all zooplankton was less than 0.5 at 1.0 mg/L of added Dinotefuran. For the functional parameter, it was judged that there was no influence in all addition systems based on statistical analyses (Fig. 7.20). The P/R ratios of all systems converged upon a similar value as that of the control system. From these results, the m-NOEC of Dinotefuran was assessed to be over 1.0 mg/L.

7.4.7 4-Chlorophenol

The effect of 4-Chlorophenol was investigated by Dr. Sugiura. 4-Chlorophenol was added at the start of microcosm culturing (day 0), and the number of organisms and amounts of respiration and production were continuously tracked. The m-NOEC of 4-Chlorophenol was estimated to be 0.15 mg/L (Sugiura 1992).

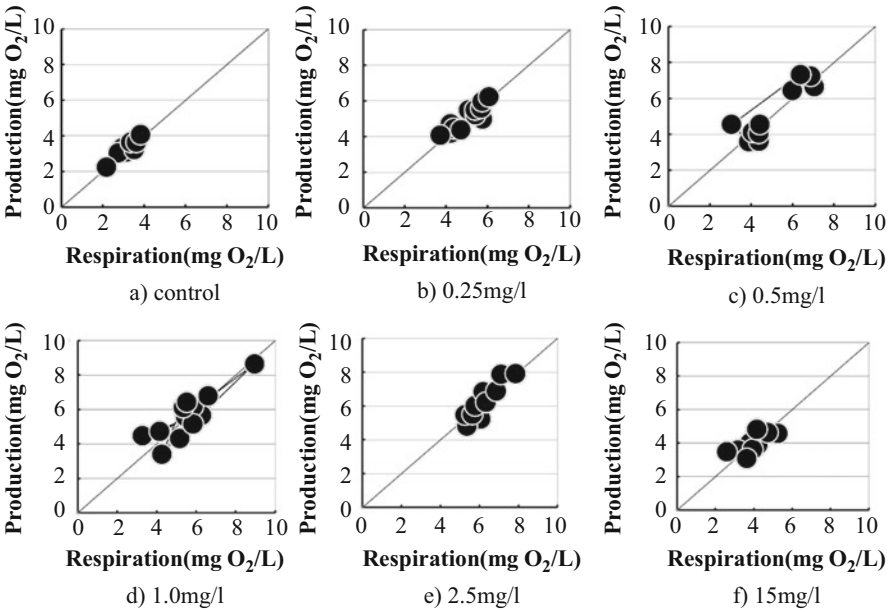

Fig. 7.20 Time course of P/R ratio in Dinotefuran-added microcosm N-system. (**a**) Control. (**b**) 0.25 mg/L. (**c**) 0.5 mg/L. (**d**) 1.0 mg/L. (**e**) 2.5 mg/L. (**f**) 15 mg/L

7.4.8 Methoxychlor

The effect of Methoxychlor was investigated by Dr. Sugiura. Methoxychlor was added at the start of the microcosm culture (day 0), and the number of organisms and amounts of respiration and production were continuously tracked. The m-NOEC of Methoxychlor was estimated to be 0.2 mg/L (Sugiura 1992).

7.4.9 β-Hexachlorocyclohexane (β-HCH)

The effect of β-Hexachlorocyclohexane (β-HCH) was investigated by Dr. Sugiura. β-Hexachlorocyclohexane was added at the start of the microcosm culture (day 0) and was adjusted to 0.1, 0.3, 1.0, and 3.0 mg/L. When 0.1 mg/L of β-HCH was added, the populations of *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Lecane* sp. increased during the early transition, when compared to the control system. Similarly, in the 1.0 mg/L addition system, the numbers of bacteria and *Cyclidium glaucoma* were higher than those of the control system. Conversely, in the 3.0 mg/L addition system, the populations of *Philodina erythrophthalma*, *Lecane* sp., and *Chlorella* sp. were lower than those of the control system. However, as the transition progressed, each species showed a tendency to approach the population size of the control system

at each concentration, and the extinction of organisms did not occur within the concentration range of the microcosm test. The time series of community metabolism also exhibited nearly the same trajectory as in the control system, and the ratio of respiration volume to production volume (P/R ratio) after the 10th day from the start of the culture also approached 1. From these results, β-HCH was not estimated to affect this system in the concentration range examined (Sugiura 1992).

7.4.10 γ-Hexachlorocyclohexane (γ-HCH)

The effect of γ-Hexachlorocyclohexane (γ-HCH) was investigated by Dr. Sugiura. At the start of the microcosm culture (day 0), γ-HCH was adjusted to 0.01, 0.1, 0.5, 1.0, and 5.0 mg/L and added to the system. As in the case of β-HCH, the numbers of *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Lecane* sp. were higher in the early stage of transition at concentrations of 0.01, 0.1, and 0.5 mg/L when compared with the control system. However, as the transition progressed, the control system population was approached in all of the addition systems. The change over time in community metabolism also exhibited almost the same trajectory as in the control system. From this result, it was assumed that γ-HCH does not affect this system in the concentration range examined (Sugiura 1992).

7.4.11 Dichlorodiphenyltrichloroethane (DDT)

The effect of Dichlorodiphenyltrichloroethane (DDT) was investigated by Dr. Sugiura. At the start of the microcosm culture (day 0), DDT was adjusted to 0.01, 0.05, 0.1, and 0.5 mg/L and added to the system. The addition of DDT did not affect the population density of the constituent organisms, nor did it appear as a change in the time series of the community metabolism relative to the control system until the addition of 0.5 mg/L (Sugiura 1992).

7.4.12 λ-Cyhalothrin

λ-Cyhalothrin is a synthetic pyrethroid insecticide. It acts on the central and peripheral nervous systems and acts to inhibit neurotransmission. On the 16th day after the start of the microcosm culture, λ-Cyhalothrin was adjusted to 1 ng/L and 10 ng/L and added to the system. As a result of performing a one-way ANOVA on the production and respiration, which are functional parameters, it was determined that there was no

statistical difference between the control system and λ-Cyhalothrin addition system at any concentration (Sugiura 1992).

7.5 Endocrine-Disrupting Chemicals

Endocrine systems are found in all vertebrate and most invertebrate species. The endocrine system is made up of glands, which secrete hormones to body fluids, and receptor cells, which detect and react to the hormones. The hormones act as chemical messengers. Hormones bind to cells that contain matching receptors in or on their surfaces, much like a key would fit into a lock. Disruption of the endocrine system can occur in various ways. Some chemicals mimic a natural hormone, fooling the body into overresponding to the stimulus or responding at inappropriate times. Other endocrine disruptors block the receptor site on a cell. Still others directly stimulate or inhibit the endocrine system and cause overproduction or underproduction of hormones. The substances that exhibit these effects are known as endocrine-disrupting chemicals (EDCs) or hormone-disrupting chemicals (HDCs). The most conspicuous EDCs are those that affect reproduction. Endocrine-disrupting chemicals have been demonstrated to markedly affect animal populations in coastal environments.

Hormone-disrupting chemicals, or environmental endocrine-disrupters, as they are also known, are the materials that are doubted to disturb the action of hormones in a living body. When taken into a living body, hormone action, such as the synthesis, storage, and secretion of hormones, is obstructed. For example, EDCs can inhibit generative functions, possibly causing malignant tumors.

7.5.1 Nonylphenol

The statistically significant difference was taken as the NOEC of 0.1 mg/L of Nonylphenol, and there was a statistically significant difference observed between the experimental microcosm and the control system with the addition of 1 mg/L. Therefore, it was determined there was influence on the ecosystem with the addition of 1 mg/L of Nonylphenol. The amounts of production (P) and respiration (R) at a Nonylphenol concentration of 0.2 mg/L were nearly the same as those of the control system, but there was a slight difference observed between concentrations of 0.1 mg/L and 0.2 mg/L (Fig. 7.21).

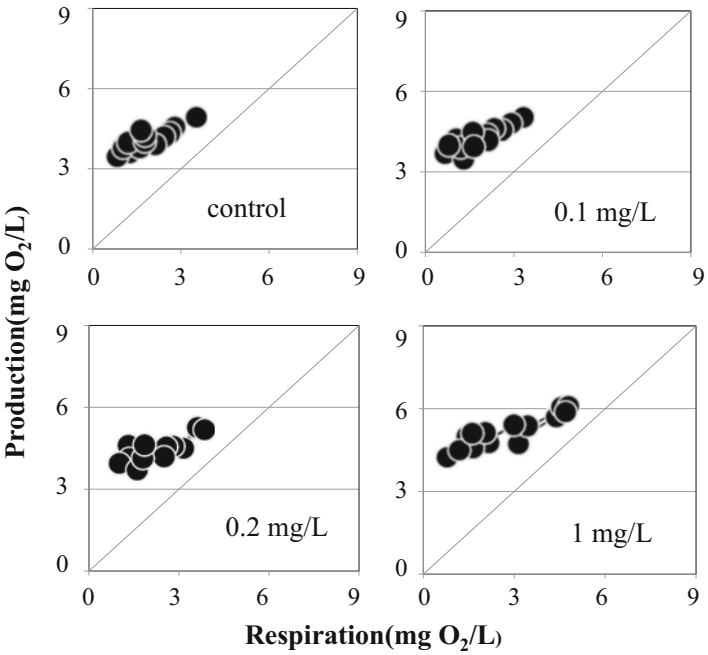

Fig. 7.21 Time course of P/R ratio in Nonylphenol-added microcosm N-system

7.5.2 Dibutyl Phthalate

Dibutyl phthalate is an organic compound, which is widely used as an additive in adhesives, print inks, and plasticizers. With the oily liquid, which has a sweet smell, alcohol, ether, and benzene are used as organic solvents. Whether this compound causes disturbance to the internal secretion of hormones is doubted. The effect of Dibutyl phthalate was investigated by Dr. Sugiura and added to the microcosm at the start of cultivation (day 0), and the populations of microorganisms and the amounts of respiration and production were tracked continuously. The m-NOEC of Dibutyl phthalate was estimated to be 1 mg/L (Sugiura 1992).

7.6 Antibiotics

Antibiotics, also called antibacterials, are a type of antimicrobial drug used in the treatment and prevention of bacterial infections. They may either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal capabilities. Antibiotics are not effective against viruses, such as the common cold or influenza; drugs that inhibit viruses are termed “antiviral drugs” or “antivirals,”

rather than "antibiotics." Antibiotics revolutionized medicine in the twentieth century. However, their effectiveness and easy access have also led to their overuse, prompting bacteria to develop resistance. This has led to widespread problems and prompted the World Health Organization (WHO) to classify antibiotic resistance as a "serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country."

The appearance of drug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), is a problem resulting from the diversity and abuse of antibiotics, and multiple-drug-resistant bacteria, called "super resistant bacteria," began appearing in 2010, quickly becoming a major societal problem. Antibiotics are used not only in medical care but also in stock raising (i.e., ranching) and the aquaculture business, particularly when mixed with bait and supplied in nurseries. Not only are antibiotics discharged as excrement, but they are also excreted from bait directly into the environment and may have a great impact on aquatic ecosystems. Therefore, it is important to understand the influences of antibiotics, including the appearance of drug-resistant bacteria, on aquatic ecosystems.

7.6.1 Oxytetracycline

The antibacterial spectrum is wide, and the antibiotic Oxytetracycline (OTC) is used in the marine products industry, most commonly including aquaculture, and resistant bacteria have been confirmed in the outdoors. However, there has been no ecosystem impact assessment of OTC reported. The test results of oxytetracycline were compared with 43 ecological influence tests (42 single-species examinations and a large-scale outdoor mesocosm). The LOEC of the large-scale outdoor microcosm was estimated to be 100 μg/L, whereas the m-NOEC of the microcosm test was 70 μg/L, and it was demonstrated that the microcosm test is sufficiently applicable for use as an OECD test. Additionally, it succeeded in determining the relative community metabolism (RCM) (i.e., the metabolic activity of the community), and a difference was observed in the metabolic activity as a result of exposure to OTC. It was also shown that the microcosm test was able to reflect the mechanisms of the antibiotic influence. Furthermore, because RCM changed with changes in the community structure, what a near menstruation state could reproduce was suggested by impossible natural ecosystem in the conventional test method, which did not include community structure, and was able to show the superiority of the microcosm test for this assessment.

Resistant bacteria appeared in the microcosm by adding the antibiotic OTC. The shape of the bacterial colony cultured in the control system in a PY nutrient medium was different from the form of the bacterial colony cultured in the nutrient medium and which had OTC, clearly to become 25 mg/L to PY nutrient medium. The colony pro-control was whitish as a whole, and the border of the colony was not clear. Two colonies, which greatly varied in shape, appeared in the system with the addition of 7 mg/L of OTC. The first colony was generally light yellow and viscous, and the second colony was white, and the border exhibited clear granulation. Because the

latter colony did not appear in the control system cultured in the PY nutrient medium, these were judged to be OTC-resistant bacteria. The test lasted for 14 days after OTC was added to the system at concentrations ranging between 0.007 mg/L and 7 mg/L. No difference was recognized in the algal population, which was evaluated from the total number of bacteria and the density of chlorophyll *a* in comparison with the control system. Additionally, the population of *Philodina erythrophthalma*, a metazoan, was not affected, the same as with algae. However, an influence was observed in the populations of OTC-resistant bacteria, *Cyclidium glaucoma*, *Lecane* sp., and *Aeolosoma hemprichi*, according to the OTC concentration over time.

The influences observed for 14 days after the addition of OTC were categorized as acute (2–4 days later), subacute (7–10 days later), and chronic (14 days later) to investigate changes in the microbial population in detail. It was determined that the system was affected when a population increased or decreased beyond the range of the control population. Because the population of *Aeolosoma hemprichi* exhibited an acute decrease with the addition of 7 mg/L of OTC, the NOEC of *Aeolosoma hemprichi* was estimated as less than 7 mg/L. Additionally, the NOEC for each was judged to be under 0.007 mg/L because increases and decreases in the populations of *Cyclidium glaucoma* and *Lecane* sp. were observed following the addition of 0.007 mg/L of OTC. Additionally, the population of *Cyclidium glaucoma* increased with OTC addition, while the population of *Lecane* sp. decreased. Because a common organism (bacteria) is assumed as the prey in this test, it is thought that the observed population changes are an outcome of the competitive interactions between organisms in the microcosm. Moreover, the NOEC of OTC-resistant bacteria was estimated to be less than 0.07 mg/L because an increase was observed in a number of bacteria with the addition of 0.07 mg/L of OTC. In this way, it is possible to estimate the NOEC for subacute and chronic influences equally. The effects of OTC on algae, all bacteria, and *Philodina erythrophthalma* were minimal, and the NOEC of each organism was greater than 7 mg/L for acute, subacute, and chronic influences. On the other hand, *Cyclidium glaucoma* and *Lecane* sp. were susceptible to OTC, and the NOEC for these organisms was less than 0.007 mg/L, except for the subacute influence (0.07 mg/L or less) of *Lecane* sp. It became clear from the population changes observed in the microcosm that the NOEC was less than 0.007 mg/L.

When high concentrations of OTC were added (7 mg/L and 0.7 mg/L), the amounts of production (P) and respiration (R) decreased in comparison with the control system (0 mg/L) over time, and the decrease in the amount of respiration was particularly large (Fig. 7.22). It is unclear why the addition of OTC decreased the amount of respiration, but it is likely that OTC binds to the intracellular ribosome, decreasing metabolic activity. When low concentrations of OTC were added (0.07 mg/L and 0.007 mg/L), a temporary increase in the amount of respiration was observed in the system with 0.07 mg/L of OTC, but the amounts of production and respiration both showed a tendency to decrease, the same as in the high-concentration systems. However, there were fewer decrements than in the high-concentration systems. The decrease in the amounts of production and respiration

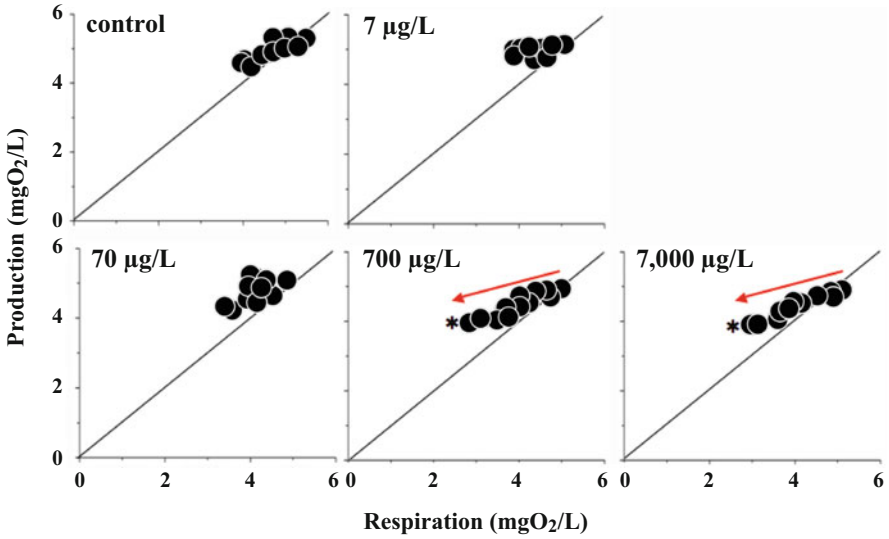

Fig. 7.22 Time course of P/R ratio in OTC-added microcosm N-system

was greater than in the control system in the lowest concentration (0.007 mg/L) system, and the m-NOEC estimated by changes in the P/R ratio was 0.007 mg/L.

The estimated NOEC was compared with the conventional value from the change in the populations mentioned above and the change in the P/R ratio. Based on the evaluation of population succession, the NOEC (greater than 7 mg/L) for the algae in this microcosm was larger than the conventional one column legal value (less than 0.11 mg/L). In the microcosm, the NOEC values for *Cyclidium glaucoma* and *Lecane* sp. were particularly low, at less than 0.007 mg/L. Evaluation by the US Environmental Protection Agency (EPA) has not been performed for these organisms, but the value (less than 0.007 mg/L) provided for the microcosm is less than the one column NOEC level judged in single-species tests of the EPA (less than 0.11 mg/L). Furthermore, the NOEC obtained from the P/R ratio was less than 0.007 mg/L, and it was revealed that it was susceptible to the addition of OTC into the microcosm.

The NOEC level determined above through the microcosm test (less than 0.007 mg/L) is less than the value (less than 0.11 mg/L) set by the US-EPA, and it is thought that this is because of the direct influence on the sensitivity of organisms to OTC, which is not considered in the EPA examination, and through the indirect influence of the interactions among organisms that were evaluated. The NOEC of OTC was estimated to be less than 0.007 mg/L, and it was suggested that the aquatic ecosystem may be influenced. In particular, it is possible that the addition of OTC, which influenced susceptible protozoans and metazoans, spread through the ecosystem through interactions among the constituent organisms.

7.7 Algal Toxins

In the aquatic ecosystem, pollution with microcystin, which are the toxins produced by high fecundity, eutrophying blue-green algae, combined with anthropogenic eutrophication, presents a major environmental problem. In eutrophic lakes during the summer season, blue-green algae such as *Microcyctis* multiply irregularly to large quantities, modifying the quality of the water and the biota of the aquatic ecosystem every year. In particular, blue-green algae produce toxins such as microcystin, and only very small amounts of these toxins that are present in lakes are derived from nature. However, an impact assessment of the aquatic ecosystem, especially the microbial ecosystem, with respect to contamination by microcystin, has not been performed. Therefore, evaluation of the influence of the toxin microcystin on microbial ecosystems, through examination of the biological interactions and material circulation among organisms and through the use of reduction functions, is important.

7.7.1 Microcystin-LR

Based on the structural parameter (population density), there was no difference observed between the populations of two microcystin-LR (where L stands for leucine and R for arginine) systems (0.1 mg/L and 1 mg/L addition) and the control system over time. The populations of *Chlorella* sp. and *Scenedesmus quadricauda*, both chlorophytes, remained stable between 10^5 N/mL and 10^6 N/mL after the addition of microcystin-LR. The population of *Tolypothrix* sp., a cyanophycean, was also stably maintained between 10^4 N/mL and 10^6 N/mL. As for the microanimals, the population of *Cyclidium glaucoma* decreased after the addition of microcystin-LR, but because this behavior was also observed in the control system, it was thought to simply reflect a natural change in the microcosm, and the population was stable between 10 Nm/L and 10^2 N/mL. Additionally, the populations of *Philodina erythrophthalma* and *Aeolosoma hemprichi* were stable and ranged from 1 N/mL to 10 N/mL.

With the addition of 0.1 mg/L and 1 mg/L of microcystin, it became clear that there was no remarkable change in the populations of this microcosm. Similarly, no remarkable changes in the P/R ratio between the two addition systems (0.1 mg/L and 1 mg/L of microcystin-LR addition) and the control system, which involved the addition of 480 mg/L of methanol, were recognized. However, there was a tendency for the amount of respiration (R) to increase in comparison with the additive-free control system, but it became clear that microcystin-LR did not have a remarkable influence on the P/R ratio under the conditions of this experiment (Fig. 7.23).

From these results, the m-NOEC of microcystin-LR was estimated to be more than 1 mg/L. This value is larger than 1 ppb, which is the guideline for concentrations in tap water established by the World Health Organization. It was made clear that microcystin-LR does not have a significant influence on the microcosm.

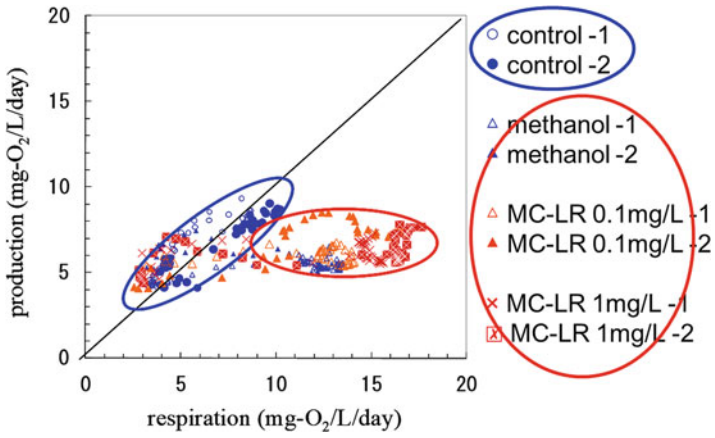

Fig. 7.23 P/R ratio in microcystin-LR-added microcosm N-system
○: without methanol as solvent for microcystin-LR
○: with methanol (480 mg/L) as solvent for microcystin-LR

7.8 Organic Matter and Organic Solvents

The effect of organic loading of the flask-sized microcosm system was investigated and assessed from the succession pattern of microbiota, the abundance in the stable state (N_{30}), and the biomass in the organic-loaded period ($B_{16–30}$) to obtain basic information from bioremediation, which uses certain essential microorganisms to improve polluted environments.

7.8.1 Polypeptone

Polypeptone, as a bacterial nutrient source, was added to the microcosm at a concentration of 5 g/L. The bacterial population density increased with absorption of polypeptone, and the culture medium was colored yellow by the 2nd day. The density of CFUs of bacteria increased to 10^9 N/mL, and CO_2 emissions also increased. *Chlorella* sp. and *Tolypothrix* sp. decreased in their population sizes immediately. Microanimals, such as *Cyclidium glaucoma* and *Philodina erythrophthalma*, became extinct, and only bacteria survived in the flask. The rate of succession in the microcosm increased in the 0.1 g/L addition system in comparison with the 0.5 g/L addition system.

Polypeptone, the substrate of the bacteria in the microcosm, was added as an organic matter load 16 days after the start of microcosm culturing. The addition quantity (quantity of the load) was assumed to be one, two, five, or ten times (i.e., 1×, 2×, 5×, or 10×) the quantity of existence in the microcosm on the 16th day after culturing began. In the 1× polypeptone loading system, bacteria multiplied rapidly

after the addition of polypeptone, and the protozoan, *Cyclidium glaucoma*, multiplied afterward. Then, the chlorophyceans, *Chlorella* sp. and *Scenedesmus quadricauda*, and the rotifers, *Lecane* sp. and *Philodina erythrophthalma*, multiplied in a more moderate fashion. In the 10x addition system, bacteria multiplied promptly after addition, and *Cyclidium glaucoma* multiplied but then suddenly decreased and perished afterward. Additionally, *Chlorella* sp., *Scenedesmus quadricauda*, *Lecane* sp., and *Philodina erythrophthalma* multiplied.

From the behaviors of the constituent organisms in the microcosm, it was observed that bacteria multiplied when organic matter was added and *Cyclidium glaucoma* multiplied as it preyed upon the bait. However, when organic matter was overloaded, because *Cyclidium glaucoma* disappeared after an increase in the concentration of organic matter, it was thought that the primary consumers (protozoans) were damaged (i.e., disappeared from the system) by too high of an addition concentration of the substrate. *Chlorella* sp. multiplied well, as evaluated by the comparison with the population (N_{30}) of the stable stage, but *Cyclidium glaucoma* perished at concentrations of greater than 250 mg/L. Organisms were shown to multiply in proportion to the increase in the concentration of polypeptone, except for the protozoan, *Cyclidium glaucoma*, and it became clear that the sensitivity of primary consumers (protozoans) to organic matter was extremely high. The rotifer, *Lecane* sp., followed by the protozoan, *Cyclidium glaucoma*, reacted sensitively based on a comparison of the population sizes before and after the addition ($B_{16–30}$) of polypeptone, and the possibility that *Lecane* sp., a bacterivore, participated as a buffer was demonstrated.

7.8.2 Acetone

When acetone is used as a solvent for a pesticide that is added to the microcosm, more than three times the respiration and production amounts are measured in the acetone addition system than are measured in the control system. Treated statistically (via ANOVA), there was an observed difference in the amounts of respiration and production between the control system and the solvent addition system on each measurement day. In the case of acetone addition, significantly higher values relative to the control system were shown for the respiration amount on the 9th day after addition and the production amount on the 7–9th days after addition. In this way, because the pesticide had very low solubility in water, it was necessary to dissolve it in organic solvents, such as acetone, but it became clear that the organic solvent itself caused an increase in the amounts of production and respiration.

7.8.3 Ethanol

When ethanol is used as a solvent for a pesticide that is added to the microcosm, more than three times the respiration and production amounts are measured in the acetone addition system than are measured in the control system. Treated statistically

(via ANOVA), there was an observed difference in the amounts of respiration and production between the control system and the solvent addition system on each measurement day. In the case of acetone addition, significantly higher values relative to the control system were shown for the respiration amount on the 9th day after addition and the production amount on the 7–9th days after addition. In this way, because the pesticide had very low solubility in water, it was necessary to dissolve it in organic solvents, such as acetone, but it became clear that the organic solvent itself caused an increase in the amounts of production and respiration.

7.8.4 Methanol

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7.8.5 Phenol

The effect of phenol was investigated and reported by Dr. Sugiura. Phenol was added at the time microcosm cultivation began (day 0), and the microorganism population and amounts of respiration and production were tracked over time. The amount of production became larger than that of the control system, but, with the addition of phenol, the amount of respiration was not observed to change with the addition of phenol when compared with the control system. The m-NOEC of phenol was estimated to be 5 mg/L (Sugiura 1992).

7.8.6 2.4-Dichlorophenol

2,4-Dichlorophenol is used as an organic, phosphorus-based insecticide and sterilizer, a phenoxy-based weed killer, and for herbicidal raw materials, and it may be generated by the resolution of a pesticide. The effect of 2,4-dichlorophenol was investigated and reported by Dr. Sugiura, and it was added to the microcosm when

cultivation began (day 0); the microorganism population and amounts of respiration and production were tracked over time. The m-NOEC of 2,4-dichlorophenol was estimated to be 10 mg/L (Sugiura 1992).

7.9 Nutrients

7.9.1 Phosphate

An ecological impact assessment of PO_4-P on the P/R ratio in an experimental microcosm system was performed. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

On the 16th day after microcosm cultivation began, 30 mg/L of phosphate, which is a nutrient source for *Chlorella* sp. and *Tolypothrix* sp., was added. This is a very high concentration in comparison with that of natural aquatic ecosystems. Phytoplankton in the microcosm performed photosynthesis and absorbed phosphate, and the culture medium was colored a deep green. *Chlorella* sp. increased to 50 times that of its original population size, and CO_2 absorption and O_2 emission increased accordingly. Bacterial abundance increased simultaneously, whereas zooplankton, such as *Cyclidium glaucoma* and *Philodina erythrophthalma*, became extinct, and their corpses were observed.

7.9.2 Ammonia

The effect of ammonia was investigated and reported by Dr. Sugiura. Ammonia was added when microcosm cultivation began (day 0), and the populations of microorganisms and amounts of respiration and production were tracked over time. The amount of production became greater than that of the control system, but, with the addition of ammonia, the amount of respiration was not observed to change in comparison to the control system. The m-NOEC of ammonia calculated from the test concentration was 0.35 mg/L (Sugiura 1992).

7.10 Metals

Metal is worldwidely used for convenience in our life. On the other hand, it caused serious problem in the society such as Minamata disease by exposure of organic mercury (Hg) and Itai-itai disease by exposure of cadmium (Cd). In addition, utilization of silver nanoparticles (AgNP) as antibacterial agents has become a new environmental problem recently because of its environmental standard which has not been regulated yet.

Because heavy metals are typically highly toxic and are not biodegradable, they can easily remain in aquatic environments.

7.10.1 Cu

Assessment data for Cu by a microcosm test were reported by Dr. Sugiura. It was clear that the system did not collapse with the addition of 1.2 mg/L of Cu during the stable stage, although it collapsed with the addition of 0.4 mg/L during the initial stage of succession. The bioactivity in the microcosm was high in the initial stage and low in the stable stage of succession, indicating that the excretion amount is also high in the initial stage and low in the stable stage. The toxicity of Cu was expressed by bonding with organic matter in the microcosm. According to this, microorganisms survived, and the system was maintained in the stable stage, even with high concentrations of Cu being added. Organisms are susceptible to disturbance under high activity conditions, while they are strong under low activities. It was suggested that the stability of the system was maintained under coexisting and mutually poor conditions. The m-NOEC of Cu was estimated to be 0.16 mg/L (Sugiura 2001, 2009).

7.10.2 Zn

The effect of zinc was investigated by Dr. Sugiura, and it was added when microcosm cultivation began (day 0); the populations of microorganisms and amounts of respiration and production were tracked over time. The amount of production became larger than that of the control system, but, with the addition of Zn, the amount of respiration was not observed to change relative to the control system. The P/R ratio was 1 when 19.2 mg/L of Zn was added and became completely over-respirated in the 153.3 mg/L addition system, and the system collapsed. The m-NOEC of Zn was estimated to be 2.4 mg/L (Sugiura 2009) (Fig. 7.24).

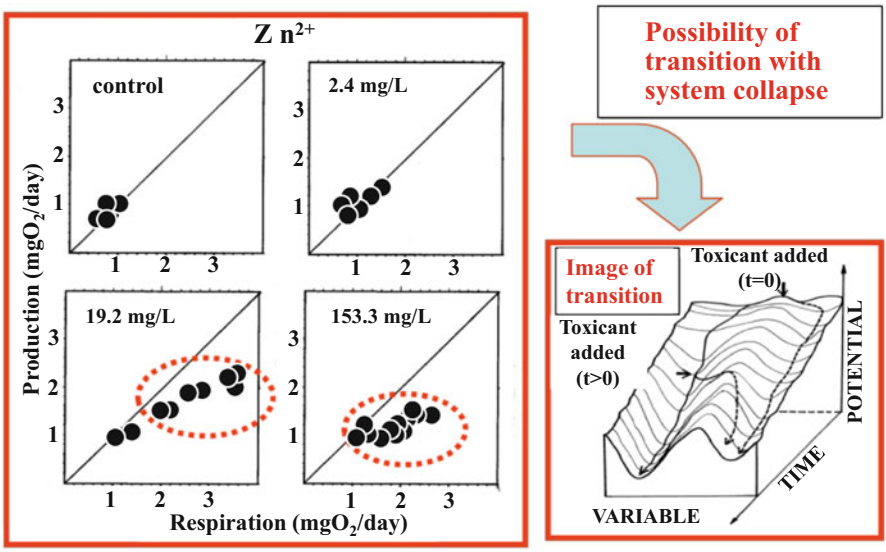

Fig. 7.24 Time course of P/R ratio in Zn-added microcosm N-system

7.10.3 Al

The concentrations of Al were 0.3, 0.6, 1.25, and 5.0 mg/L at the start of microcosm cultivation (day 0), and Al^{3+} was added by adjusting the concentration of $AlCl_3$. The initial concentration of Al^{3+} addition except 0.5–1.0 mg/L of systems; with respect to the population's metabolic rate, a change was observed over time that differed from the behavior of the control system. In particular, the concentration of Al^{3+} passed at 5 mg/L, and the state of the time change was different, and the extinction of organisms in the system was observed. The P/R ratio was 1 when 0.6 mg/L of Al was added, and it became completely over-respirated with the addition of 1.25 mg/L of Al, and the system collapsed (Fig. 7.25). The m-NOEC of Al was estimated to be 0.3 mg/L. These macrocosm tests were conducted by Dr. Sugiura (Sugiura 2001, 2009).

7.10.4 Cd

The effect of cadmium was investigated and reported by Dr. Sugiura. Cadmium was added at the time when microcosm cultivation began (day 0), and the populations of microorganisms and amounts of respiration and production were tracked over time. With the addition of Cd, the amounts of respiration and production became larger than in the control system. The m-NOEC of Cd was estimated to be 0.5 mg/L (Sugiura 2009).

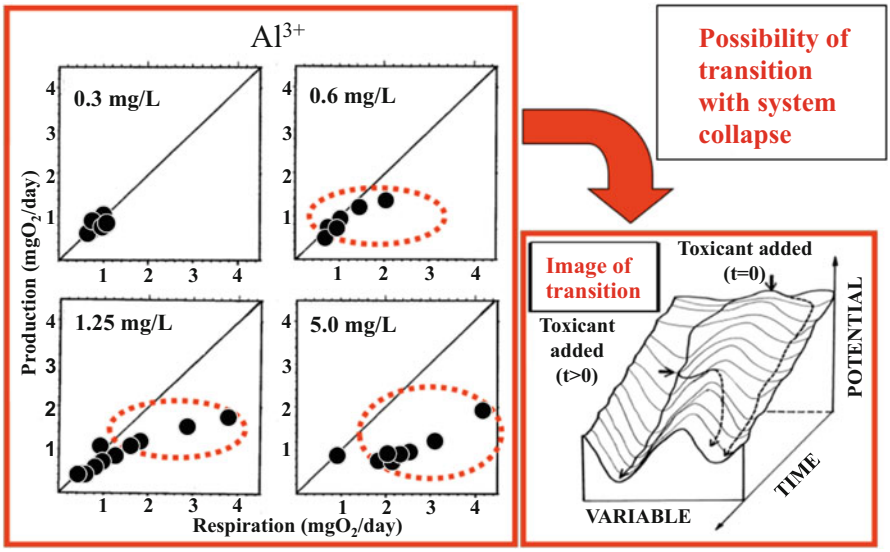

Fig. 7.25 Time course of P/R ratio in Al-added microcosm N-system

7.10.5 Mn

The addition concentration of Mn was adjusted to 0.1, 0.25, 0.5, 1.0, and 5.0 mg/L, and it was added on the 16th day of the stable state into the microcosm. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

An evaluation of the risk of Mn on the structural parameter was conducted by comparison with the behavior of microorganisms in the control system 14 days after the addition of Mn. It was clear that the protozoan, *Cyclidium glaucoma*, was greatly affected at a concentration of 1.0 mg/L of Mn from the behaviors of representative species in the microcosm (Fig. 7.26). In other words, it was shown in the multispecies system that the influence varied according to microbial species. In N_{30}, the microbial community was divided into two groups, species that increased in abundance (microalgae) and those that decreased in abundance (microanimals). These groups were divided into two as N_{30} in $B_{16–30}$. Using the functional parameter, evaluation of the Mn risk was conducted in comparison with the behavior of the P/R ratio in the control system 14 days after the addition of Mn. An influence was observed at a concentration of 1.0 mg/L of Mn, especially in the respiration amount,

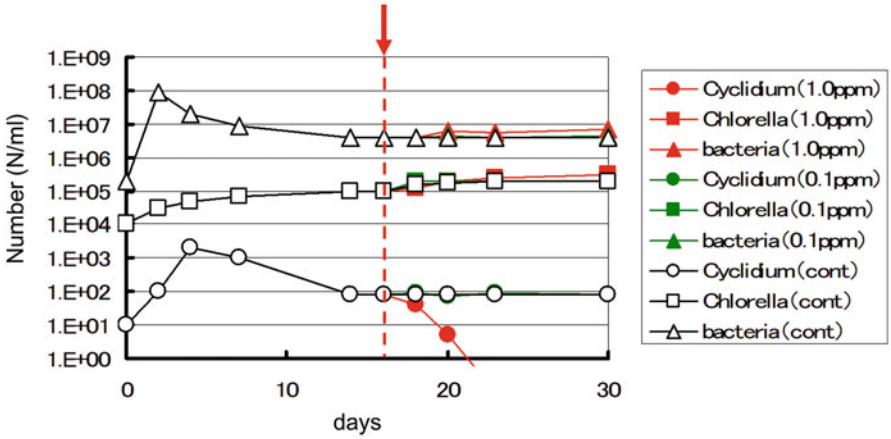

Fig. 7.26 Time course of population (representative species) in Mn-added microcosm N-system

and the P/R ratio decreased but recovered to ~1 (i.e., in the 1.0 mg/L Mn addition system, temporary toxicity occurred, but it was not chronic).

The m-NOEC of Mn was less than 1.0 mg/L, and a high sensitivity of the microcosm test was shown in comparison with the single-species test. The effectiveness of the assessment using the P/R ratio of the microcosm was indicated because of its similarity to the patterns of organismal abundance. However, further investigation of the m-NOEC calculation is needed.

7.10.6 Mg

The risk assessment of Mg as a sediment remediation material in aquatic ecosystems was conducted using a flask-sized microcosm system, based on the P/R ratio and the succession of microbial biota in comparison with the control system. The impact assessment of Mg was estimated from both the P/R ratio, as the functional parameter, and microbiota, as the structural parameter, in the microcosm. The environmental impact and ecological risk of these materials were estimated from comparison with the no-addition system (control) in both assessment methods described below. The P/R ratio was calculated from the amount of oxygen produced during the daytime, the production rate, and the amount of oxygen consumed at night, the respiration rate. When the value of the P/R ratio is near 1, the ecosystem is in a stable state, but when the value is far from 1, the ecosystem is in a state of succession or collapse (Odum 1983; Odum and Horne et al. 1983). In the case in which the P/R ratio is ~1, the impact risk of Mg was assessed as not serious, but a risk is involved for cases in which values are far from 1. The state of the microbiota was estimated from (1) the succession pattern of each microorganism, (2) the abundance at 30 days after cultivation began (N_{30}), and (3) the biomass during the period from the addition of

Mg to the end of cultivation ($B_{16–30}$). The regular succession pattern is similar. After cultivation began, bacteria grew rapidly using a polypeptone in the basal medium, and after that the protozoan *Cyclidium glaucoma* predated these bacteria and increased its abundance. The chlorophyceans, *Chlorella* sp. and *Scenedesmus quadricauda*, grew via photosynthesis under artificial light. After that, the other microorganisms increased their numbers through microbial interactions, such as prey-predator interactions, and the succession of microbiota into the stationary phase 14 days after cultivation began. The succession pattern of the P/R ratio in the period from the day of Mg addition to the final day of microcosm cultivation was compared with the control system. The results showed that the respiration rate (R rate) was greatly affected in the case of a 10.0 mg/L concentration of Mg injection in comparison with the production rate (P rate) (i.e., the respiration rate increased from 0.9 mg/day on the 16th day to 4.3 mg/day on the 18th day (maximum) and recovered to ~1.1 mg/day by the 24th day). Similarly, the production rate increased from 1.2 mg/day on the 16th day to 2.2 mg/day on the 18th day (maximum) and recovered to ~1.1 mg/day by the 24th day. The P/R ratio temporarily decreased from 1 to 0.5, and, after that, the P/R ratio recovered to ~1. Based on the results from statistical analyses, there was a significant difference between the system with 10.0 mg/L of Mg added and the control ($p < 0.05$) regarding the R rate in the period between the 16th day and the 24th day and the P/R ratio during the period between the 16th day and the 21st day. Conversely, there was no significant influence on the P/R ratio observed at a Mg concentration of 5.0 mg/L ($p < 0.05$). From this, the 10.0 mg/L concentration of Mg was considered toxic, although not chronic (but temporary), with respect to the P/R ratio as the functional parameter.

From an evaluation of the succession pattern of microorganisms as the structural parameter, the protozoan, *Cyclidium glaucoma*, and oligochaete, *Aeolosoma hemprichi*, were greatly influenced by the addition of 10.0 mg/L of Mg, and these two species decreased in their abundance in the 10.0 mg/L addition system. Other microanimals, such as the rotifers, *Lecane* sp. and *Philodina erythrophthalma*, also decreased in comparison with the control system. Thus, the influence of Mg differed for different species under coexisting culture conditions, such as those in the microcosm. Conversely, there was no significant difference observed in the abundance of organisms, when compared with the control system, at Mg concentrations of under 5.0 mg/L. As for the water quality, the pH rose with the addition of Mg, but the value of the pH was approximately 8.5–9.5 in all experiments. From an estimation of N_{30} at a concentration of 10.0 mg/L of Mg, the microorganisms were divided into two groups, those with increasing abundance and those with decreasing abundance. The former group contained algae as the producers (*Chlorella* sp., *Scenedesmus quadricauda*, and *Tolypothrix* sp.) and bacteria as the decomposers (*Pseudomonas putida*, *Acinetobacter* sp., *Bacillus cereus*, coryneform bacteria, etc.), and the latter group contained protozoans and metazoans as the consumers (*Cyclidium glaucoma*, *Lecane* sp., *Philodina erythrophthalma*, and *Aeolosoma hemprichi*). From an estimation of $B_{16–30}$, the microorganisms were divided into two groups, the increasing abundance and the decreasing abundance groups, the same as for N_{30}. The m-NOEC of Mg was estimated to be more than 10.0 mg/L.

7.10.7 Ca

An ecosystem impact assessment of Ca was conducted using both the P/R ratio as the functional parameter and the microbiota as the structural parameter in the microcosm. The environmental impacts and ecological risks of these materials were estimated from comparison with the no-addition system (control) in both assessment methods. The succession pattern of the P/R ratio during the period that began from the day Ca was added and lasted until the final day of microcosm cultivation was compared with that of the control system. The results showed that the P rate was more strongly affected at a 5.0 mg/L concentration of Ca in comparison with the R rate (i.e., the P rate decreased from 1.1 mg/day on the 16th day to 0.6 mg/day on the 21st day (maximum) and recovered to ~1.0 mg/day by the 24th day). Meanwhile, the R rate increased from 0.9 mg/day on the 16th day to 2.1 mg/day on the 18th day (maximum) and recovered to ~1.2 mg/day by the 23rd day. The P/R ratio temporarily decreased from 1.2 to 0.4, and, after that, it recovered to ~1 by the 23rd day. The microcosm was considered to be influenced by Ca concentrations of 5.0 mg/L and 10.0 mg/L in this study. According to the results of statistical analysis, there was a significant difference among the systems with 5.0 mg/L and 10.0 mg/L of added Ca and the control ($p < 0.05$). With the P/R ratio as the functional parameter, the 5.0 mg/L concentration of Ca was considered toxic, although not chronically. From an estimation of the succession pattern of microorganisms as the structural parameter, *Cyclidium glaucoma* and *Lecane* sp. were greatly influenced by the addition of 5.0 mg/L of Ca, and these two species decreased in their abundance with the addition of 5.0 mg/L of Ca, but other microanimals, such as the rotifer, *Philodina erythrophthalma*, decreased in comparison with the control microcosm. From an estimation of N_{30} at a concentration of 5.0 mg/L of Ca, the microorganisms were divided into two groups, those of increasing abundance and decreasing abundance. The former group contained bacteria as the decomposers, and the latter contained protozoans and metazoans as the consumers. Algae, as the producers, were divided into two groups; the abundance of the chlorophyceans, *Chlorella* sp. and *Scenedesmus quadricauda*, decreased, and the abundance of cyanophycean, *Tolypothrix* sp., increased. This phenomenon was caused by the growth inhibition of microanimals, especially *Aeolosoma hemprichi*, by the sudden increase in pH (from 8.2 to 11.1 just after the addition of 5.0 mg/L of Ca) and the photosynthetic inhibition of microalgae, especially *Chlorella* sp. and *Scenedesmus quadricauda*, by the light inhibition of Ca. The oligochaete, *Aeolosoma hemprichi*, has a strong jaw for eating and destroying flocs contained in bacteria, algae, other organisms, and detritus (Inamori et al. 1990). It was thought that *Aeolosoma hemprichi* was damaged in its physiological activity by the increase in pH; subsequently, *Tolypothrix* sp. escaped from the predation by *Aeolosoma hemprichi*. Under microscopic observation, it was confirmed that *Aeolosoma hemprichi* predated flocs in *Tolypothrix* sp., bacteria, and detritus in the control microcosm. The coefficients of variation (standard deviation/mean, expressed as percentages) for the abundance of each microorganism in the microcosm during the

cultivation period were 10% or less (50 independent experiments). This value is almost the same as previously reported values (e.g., Inamori et al. 1992; Kurihara 1978a, b).

From these outcomes, the effects of Mg and Ca on the aquatic ecosystem as sediment remediation materials both indicate powerful nutrient removal (especially for phosphorus) by bonding with PO_4-P through chemisorption in the water eluted from the eutrophied sediment (Murakami et al. 2000), differing in both influential concentrations and target microorganisms. The impact risk to the aquatic ecosystem of Mg was estimated as half that of Ca from the viewpoint of the concentration that had any influence on the microorganisms in the microcosm. The m-NOEC was estimated to be less than or equal to 10.0 mg/L for Mg and as less than or equal to 5.0 mg/L for Ca, from the viewpoint of changes in the P/R ratio, which is considered to be the functional parameter of the microcosm. From the biotic succession, as the structural parameter, the m-NOEC was considered to be less than or equal to 5.0 mg/L for Mg and much lower than 5.0 mg/L for Ca, with the abundance of protozoans, rotifers, and oligochaetes obviously decreasing and not recovering. However, the P/R ratio of the microcosm was maintained at the value of 1, notwithstanding the different biota, which consisted of producers, consumers, and decomposers, as the basic components of the ecosystem. Considering that the P/R ratio of the natural ecosystem in the stable state converges to almost 1 (Odum 1983), the m-NOEC should be estimated by the P/R ratio (functional parameter) rather than by the biotic succession (structural parameter). Further discussion and experimental data are necessary to better understand this estimation method.

7.10.8 Ni

Nickel is used for alloying and plating, and it may become mixed with tap water by elution from mine wastewater and nickel plating. The toxicity of Ni is different from a chemical form in the properties of matter; the oral toxicity is relatively low and is at the same level as the requisiteness of other metals, such as Cu, Co, and Zn, but the toxicity of inhaled nickel carbonyl is high, and the fatal dose for *Homo sapiens* has been estimated as 30 ppm/30 min. For Ni and its compounds, the targeted value for water quality management is less than 0.01 mg/L (temporarily).

Nickel was supplied for assessment, and the no-addition system (control) and addition systems (0.1, 1.0, 5.0, and 10 mg/L) were adjusted and added 16 days after culturing began. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As the result of the gnotobiotic-type microcosm test (N-system), after evaluating the ecosystem risk from the change in the P/R ratio as the functional parameter, no influence was observed at 0.1 mg/L of Ni addition, but respiration increased with 1.0 mg/L of addition, and the P/R ratio was greater than 1. From this, it was thought that Ni acted to increase the amount of respiration in the system. Microbiota in the microcosm were divided into two groups, those of increased abundance (microalgae) and those of decreased abundance (microanimals) in comparison with the control system, using the abundance as the structural parameter. The respiration activity *per* microanimal population rose, and the production activity *per* microalgae decreased with an increasing concentration of Ni, based on the evaluation of changes in the DO concentration *per* individual, because $B_{16–30}$ revealed a decrease in the microanimal population with the addition of Ni.

From these outcomes, the m-NOEC of Ni was estimated to be between 0.1 and 1.0 mg/L. Additionally, as the result of the naturally derived microcosm test, the P/R ratio (functional parameter) converged to approximately 1, but the microbiota in the control (no-addition system) were not stable, and the abundance of organisms (structural parameter) also differed. Reproducibility was not maintained in all of the systems. During the culture period, *Chlorella* sp., *Nitzschia* sp., *Monoraphidium contortum*, and *Aulacoseira granulata* ordinarily appeared, but no characteristic behavior linked to Ni addition was observed. As the result of the stress-selected-type microcosm test, only 7 stable systems out of the 40 total systems were established. Even in the control system, the reproducible stability was insufficient. No remarkable change was observed, regardless of the concentration of the Ni addition, and the P/R ratio varied near 1 and was stable. However, a clear decrease in population occurred at a 1.0 mg/L concentration of Ni, and it was shown that *Nitzschia* sp. and *Monoraphidium contortum* were particularly affected. It was predicted that diatomaceans were affected more than chlorophyceans.

7.10.9 Co

Cobalt was supplied for impact assessment to the no-addition system (control) and addition systems (1, 1.5, 2, 4, 6, 8, and 10 mg/L concentrations) and was added 16 days after the start of culturing. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As a structural parameter, the acute influence $B_{16–20}$ (0–4 days after loading), subacute influence $B_{20–23}$ (5–7 days after loading), chronic influence $B_{23–30}$ (8–14 days after loading), and $B_{16–30}$ as abundance were estimated. As a result, microorganisms did not perish during the period of acute influence, but extinction

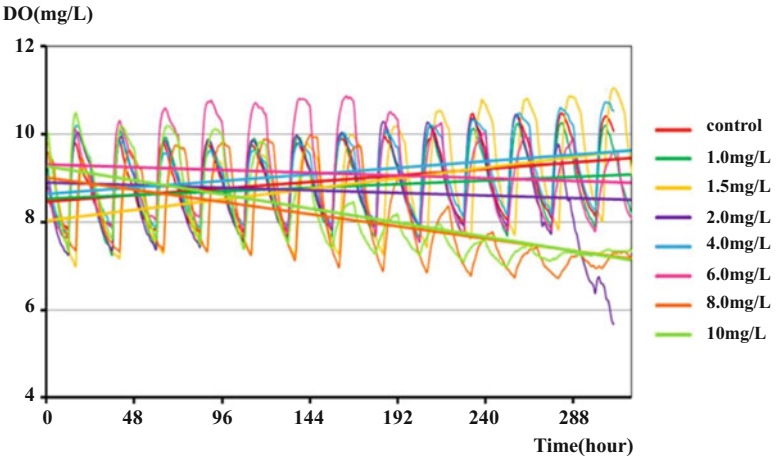

Fig. 7.27 Time course of DO in Co-added microcosm N-system

was observed during the periods of subacute influence and chronic influence. Additionally, it was revealed that many populations decreased in the high-concentration systems from $B_{16–30}$. As a functional parameter, the amplitude of the DO was damped by addition concentrations of more than 8 mg/L, and it is thought that the system collapsed (Fig. 7.27). The P/R ratios in all systems were stable at ~1. From these results, the m-NOEC of the structural parameter was estimated to be 1.5 mg/L of Co because microorganisms in the microcosm perished in more than 2 mg/L during the period of chronic influence. Meanwhile, the m-NOEC of the functional parameter was estimated to be 6 mg/L because the system collapsed with concentrations of more than 8 mg/L of Co from the viewpoint of changes in the DO concentration over time.

7.10.10 Ni and Co

Cobalt and Ni were supplied for assessment, and a no-addition system (control) and complexed addition systems (Co 0.2 mg/L + Ni 0.02 mg/L, Co 0.4 mg/L + Ni 0.04 mg/L, Co 0.6 mg/L + Ni 0.06 mg/L, Co 0.8 mg/L + Ni 0.08 mg/L, Co 1 mg/L + Ni 0.1 mg/L, Co 3 mg/L + Ni 0.3 mg/L, Co 5 mg/L + Ni 0.5 mg/L, and Co 10 mg/L + Ni 1 mg/L) were adjusted and loaded 16 days after culturing began. The addition concentration was set on the basis of the m-NOEC of Co and Ni. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of the abundance and population density (N_{30}) on the 30th day. The DO

concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As the structural parameter, $B_{16–30}$, the abundance 14 days after the addition of Co, was evaluated. Microorganisms in the microcosm did not perish during the period of acute influence, but extinction was observed during the periods of subacute influence and chronic influence. Increases and decreases in the populations of each microorganism were observed with Co addition, and the tendency for populations to slightly increase in a low-concentration system was recognized in *Philodina erythrophthalma*, but large decreases in population were observed in *Cyclidium glaucoma* and *Lecane* sp. Additionally, it was revealed that many populations decreased in the high-concentration systems from $B_{16–30}$. It is thought that the population ratio changed due to differences in the Co-resistance of each microbial species. As a functional parameter, the amplitude of the DO damped in the 5 mg/L of Co + 0.5 mg/L of Ni system, and it is thought that the system collapsed. The P/R ratio in all systems was stable at approximately 1.

From these results, the m-NOEC of the structural parameter was estimated to be 1 mg/L of Co + 0.1 mg/L of Ni because microorganisms in the microcosm perished at this concentration during the period of chronic influence. Meanwhile, the m-NOEC of the functional parameter was estimated to be 5 mg/L of Co + 0.5 mg/L of Ni because the system collapsed at concentrations higher than this from the viewpoint of the changes in the concentration of DO. In comparison with Co-only addition system, the m-NOEC was nearly equal, but the biomass ratio of the zooplankton largely decreased in the low-concentration system.

7.10.11 Cs

Cesium was supplied for assessment, and a no-addition system (control) and addition systems (0.1, 0.5, 1, and 2 mg/L) were adjusted and added as CsCl 16 days after the start of cultivation. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of the abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As a structural parameter, although a decrease was observed in the population of the protozoan, *Cyclidium glaucoma*, which was the primary consumer, no major change was observed for the entire microcosm. However, *Cyclidium glaucoma* perished on the 30th day (14th day after addition) at a Cs concentration of 1 mg/L, and an immediate decrease in the population after addition was observed at a concentration of 2 mg/L. From this, the m-NOEC was estimated to be less than 1 mg/L for Cs. Additionally, it was thought that there was a possibility of metastasis of the system due to Cs addition because the microanimals, such as *Philodina*

erythrophthalma, and microalgae, such as *Chlorella* sp., coexisted after *Cyclidium glaucoma* disappeared from the system and maintained a constant population. As a functional parameter, from the time series of the DO concentration in the microcosm, a 0.1 mg/L addition of Cs increased the activity and coped with the Cs load, and the 1 mg/L addition system recovered its activity through microbial interactions, material circulation, and energy flow among the microorganisms in the microcosm after the activity decreased due to the addition of Cs. Because the P/R ratio (functional parameter) is ~1, and the system was estimated to be stable, all addition systems were estimated as stable. From these results, with respect to the P/R ratio, the ecosystem function in the microcosm was judged to be stable when Cs addition concentrations were less than 1 mg/L. As a result of having analyzed the ecosystem influence of the Cs from the P/R ratio and microbial abundance (structural parameter), and because *Cyclidium glaucoma* was not observed, though the activity of the system was restored at a Cs concentration of 1 mg/L, it was thought that differential structuring of the system occurred.

From these outcomes, the m-NOECs of both the structural parameter and functional parameter were estimated to be less than 1 mg/L of Cs. Additionally, when it was assumed that a radiation of 3000 Bq was emitted from 1 mg of Cs based on the ^{137}Cs data from the Chernobyl nuclear power plant accident (Iimoto 2012; Minai et al. 2011), 0.02 mg of Cs existed in a 200 ml volume of microcosm culture fluid by the addition of 0.1 mg/L of Cs, and the half-life of ^{137}Cs was applied to calculate the type of radiation exposure dose in the microcosm (Iimoto 2012) for 30 years; the radiation exposure dose, D, was approximately 30 Sv. That is, in the microcosm, approximately 30 Gy/day would be irradiated at a Cs concentration of 0.1 mg/L. It was shown that there was a difference of ~10 times the radiation exposure (hot run) and m-NOEC of the metal load (cold run) in the microcosm when following the conversion mentioned above because there was some influence at the 23 Gy/day dose rate and almost no influence at the 10 Gy/day dos rate observed from the gamma beam irradiation experiment, and it was estimated that the radiation exposure had an influence that was approximately ten times as strong as that of the metal load. Here, the ecosystem influence of the addition of Cs was analyzed using a cold run, using the cold Cs, but it is difficult to convert values from Bq to Gy and Sv because they rely on different definitions for radioactive Cs, and further examination is necessary to complete the ecosystem impact assessment of Cs pollution.

7.10.12 I_2

Iodine was supplied for assessment, and a no-addition system (control) and addition systems (8 and 10 mg/L) were adjusted and added 16 days after the start of culturing. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the

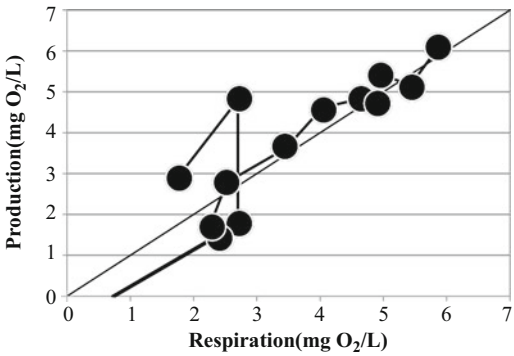

Fig. 7.28 Time course of P/R ratio in 10 mg/L of I_2-added microcosm N-system

ratio of the abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As a structural parameter, a decrease was observed at a concentration of 8 mg/L of I_2 in the populations of *Cyclidium glaucoma*, *Lecane* sp., *Scenedesmus quadricauda*, and *Tolypothrix* sp., but no microorganisms in the microcosm perished. Extinction was observed for *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Aeolosoma hemprichi* at an I_2 concentration of 10 mg/L, and the populations of *Scenedesmus quadricauda* and *Tolypothrix* sp. also decreased. Additionally, the tendency for only *Lecane* sp. to increase in comparison with other zooplanktons in N_{30} was recognized. These were influences upon the microcosm due to the inhibition of photosynthesis by I_2, and it was thought that extinction did not occur because the phytoplankton had a high ability to acclimate. As a functional parameter, bioactivity was observed at an I_2 concentration of 8 mg/L, and the P/R ratio was relatively stable. Activity decreased with the addition of 10 mg/L of I_2, and the P/R ratio measured just as this began was unstable, but it stabilized after the 22nd day of culturing (Fig. 7.28). Both production and consumption decreased, but the system remained stable. It was thought that the microbial interactions were responsible for recovery of the system after it collapsed under the influence of I_2. From these outcomes, it was estimated that the m-NOECs of both the structural parameter and functional parameter were present in the range between 8 mg/L and 10 mg/L because a tendency toward decreasing populations of both producers and consumers was recognized, whereas the P/R ratio of the system was recovered after the addition of I_2.

7.10.13 Cs and I_2

Cesium and I_2 were supplied for assessment, and a no-addition system (control) and complexed addition systems (0.5 mg/L of Cs + 1.5 mg/L of I_2, 1.0 mg/L of Cs + 3.0 mg/L of I_2, 1.5 mg/L of Cs + 4.5 mg/L of I_2, 2.0 mg/L of Cs + 6.0 mg/L of I_2, 2.5 mg/L of Cs + 7.5 mg/L of I_2, 0.25 mg/L of Cs + 0.75 mg/L of I_2, 2.7 mg/L

of Cs + 8.0 mg/L of I_2, and 8.0 mg/L of Cs + 2.7 mg/L of I_2) were adjusted and loaded 16 days after culturing began. The addition concentrations were set on the basis of the m-NOECs of Cs and I_2. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of the abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R). As a result, the m-NOEC of the structural parameter was estimated to be 1.0 mg/L of Cs + 3.0 mg/L of I_2. In comparison with the I_2-only addition system, the *Aeolosoma hemprichi* population was recognized as having a tendency to increase.

7.10.14 Sr

Strontium was supplied for assessment, and a no-addition system (control) and addition systems (0.1, 0.3, 0.4, 0.5, 1, 4, 6, 7, 8, and 10 mg/L) were adjusted and added 16 days after culturing began. The endpoints were abundance (structural parameter) and the concentration of DO (functional parameter), and the population was measured using an optical microscope and counted from the start of culturing on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30; it was evaluated from the results of $B_{16–30}$ (days 16–30), which was the ratio of abundance and population density (N_{30}) on the 30th day. The DO concentration was measured continuously from the 16th day, and the P/R ratio was calculated from the amounts of production (P) and respiration (R).

As a structural parameter, after the addition of Sr, all of the microorganisms in the microcosm existed at a Sr concentration of 0.3 mg/L, but *Cyclidium glaucoma* perished with the addition of 0.4 mg/L. Additionally, other zooplanktons were observed to decrease in abundance with increased Sr concentrations. The population of the phytoplankton, *Scenedesmus quadricauda*, increased, and *Tolypothrix* sp. also increased from double to ~3.5 times its initial abundance. These species increased with higher concentrations of Sr. It was thought that Sr worked to decrease the abundance of zooplankton and to multiply phytoplankton. As a functional parameter (the P/R ratio), the activity increased and was not disturbed at concentrations of 7 mg/L, but it was unbalanced with the addition of 8 mg/L of Sr near the end of the 25th day. For the N30 of the 7 mg/L and 8 mg/L concentrations, phytoplankton increased in both concentrations. It was thought that there was a mechanism that caused reduction in zooplankton activity at high concentrations. Additionally, the P/R ratio collapsed on the consumption side at a concentration of 8 mg/L (Fig. 7.29). Because the abundance of zooplankton decreased, the multiplication of phytoplankton was affected and decreased in its activity, and it is thought that phytoplankton consumed more than they produced. From these outcomes, the m-NOEC of the structural parameter was estimated to be 0.3 mg/L, and the m-NOEC of the

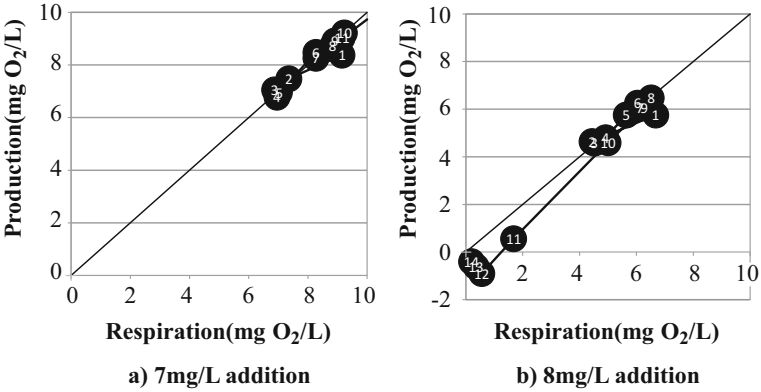

Fig. 7.29 Time course of P/R ratio in Sr-added microcosm N-system. (**a**) 7 mg/L addition. (**b**) 8 mg/L addition

functional parameter was estimated to be 7 mg/L. The toxicity of Sr affected zooplankton consumers, and the activity of phytoplankton decreased at high concentrations.

7.10.15 Silver Nanoparticles (AgNP)

In recent years, silver nanoparticles (AgNP) have come to be widely used as antibiotics and sterilizers, but information is not available on the influence these particles may have on aquatic ecosystems through drainage into river water after use. Though the metal (Ag) is known to exhibit serious toxicity in aquatic ecosystems that contain biological interactions, material circulation, and energy flow, the environmental risk remains poorly understood. The microcosm, which is the model microbial ecosystem consisting of producers, consumers, and decomposers, is useful for evaluating the environmental risk to an ecosystem system with complex interactions among organisms, rather than the toxic risk evaluation for single species at an ecosystem level, and positioning as a standard docimasy becomes important. Here, we conducted an environmental impact assessment of AgNP on microbial community function and structure, using the experimental flask-sized microcosm system.

To assess its influence, AgNP was added at several concentrations on the 16th day after microcosm cultivation began. Plankton observation using an optical microscope was conducted at the start of culturing, on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30, and the kinds of changes in a flask for each addition concentration as the structural parameter were observed. In addition, DO was measured consecutively from the 16th day during the culture period to evaluate the function side by the P/R ratio as the function parameter.

The appearance of the microcosm succeeded from green in the non-addition system (control system) to a strong gray with the increasing addition concentration

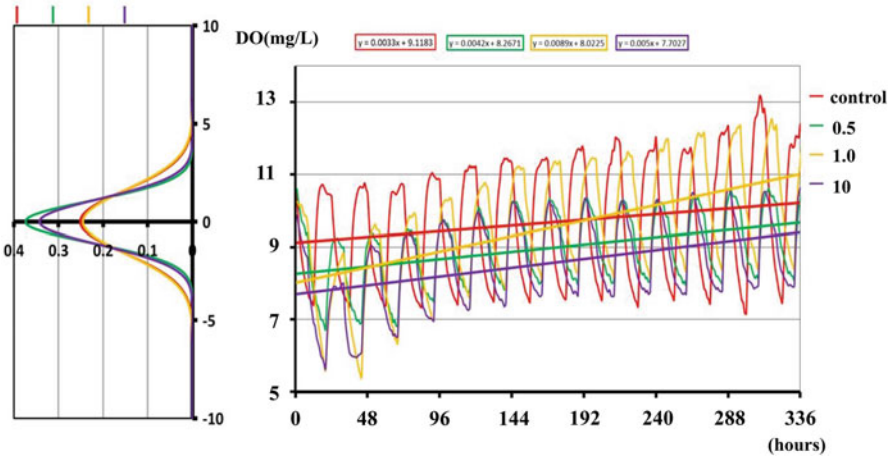

Fig. 7.30 Time course of DO in AgNP-added microcosm N-system

of AgNP, and a large influence was produced in the microcosm. To focus on the P/R ratio, the amplitude of the DO concentration tended to decline with increasing of AgNP concentrations, as shown in Fig. 7.30. The activity of the microcosm decreased with the addition of 0.5 mg/L of AgNP, and the activity of the system ceased with 10 mg/L of added AgNP. A large population decrease in the producers was not observed with the addition of AgNP for the chlorophyceans, *Chlorella* sp., and *Scenedesmus quadricauda*, but the population decreased and the individual lengths shortened for the cyanophycean, *Toplypothrix* sp. with AgNP addition. The populations of the consumers, *Cyclidium glaucoma* and *Aeolosoma hemprichi*, decreased at concentrations of 0.5 mg/L of AgNP, and the abundance of *Lecane* sp. slightly decreased in the 1.0 mg/L of AgNP addition system. Both *Cyclidium glaucoma* and *Aeolosoma hemprichi* perished, and the abundance of the rotifers, *Philodina erythrophthalma* and *Lecane* sp., decreased with the addition of 10 mg/L of AgNP (Fig. 7.31).

7.11 Irradiation

The lower-energy, longer wavelength part of the spectrum, including visible light, infrared light, microwaves, and radio waves, is nonionizing; its main effect when interacting with tissue is heating. This type of radiation only damages cells if the intensity is high enough to cause excessive heating. Ultraviolet radiation has some features of both ionizing and nonionizing radiation. While the part of the ultraviolet spectrum that penetrates the Earth's atmosphere is nonionizing, this radiation does far more damage to many molecules in biological systems than can be accounted for by the effects of heating, sunburn being a well-known example. These properties are

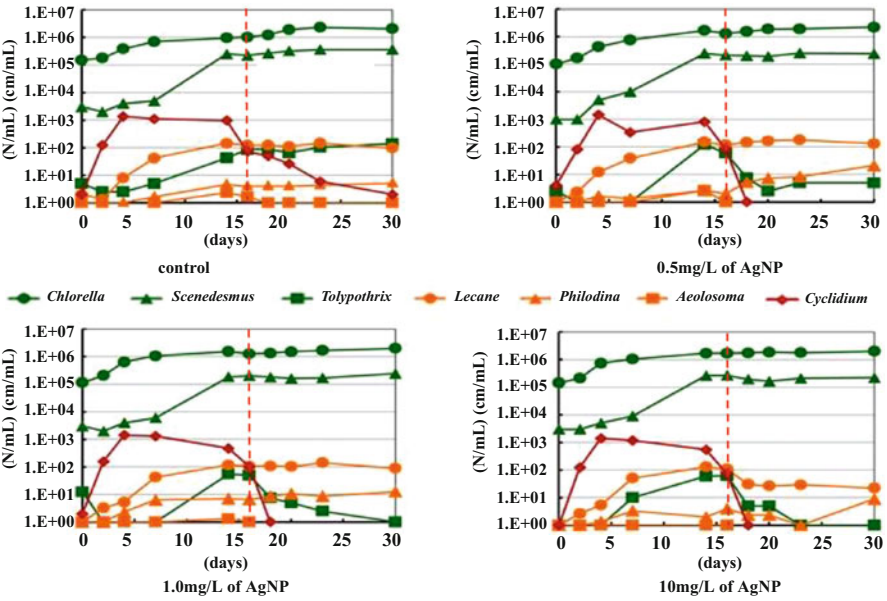

Fig. 7.31 Time course of population dynamics in AgNP-added microcosm N-system

derived from the ability of ultraviolet radiation to alter chemical bonds, even without having quite enough energy to ionize atoms.

Across the globe, the need for nuclear power generation is discussed as part of energy policies. The nuclear power plant accident during the earthquake disaster in eastern Japan, that is, the Tohoku earthquake and the resulting tsunami that affected power plants including Fukushima Daiichi, was the beginning of much discussion. With respect to nuclear power generation, the inspection and accumulation of knowledge about the safety for and effects of radiation on human beings, animals, plants, and the aquatic environment are necessary. The monitoring of pollution from fishery by-product pollution, soil pollution, and the accumulation of sewage grime was accomplished using radiological vegetables after the nuclear plant accident, and further analysis is now being pushed forward. However, consideration of the kind of influence radiation has on the small animals and phytoplankton constituting the base of the aquatic ecosystem, so-called microbes, remains limited. Here, the influence of radiation on the aquatic ecosystem is described.

7.11.1 γ-Ray (^{137}Cs)

To characterize the indirect effects of ionizing radiation on aquatic microbial communities, the effects of acute γ-irradiation were investigated in a microcosm (Fig. 7.32). Population changes in the constituent organisms were observed over

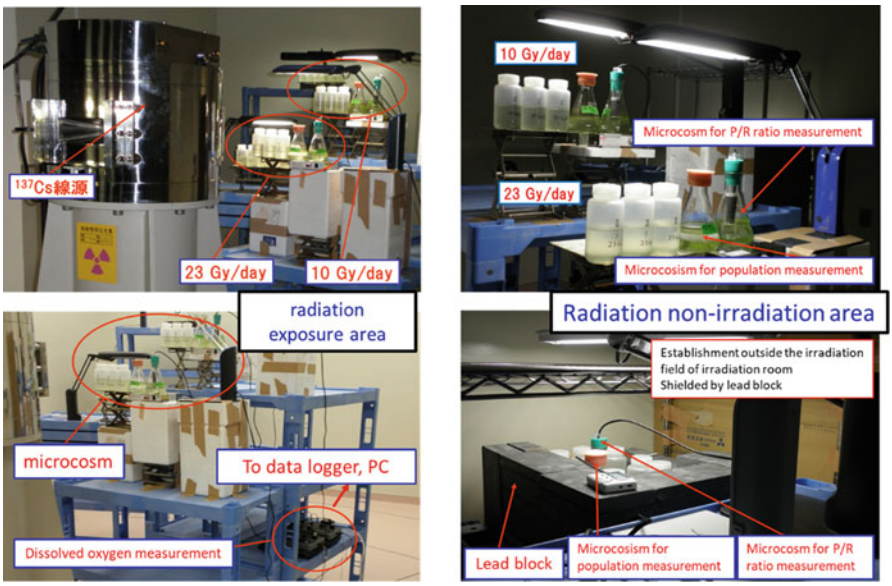

Fig. 7.32 Environmental impact risk assessment of irradiation on microcosm N-system

160 days after irradiation. The prokaryotic community structure was also examined by denaturing gradient gel electrophoresis (DGGE) of 16S rDNA. Principal response curve analysis revealed that the populations of the microcosm as a whole were not significantly affected at 100 Gy while they were adversely affected at 500–5000 Gy in a dose-dependent manner. However, some effects on each population, including each bacterial population detected by DGGE, did not depend on radiation doses, and some populations in the irradiated microcosm were larger than those of the control system. These unexpected results are regarded as indirect effects through interspecies interactions, and possible mechanisms are proposed originating from population changes in other organisms coexisting in the microcosm. For example, some indirect effects on consumers and decomposers likely arose from interspecies competition within each trophic level. It is also likely that predator-prey relationships between producers and consumers caused some indirect effects on producers.

The effects of acute γ-irradiation were investigated in the aquatic microcosm. At 100 Gy, populations were not affected in any taxa. At 500–5000 Gy, one or three taxa died out, and populations of two or three taxa decreased over time, while that of *Tolypothrix* sp. increased. This *Tolypothrix* sp. increase was likely an indirect effect due to interspecies interactions. Principal response curve analysis revealed that the main trend in the effects was a dose-dependent population decrease. For a better understanding of radiation risks in aquatic microbial communities, effect doses of γ-rays, compared with copper, herbicides, and detergents, were evaluated using a radiochemoecological conceptual model and the effect index for the microcosm. The populations of all microorganisms in the microcosm were stable under the irradiation conditions during an experimental period in a gamma beam irradiation experiment.

The cell count (CFU) of bacteria at 10 Gy/day (low dose of irradiation system) decreased more than in the control condition after irradiation began on the 19th day, but no influence was observed in other species. At 23 Gy/day (high dose of irradiation system), the abundance of *Tolypothrix* sp. increased to more than that of the control system after the 28th day. The bacteria decreased after the 4th day, and *Lecane* sp. decreased on the 45th day, but no influence was observed in other microorganisms. Culturing of the microcosm was conducted under a light and dark period for 12 h each, but, as for DO (functional parameter), the value of the P/R ratio was stable at approximately 1 under both control and irradiation conditions during the experimental period; no remarkable influence of gamma beam irradiation was recognized, and the behavior of DO was very stable (Fig. 7.33).

The structural parameter of the microcosm system was affected when the populations of certain microorganisms decreased or increased, but the functional parameter that assumed the P/R ratio by consecutive irradiation of the gamma beam did not come under influence. Mechanistically, even if a population increases or decreases, the metabolic activity of the population was not affected, and accordingly the possibility that the metabolic activity of an individual changed was suggested. For example, it is thought that the metabolic activity of an individual that survived rose when a population declined. Additionally, when the population of a certain organism increased or decreased, and the metabolic activity of the population changed, the metabolic activity of organisms with similar ecological functions changed accordingly, and the possibility that the metabolic activity of the population was not affected is considered. In other words, it is thought that when the population of an organism decreased, and its metabolic activity decreased, other organisms' populations rose using a surplus of resources. By either mechanism, the results obtained suggest the ability of the microcosm to be maintained functionally at the population or community level, even in the case in which the microcosm has been structurally affected (Fuma et al. 2010).

It is thought that the possibility that a real ecosystem will be bombed at equivalent dose rates to these experiments (i.e., 10 Gy/day and 23 Gy/day radiation) is extremely low. Serious atomic energy accidents do occur, and the inappropriate disposal of high-level atomic waste has been practiced, but most of the associated dose rates are less than the values used in this experiment. For example, in the Chernobyl nuclear power plant accident, which occurred in the former Soviet Union (present-day Ukraine) in 1986 and was said to be the worst nuclear disaster ever, the maximum dose rate that a fish received was only 0.03 Gy/day. Additionally, in the Mayak nuclear compound, also in the former Soviet Union (present-day Russia) and south of the Ural region, it is thought that an individual fish received a maximum dose of radiation equal to 0.6 Gy/day due to the inappropriate disposal of radioactive waste into the Techa River from 1950 to 1951, and a maximum of 0.1 Gy/day was provided in the Kyshtym accident due to malfunctioning of the cooling facilities, which occurred in 1957. The only example of a natural environment being bombed with dose rates greater than those examined in this experiment is due to the inappropriate disposal of large quantities of radioactive waste to Lake Karachay in the southern Ural Mountains from 1951 through 1952, when the dose rate was

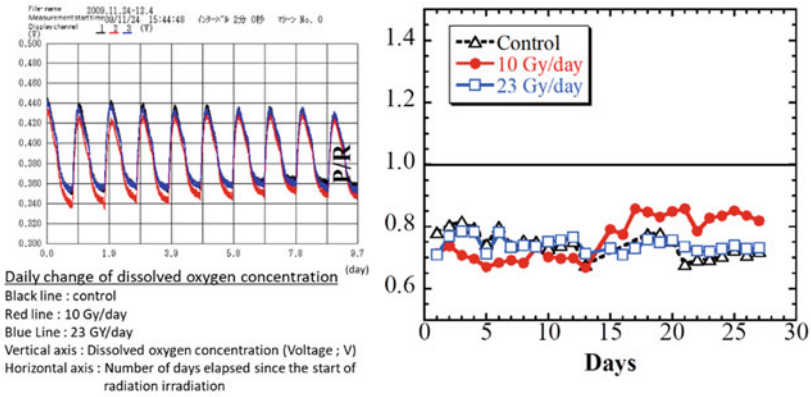

Fig. 7.33 Assessment from P/R ratio of γ-ray (^{137}Cs)

estimated to reach 300–800 Gy/day, if it was assumed that the fish survived. Therefore, the radiation risk for serious damage to aquatic microbial ecosystems is expected to be low, even if the accidents at atomic energy facilities and the inappropriate disposal of radioactive waste are considered, because a change in population was observed with some microorganisms in a microcosm, without any concomitant influence on the P/R ratio of the microcosm, in the 23 Gy/day experiments (Fuma et al. 2012) (Fig. 7.33).

Using another aquatic microcosm, a more simplified system, the effects of chronic γ-irradiation were investigated in the microcosm consisting of flagellate alga, *Euglena gracilis* Z, as producers; the ciliate protozoan, *Tetrahymena thermophila* B, as consumers; and the bacterium, *Escherichia coli* DH5α, as decomposers. At a dose rate of 1.1 Gy/day, no effects were observed. At a dose rate of 5.1 Gy/day, the population of *Escherichia coli* showed a tendency to be lower than that of the control system. At dose rates of 9.7 Gy/day and 24.7 Gy/day, a population decrease was observed in *Escherichia coli*. *Euglena gracilis* and *Tetrahymena thermophila* died out after a temporary population decrease, and the abundance of *Tetrahymena thermophile* subsequently increased. It is likely that this temporary population increase was an indirect effect of interspecies interactions. The effect dose rates of γ-rays were compared with the effect concentrations of some metals using a radiochemoecological conceptual model and the effect index for the microcosm. Comparison of these community-level effects with environmental exposure data suggests that ionizing radiation, Gd, and Dy pose low risks to aquatic microbial

communities, while Mn, Ni, and Cu pose considerable risks. The effects of chronic irradiation were smaller than those of acute irradiation, and an acute-to-chronic ratio was calculated to be 28 by dividing an acute dose by the chronic daily dose rate at which the effect index was 10%. This ratio would be useful for community-level extrapolation from acute to chronic radiation effects.

It is necessary to evaluate the combined effects of ionizing radiation and other toxic agents on ecosystems because ecosystems are exposed to various factors. The combined effects of γ-rays and acidification on an experimental model ecosystem (i.e., the microcosm) that mimicked aquatic microbial communities were investigated. Microcosms, consisting of *Euglena gracilis* Z as a producer, *Tetrahymena thermophila* B as a consumer, and *Escherichia coli* DH5α as a decomposer, were loaded by the following treatments: (1) irradiation with 100 Gy ^{60}Co γ-rays; (2) acidification of the culture medium to a pH = 4.0, with a mixture of 0.1 N of HNO_3 and 0.1 N of H_2SO4 (1:1, v/v), which mimicked acid rain; and (3) irradiation with 100 Gy γ-rays followed by the acidification of the culture medium (pH = 4.0). The γ-irradiation induced a temporary decrease in the cell density of *Escherichia coli* but did not affect the cell densities of the other species. The concentrations of chlorophyll *a* and ATP in the microcosm were not affected by γ-irradiation, and chlorophyll *a* concentrations in *Euglena gracilis* cells were also not affected. Acidification significantly decreased the cell density of *Tetrahymena thermophila*, slightly decreased the cell density of *Escherichia coli*, and slightly increased the cell density of *Euglena gracilis.* The concentrations of chlorophyll *a* and ATP in the microcosm were increased by acidification, although chlorophyll *a* concentrations in *Euglena gracilis* cells decreased. The combined exposure to γ-rays and acids temporarily decreased the cell density of *Escherichia coli*, significantly decreased the cell density of *Tetrahymena thermophila*, and slightly increased the cell density of *Euglena gracilis.* The concentrations of chlorophyll *a* and ATP in the microcosm were increased by this combined exposure, although chlorophyll *a* concentrations in *Euglena gracilis* cells decreased. It was therefore concluded that the combined exposure to γ-rays and acids had additive effects on cell densities, chlorophyll *a* and ATP concentrations in the microcosm, and chlorophyll *a* concentrations in *Euglena gracilis* cells (Fuma et al. 2010).

7.11.2 γ-Ray (^{60}Co)

^{60}Co γ-rays were used to irradiate the microcosm at various stages of biological succession and with various strengths, and the microcosm was transferred to a fresh culture medium after a certain period of time. In the case of irradiation during the initial stage or young stage, with various strengths, all microorganisms in the microcosm became extinct at 3000 R and the system collapsed; it did not recover by being transferred to a fresh culture medium. In contrast, the system did not collapse with 1000,000 R irradiation during the stable stage. In the transferred, fresh culture medium after 1 day since irradiation, there was an increased volume

of biomass, which was indicated to be higher in 1000,000 R <100,000 R <10,000 R, and close to that of the control system. However, in the case of transferring after 22 days, the 1,000,000 R irradiated system showed a similar increase in the volume of biomass as observed in the control system. This indicates that damage from irradiation was recovered after 22 days. The fact the mature biota had high fecundity means that microorganisms survived under strong irradiation, and these surviving individuals recovered to increasing conditions again after 22 days.

The microorganisms in the microcosm N-system were not influenced by irradiation stronger than that just after the Fukushima nuclear power plant accident in Japan. This phenomenon makes sense considering that microorganisms first appeared on the early Earth, which was exposed to very strong irradiation. It is important to generate new findings for environmental protection and restoration that some microorganisms have the ability to resist the influence of strong irradiation.

7.12 Microbial Pesticides

These days, the residues and accumulation of chemical pesticides have become one of the most serious problems all over the world. At the same time, biological prevention such as the use of microbial pesticides, which include natural enemies, is revisited, and, in Europe and the USA, microbial pesticides have already been made practicable. They are obtained from organisms including plants, bacteria and other microbes, fungi, nematodes, etc. They are often important components of integrated pest management (IPM) programs and have received much practical attention as substitutes for synthetic chemical plant protection products (PPPs).

Microbial pesticides are one of many biological preservation methods, and their main components are bacteria, fungi, viruses, protozoans, and nematodes, which may or may not be. These microbial pesticides are considered safer for the environment than chemical pesticides because of their natural origins. Furthermore, not only natural microbial pesticides but also genetically engineered ones have been developed, and more capitalization has been done. Note, however, that how the microbial pesticide behaves in nature and what effects will happen have not been made clear. Therefore, it is very important to make clear the proliferation and decay of microbial pesticides in natural ecosystems and to obtain the basal information for environmental assessment for the field release of microbial pesticides.

7.12.1 Bacillus thuringiensis *subsp.* aizawai *KH*

In this section, we focus on the environmental impact assessment of microbial pesticides, which constitute the lowest prey species in the microcosmic food chain. *Bacillus thuringiensis* subsp. *aizawai* KH was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, as a bacterial pesticide, and its

influence on the structure and function of the ecosystem was examined. This strain has Sm^r and Rf^r as markers. *Bacillus thuringiensis* subsp. *aizawai* KH rapidly decreased at 1, 10, or 100 times inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate, *Cyclidium glaucoma*) in the microcosm increased. *Bacillus thuringiensis* subsp. *aizawai* KH has shown to be a suitable food source for constituent heterotrophic groups such as protozoan ciliates, including *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Aeolosoma hemprichi*, in prey-predator interaction tests, which were considered to be dominated by these predatory actions. By comparison, in N_{30}, no significant difference was observed between the system with *Bacillus thuringiensis* subsp. *aizawai* KH added and the system without it (control system).

Bacillus thuringiensis subsp. *aizawai* KH, as a microbial pesticide, showed the same behavior as *Bacillus cereus* MC, one of the indigenous bacteria acting as a control. That is, these foreign bacteria decreased in the microcosm and did not strongly influence the indigenous microorganisms in the microcosm. As described in Fig. 7.4, the population density of *Bacillus thuringiensis* subsp. *aizawai* KH was 7.8×10^7 CFU/mL in the injected day (16th day) and decreased to 1.1×10^6 CFU/mL 2 days after (18th day), 2.2×10^4 CFU/mL 7 days after (23rd day), and 9.8×10^3 CFU/mL 14 days after (30th day). In a manner similar to the population density of *Bacillus thuringiensis* subsp. *aizawai* KH, the spores did not increase in abundance but survived at a fixed density of 5.8×10^2 N/mL after injection into the microcosm. This was because heterotrophs disliked the *Bacillus thuringiensis* spores as a food source, and vegetative cells produced spores without interruption. It was made clear that the predator-prey interaction between *Bacillus thuringiensis* and heterotrophs played a significant role in the proliferation and decay of microbial pesticides. After the injection of *Bacillus thuringiensis* and *Bacillus cereus*, a rapid increase in the number of protozoa, especially *Cyclidium glaucoma*, was observed. This suggested that the predator-prey interaction between foreign bacteria injected, such as microbial pesticide and protozoa, existed. This suggests that the proliferation and decay of microbial pesticides were greatly affected by the predation of heterotrophs, especially protozoa, which inhabited the microcosm system.

From an estimation of the succession pattern of microorganisms as the structural parameter, the protozoa *Cyclidium glaucoma* was strongly influenced by *Bacillus thuringiensis* subsp. *aizawai* KH, and this protozoa increased in its abundance under the *Bacillus thuringiensis* addition, and other microorganisms, such as the rotifers, *Lecane* sp. and *Philodina erythrophthalma*, also increased compared with the control microcosm. Thus, the influence of *Bacillus thuringiensis* was different in different microorganisms in coexisting culture conditions, such as the microcosm system. The water quality, that is, the pH, rose with the *Bacillus thuringiensis* addition, but the value of the pH was approximately 8.5 to 9.5 across all experiments. From an estimation of N_{30} using the *Bacillus thuringiensis* addition, all species of microorganisms increased in abundance. From an estimation of $B_{16–30}$, the same as in N_{30}, all microorganisms increased (Fig. 7.34).

Moreover, almost the same behavior was seen from the introduced *Bacillus thuringiensis* subsp. *aizawai* KH as bacterial microbial pesticide was observed in a

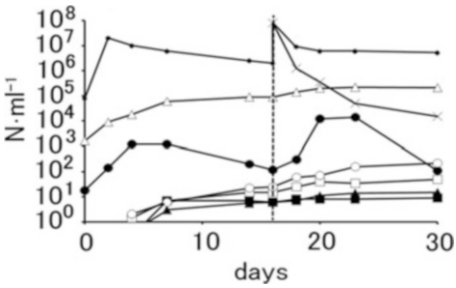

Fig. 7.34 Population dynamics in *B.t. aizawai* KH-added microcosm N-system

natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water including natural microorganisms and nutrients.

7.12.2 Bacillus thuringiensis *subsp.* kurstaki

The environmental impact risk assessment of microbial pesticides, which constitute the lowest prey species in the microcosmic food chain, was studied. *Bacillus thuringiensis* subsp. *kurstaki*, as a bacterial pesticide, was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and the influence on the structure and function of the ecosystem was examined. *Bacillus thuringiensis* subsp. *kurstaki* rapidly decreased at 1, 10, or 100 times the inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the number of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm increased. *Bacillus thuringiensis* subsp. *kurstaki* has been shown to be a suitable food source for constituent heterotrophic groups such as protozoan ciliates, including *Cyclidium glaucoma*, *Philodina erythrophthalma*, and *Aeolosoma hemprichi* in prey-predator interaction tests, which were considered to be dominated by these predatory actions. By comparison in N_{30}, no significant difference was observed between the system with *Bacillus thuringiensis* subsp. *kurstaki* added and the system without it (control system).

7.12.3 Pseudomonas fluorescens *IID5115*

Pseudomonas fluorescens IID5115, as a bacterial pesticide, was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Pseudomonas fluorescens* IID5115 rapidly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer,

the protozoan ciliate *Cyclidium glaucoma*) in the microcosm increased. *Pseudomonas fluorescens* IID5115 has been shown to be a suitable food source for constituent heterotrophic groups. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas fluorescens* IID5115 added and the system without it (control system).

Pseudomonas fluorescens IID5115, as a bacterial pesticide, was added in 10^7 N/mL; it decreased slightly after suddenly falling to 10^3 N/mL on the 30th day and disappeared finally. Meanwhile, the population density of the ciliate, *Cyclidium glaucoma*, increased from 10^2 N/mL to 10^4 N/mL. It was suggested that the predation pressure of microorganisms has a strong influence on the proliferation and decay of indigenous bacteria introduced into the ecosystem, and the results of the predator-prey interaction monoxenic culture test was lined.

7.12.4 Beauveria bassiana *F18-4B*

Beauveria bassiana F18-4B, as a fungal pesticide, was added at one and ten times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Beauveria bassiana* F18-4B slowly decreased at 1- or 10-times inoculation concentrations in viable counts using the selective media method, after microcosm inoculation, and the number of microorganisms in the microcosm increased. *Beauveria bassiana* F18-4B has been shown to be a suitable food source for *Philodina erythrophthalma* and *Aeolosoma hemprichi*, in predator-prey interaction tests, which was considered to be dominated by these predatory actions. By comparison, in N_{30}, no significant difference was observed between the system with *Beauveria bassiana* F18-4B added and the system without it (control system). Moreover, nearly identical behavior of the introduced *Beauveria bassiana* F18-4B, as a fungal microbial pesticide, was observed in a natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water, including natural microorganisms and nutrients. Irrespective of the kind of natural environmental water, all fungal pesticides decreased and disappeared in the system finally after it was added at 10^5 N/mL. The individual number of micro flagellates, such as *Chlamydomonas* sp. and *Monas* sp., in the fungal pesticide addition system increased from 10^2 N/mL to 10^3 N/mL; compared with the control system, the influence of the predation pressure by heterotrophs was confirmed. Microbial pesticides, both fungal and bacterial, exhibited a decreasing trend in the systems from which an organismal factor was removed through filtration processing, but the survivorship period was clearly lengthy, and the influence of the interaction between the microbes and the indigenous microorganisms was also confirmed. For indigenous microorganisms to become so varied in the eutrophied aquatic environment, a survivorship period of microbial pesticide tended to become short, so that the degree of water pollution was high, and a small amount of chemicals which caused the water contamination restrained the life and reproduction of the microbial pesticide. Organic matter also

had a high degree of pollution, the influence of the interaction by which the added microbial pesticide acted as competition with a settled microorganism and decreased. Or it was supposed that the physicochemical-like factor, which is the water quality, as well as the interaction between the organisms in the behavior of microbial pesticides is closely related.

7.12.5 Verticillium lecanii *F126-12-3M*

Verticillium lecanii F126-12-3M, as a fungal pesticide, was added at one and ten times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Verticillium lecanii* F126-12-3M slowly decreased at 1- or 10-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of small microorganisms in the microcosm increased. *Verticillium lecanii* F126-12-3M has been shown to be a suitable food source for only *Philodina erythrophthalma* and *Aeolosoma hemprichi* in the predator-prey interaction test, which was considered to be dominated by these predatory actions. By comparison, in N_{30}, no significant difference was observed between the system with *Verticillium lecanii* F126-12-3M added and the system without it (control system).

7.12.6 Metarhizium anisopliae *M7*

Metarhizium anisopliae M7, as a fungal pesticide, was added at one and ten times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Metarhizium anisopliae* M7 slowly decreased at 1- or 10-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms in the microcosm increased. *Metarhizium anisopliae* M7 has been shown to be a suitable food source for constituent heterotrophic groups such as metazoan *Philodina erythrophthalma* and *Aeolosoma hemprichi*. By comparison, in N_{30}, no significant difference was observed between the system with *Metarhizium anisopliae* M7 added and the system without it (control system).

7.13 Genetically Modified Bacteria

With the advancement of genetic engineering, the release of useful new microorganisms (e.g., genetically modified bacteria, genetically engineered microorganisms) to the environment has been investigated in various fields. From the viewpoint of environmental protection, their safe use should be confirmed before regular environmental release. It is essential to elucidate the behavior of GEMs in

natural ecosystems such as rivers, lakes, and marshes and in artificial ecosystems including activated sludge and biofilms. Especially, interactions among microorganisms, such as prey-predator interactions, are significant (Sudo et al. 1990). Using a microcosm system for the estimation of the proliferation and decay of GEMs is a useful method, because the microcosm has very high reproducibility and reflects the natural ecosystem.

7.13.1 Escherichia coli *HB101/pBR325*

Escherichia coli is one species of bacillus of gram-negative rods and is classified as facultative anaerobic bacteria. These bacteria are one of the major species that are present not only in the environment but also in the digestive tract of warm-blooded animals (birds and mammals), especially humans. Their size is usually 0.4–0.7 μm along the short axis and 2.0–4.0 μm along the long axis, but the long axis can be shortened, and some cells are nearly spherical. It is a model organism representative of bacteria, and it is used as a material in various studies, such as in genetic engineering, and also for the production of chemical substances through gene incorporation. Here, we focused on the competitive relationship among four taxa of bacteria, which are the lowest organisms on the microcosmic food chain. *Escherichia coli* HB101/pBR325 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The pBR325, a 5.9-kbp-sized vector plasmid, which is widely used for gene manipulation, is a non-transmissible plasmid, coding Cm^r, Tc^r, and Ap^r and having Eco *RI*, Bam *HI*, Hind *III*, and Sal *I* sites on its nucleotide sequence.

Escherichia coli HB101/pBR325 rapidly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm increased. *Escherichia coli* HB101/pBR325 has been shown to be a suitable food source for constituent heterotrophic groups in the microcosm. By comparison, in N_{30}, no significant difference was observed between systems with *Escherichia coli* added and systems without it (control system). *Escherichia coli* HB101/pBR325, as GEMs, decreased in its abundance in the same manner as *Bacillus thuringiensis*. The population density of *Escherichia coli* was 3.6×10^7 CFU/mL on the injected day (16th day) and rapidly decreased to 2.3×10^5 CFU/mL 2 days after (18th day), 7.2×10^3 CFU/mL 7 days after (23rd day), and 6.8×10^3 CFU/mL 14 days after (30th day). The predation effect of the protozoan *Cyclidium glaucoma* was observed at the same time. This indicates that *Escherichia coli* HB101/pBR325 was a suitable food source for the indigenous heterotrophs in the microcosm.

From an estimation of the succession pattern of microorganisms, *Cyclidium glaucoma* was strongly influenced by *Escherichia coli* HB101/pBR325, especially since this protozoan's density increased compared to other taxa, such as rotifer *Philodina erythrophthalma*, which slightly increased in abundance under these conditions, and compared with the control microcosm. From an estimation of N_{30}

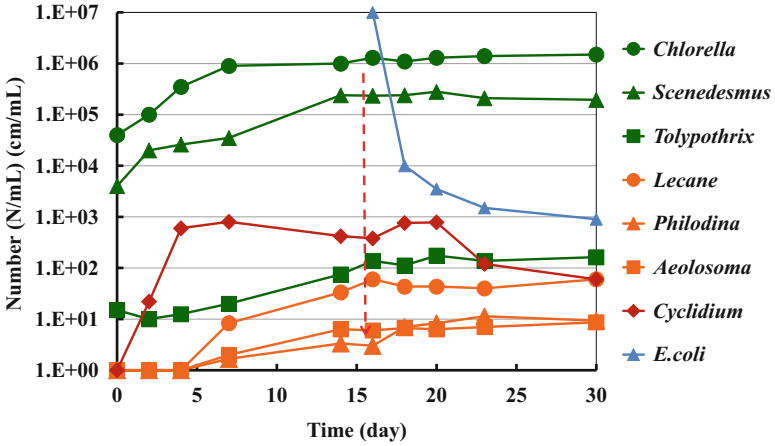

Fig. 7.35 Prosperity and decay of *E.coli* HB101/pBR325 in microcosm N-system

with *Bacillus cereus* as one of the indigenous bacteria additions, all species of microorganisms increased in abundance. From an estimation of $B_{16–30}$, all microorganisms increased similarly to N_{30}. In the case of the *Escherichia coli* addition, the trends of N_{30} and $B_{16–30}$ were almost the same as in the case of the *Bacillus cereus* addition. In addition, from the attenuation patterns of other foreign bacteria as GEMs, the patterns were divided into three types, that is, (a) rapidly decreasing, (b) slowly decreasing, and (c) slightly decreasing. *Bacillus thuringiensis* was classified as type b, and *Escherichia coli* was type a or b. Thus, the fate of foreign bacteria (alien species) can be assessed from the viewpoint of their attenuation pattern using a microcosm test (Fig. 7.35).

In the P/R ratio, which is the ratio of the production and consumption of dissolved oxygen, the consumption activity of dissolved oxygen (DO) increased as the amount of added *Escherichia coli* HB101/pBR325 increased, but eventually the non-additive system (control system) converged on the same level, and the P/R ratio was also stabilized at about 1. For this reason, the introduction of *Escherichia coli* HB101/pBR325 was judged as having no influence on the ecosystem resulting from the variation in the structural type of the structural parameter and the decline of the DO value of the functional parameter. Even if the lowest species of the food chain invades a microcosm, it was evaluated that there was no major impact on the ecosystem. When introducing the lowest species on the food chain (*Escherichia coli* HB101/pBR325) into the microcosm, both structural and functional parameters converge to the same extent as in the control system (non-additive system), and the existing ecosystem is weakly affected.

7.13.2 Escherichia coli *S17-1/pSUP104*

Escherichia coli S17-1/pSUP104 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The pSUP104 is a mobilized transmissible plasmid through the chromosome.

Escherichia coli S17-1/pSUP104 rapidly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, *Cyclidium glaucoma*) in the microcosm increased. *Escherichia coli* S17-1/pSUP104 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Escherichia coli* added and the system without it (control system). Moreover, a nearly identical behavior of introduced *Escherichia coli* S17-1/pSUP104, as a GEM, was observed in a natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water including natural microorganisms and nutrients.

7.13.3 Escherichia coli *C600/RP4*

Escherichia coli C600/RP4 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The RP-4, 56-kbp-sized vector plasmid, which is widely used for gene manipulation, is a self-transmissible plasmid, coding Ap^r, Tc^r, and Km^r and having an Eco *RI* site on its nucleotide sequence.

Escherichia coli C600/RP4 rapidly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, *Cyclidium glaucoma*) in the microcosm increased. *Escherichia coli* C600/RP4 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Escherichia coli* added and the system without it (control system).

7.13.4 Escherichia coli *S17-1/pCRO1*

Escherichia coli S17-1/pCRO1 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The pCRO1, with a 1580 bp fragment size, is a mobilized transmissible plasmid through the chromosome, coding Cr^r on its

nucleotide sequence. This plasmid was prepared by insertion between the ECO *RI* and Hind *III* sites of pBR322.

Escherichia coli S17-1/pCRO1 rapidly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm increased. *Escherichia coli* S17-1/pCRO1 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Escherichia coli* added and the system without it (control system). Moreover, a nearly identical behavior of the introduced *Escherichia coli* S17-1/pCRO1, as a GEM, was observed in a natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water including natural microorganisms and nutrients.

7.13.5 Pseudomonas aeruginosa *PAO1/pCRO1*

Pseudomonas aeruginosa PAO1/pCRO1 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The pCRO1, with a 1580 bp fragment size, is a mobilized transmissible plasmid through the chromosome, coding Cr^r on its nucleotide sequence. This plasmid was prepared by insertion between the ECO *RI* and Hind *III* sites of pBR322.

Pseudomonas aeruginosa PAO1/pCRO1 slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Pseudomonas aeruginosa* PAO1/pCRO1 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas aeruginosa* added and the system without it (control system). Moreover, a nearly identical behavior of the introduced *Pseudomonas aeruginosa* PAO1/pCRO1, as a GEM, was observed in a natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water including natural microorganisms and nutrients.

7.13.6 Pseudomonas putida *PpY101/pSR134*

Pseudomonas putida PpY101/pSR134 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. The pSR134 codes Hg^r on its nucleotide sequence. *Pseudomonas putida* PpY101/pSR134 slowly decreased at 1-, 10-, or

100-times inoculation concentrations in viable counts using the selective media method, following microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Pseudomonas putida* PpY101/pSR134 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas putida* added and the system without it (control system).

7.13.7 Pseudomonas putida *MC/pBR325*

Pseudomonas putida MC/pBR325 was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Pseudomonas putida* MC is one of the indigenous bacteria in the microcosm N-system. The pBR325, a 5.9-kbp-sized vector plasmid, which is widely used for gene manipulation, is a non-transmissible plasmid, coding Cm^r, Tc^r, and Ap^r and having Eco *RI*, Bam *HI*, Hind *III*, and Sal *I* sites on its nucleotide sequence. *Pseudomonas putida* MC/pBR325 slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. Pseudomonas putida MC/pBR325 has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas putida* added and the system without it (control system).

7.13.8 Pseudomonas paucimobilis *BHC+*

Pseudomonas paucimobilis BHC+ was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Pseudomonas paucimobilis* is a BHC+-degrading bacterium. *Pseudomonas paucimobilis* BHC+ slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Pseudomonas paucimobilis* BHC+ has been shown to be a suitable food source for constituent heterotrophs in the microcosm. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas paucimobilis* added and the system without it (control system).

7.14 Biomanipulation

Changing the fish population of bodies of water as a part of watershed management can facilitate desirable changes in aquatic ecosystems suffering from eutrophication, which is characterized by phytoplankton dominance, thus aiding ecosystem restoration, an application of restoration ecology. In ponds or lakes, alternative stable conditions (i.e., one with high algal populations, little other plant life, and turbid water) and another with low algae populations, a diverse plant population, and clear water may exist. In addition, to prevent excess nutrients such as phosphorus and nitrates, the removal of certain fish species adapted to turbid water may facilitate change from one steady state to the other, through the application of dynamical systems theory. Fish species may be removed by means of poisoning, harvesting, or the introduction of predatory species. Since a different fish community will result from these processes, it will affect recreational and commercial fishermen whose cooperation is important.

Biomanipulation technology attracts attention as a method for improving the quality of the water. Biomanipulation is a method used to introduce a creature, but this method poses a danger by collapsing existing ecosystems. Therefore, the environmental assessment needed to perform biomanipulation is important. The experiments below were carried out to investigate the basic ecosystem impact statement with respect to the water quality improvement from the biomanipulation. This study performed the basic environmental assessment, which focused its attention on ecosystem function as the P/R ratio using a microcosm. The risk assessment of top-down control and bottom-up control as a biomanipulation method on an aquatic ecosystem was conducted using a flask-sized microcosm system and its production/respiration ratio and succession of microbial biota, in comparison with a control system.

7.14.1 Cyclidium glaucoma *(Primary Consumer in the Microcosm N-System)*

In this experiment, the microcosm was loaded with *Cyclidium glaucoma*, which is a primary predator in this gnotobiotic microcosm. For on-site biomanipulation, either the top predator or primary predator was introduced into a eutrophied ecosystem to ablate the irregular phytoplankton growth, such as in an algal bloom. As the structural parameter, the population of *Cyclidium glaucoma* decreased after the 20th day and converged equivalently with the control on the 30th day (Fig. 7.36). As for both microcosms, all microorganisms did not disappear from the system, and it was thought that the predator-prey interactions between the microbe and its prey functioned. As the functional parameter, the 10-times loaded system showed the same behavior as the control system. However, the amplitude (activity) of the 10-times loaded system is larger than that of the control system. The *Cyclidium glaucoma*-loaded system keeps its function as an ecosystem. However, this system caused a structural change. As the functional parameter, any P/R ratio of a loaded system becomes approximately

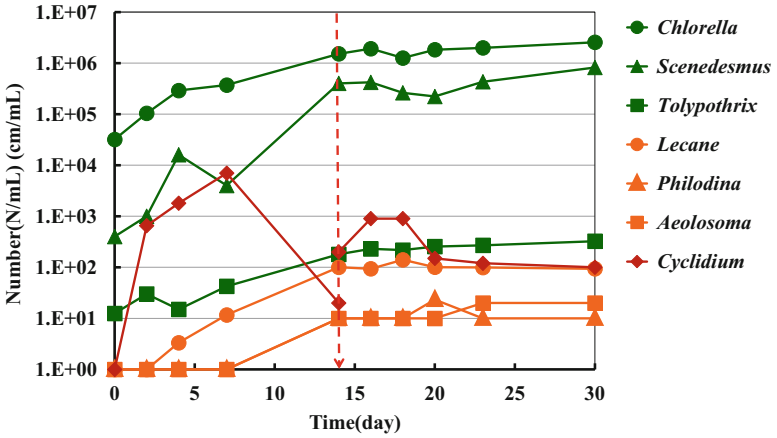

Fig. 7.36 Population dynamics in ten times of *Cyclidium*-added microcosm N-system

1. However, the activity of the system increased with the introduction of these loadings. As the structural parameter, the introduction of *Cyclidium glaucoma*, the primary predator, produces an activity change to the 10-times load, and it did not lead to the collapse of the ecosystem function.

7.14.2 Lecane *sp. (Primary Consumer in the Microcosm N-System)*

Lecane sp., one of the primary predators in the microcosm, was introduced into the microcosm. As the structural parameter, the population of *Lecane* sp. did not show significant change, but *Cyclidium glaucoma*, the primary consumer, increased to ten times that of the control and decreased after the 20th day. This was because the indigenous bacteria (decomposer and prey) took the metabolites of *Lecane* sp. and increased and bacteria-feeding microorganisms grew. However, because the specific growth rate (μ) of *Cyclidium glaucoma* (2.8/day) was larger than that of *Lecane* sp. (0.31/day), *Cyclidium glaucoma* increased. As the functional parameter, any P/R ratio of a loaded system becomes approximately 1. However, the activity of the system increased with the introduction of these loadings. As the structural parameter, the introduction of *Lecane* sp. produces an activity change to the 10-times load, and it did not lead to the collapse of the ecosystem function.

7.14.3 Philodina erythrophthalma *(Top Predator in the Microcosm N-System)*

Philodina erythrophthalma (one of the top predators in microcosm) was introduced into the microcosm as the introduced species for top-down biomanipulation. As a

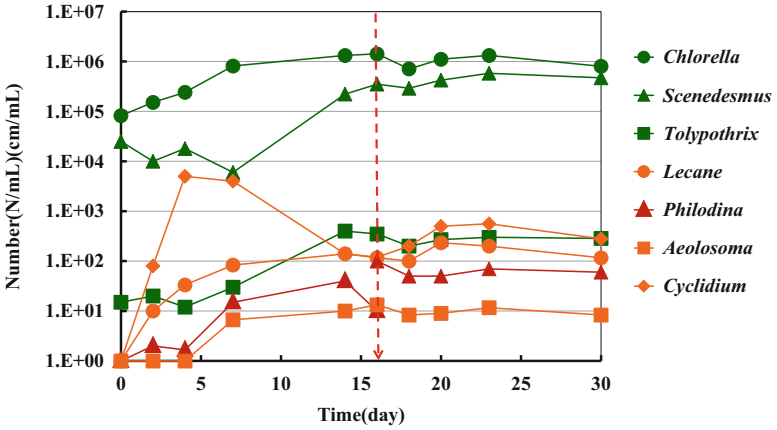

Fig. 7.37 Population dynamics in ten times of *Philodina*-added microcosm N-system

result of the mass addition of *Philodina erythrophthalma*, the color of the culture medium changed suddenly from light green to brown, and a brown solid accumulated at the bottom of the flask. Under microscopic observation, there were recognizable decolorized dead cells of *Chlorella* sp. Everywhere, and many were observed as crumbling. This is due to the excretion of much *Philodina erythrophthalma* which predated on *Chlorella* sp. At this point, both *Philodina erythrophthalma* and *Chlorella* sp. became extinct, and only bacteria and *Tolypothrix* sp. survived. As a result, in the flask with *Philodina erythrophthalma* introduced as the top predator species, *Chlorella* sp. decreased its population density by 45%, but *Scenedesmus quadricauda* did not. Although both *Aeolosoma hemprichi* and *Philodina erythrophthalma* are top predators in the microcosm N-system, *Aeolosoma hemprichi* is an aggregate feeder, while *Philodina erythrophthalma* is a bacterial feeder. Therefore, the effect of the top predator's predation type contributes significantly to the ecosystem of the microcosm. The top predator may keep down a proper population of microalga, and, in the case of top predator introduction, a possibility that could ameliorate algal blooms was demonstrated.

As a structural parameter, in either case, it is thought that the predator-prey interaction was constructed equally in the control system, without being removed from a system. The population of *Philodina erythrophthalma* slightly decreased in either 1-times quantity or 10-times quantity additions to a system by the 30th day while repeating the increase and decrease after addition together (Fig. 7.37). Additionally, for the decrease in chlorophyceans, the food source of rotifers, following the introduction of *Philodina erythrophthalma*, *Chlorella* sp. in the 10-times quantity addition system decreased by 45%, but *Scenedesmus quadricauda* did not decrease. Conversely, it is thought that the bacterial feeders *Cyclidium glaucoma* and *Lecane* sp. increased their populations mainly by having been dismantled as a metabolism by-product after *Cyclidium glaucoma* is added at ten times the quantity in the system,

and *Cyclidium glaucoma* and *Lecane* sp. increase in the 1-times quantity addition system; *Chlorella* sp. was preyed on by *Philodina erythrophthalma*. It is thought that a 1-times quantity addition of *Philodina erythrophthalma* is enough to control a population of the chlorophyceans because there is no difference seen in the decrease in *Chlorella* sp. between the 1-times quantity and 10-times quantity from these additions and from the comparison with $B_{16–30}$.

As a functional parameter, from the comparison of DO and the P/R ratio, because the amplitude could be confirmed in both systems, production and respiration were shown to function in accordance with the light and dark period in the system. In addition, an activity increase was observed with a quantity of addition, same as in other addition systems. The P/R ratio converged at P/R = 1, and it followed that the ecosystem function did not come to change with the 10-times quantity addition of *Philodina erythrophthalma*.

7.14.4 Aeolosoma hemprichi *(Top Predator in the Microcosm N-System)*

The microcosm was loaded with *Aeolosoma hemprichi*, which is the top predator in this gnotobiotic microcosm. For on-site biomanipulation, the top predator and/or primary predator were introduced into the eutrophied ecosystem to reduce irregular phytoplankton growth, such as an algal bloom. As the structural parameter, the green algae *Chlorella* sp. and *Scenedesmus quadricauda* largely decreased after the addition of *Aeolosoma hemprichi*, in a 10-times load system. The decrease of *Chlorella* sp. and *Scenedesmus quadricauda* depends on the predation of *Aeolosoma hemprichi*. The 10-times load system was effective for large decreases of the phytoplankton. *Cyclidium glaucoma*, the primary predator, was increased to about five times, but *Lecane* sp. just decreased in the community with three kinds of zooplankton as predators. Because the growth rate of the bacteria was the highest compared to the other constituent microorganisms, *Cyclidium glaucoma* preyed on the increased bacteria, and it was thought that *Cyclidium glaucoma* populations increased. As the functional parameter, the amplitude of the 10-times loaded system is larger than the 1-times loaded system. In a natural ecosystem, creatures with an ecological position equivalent to that of *Aeolosoma hemprichi* and *Cyclidium glaucoma* could potentially be introduced in on-site biomanipulation. It was shown that the introduced ecosystem recovers to a state similar to before the introduction by keeping down microorganisms and the detritus when indigenous phytoplankton decreased within a system.

When *Aeolosoma hemprichi*, the top predator, was introduced, the green algae and cyanophyceans did not decrease in the 1-times loaded system under its predation. However, a population of the green algae was reduced to half just after the addition in the 10-times load. Therefore, it was suggested that the introduction of the top predator (top-down) had an influence on the ecosystem structure (Fig. 7.38).

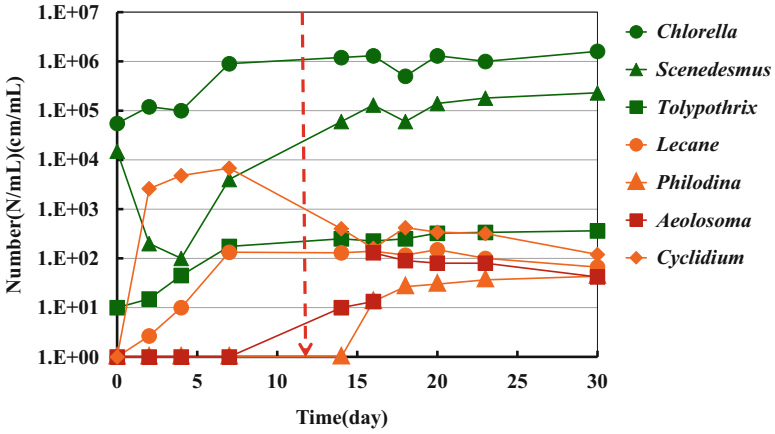

Fig. 7.38 Population dynamics in ten times of *Aeolosoma*-added microcosm N-system

However, the introduced ecosystem recovers to a state similar to that before the introduction by keeping down the introduced microorganisms and the detritus when the indigenous phytoplankton decreases within a system. It is necessary to remove the introduced microorganisms and detritus out of the system through the on-site biomanipulation. The microcosm was also effective as a tool for the environmental risk assessments, which targeted ecosystem function.

7.14.5 Moina macrocopa *(Alien Species)*

Moina macrocopa is filter-feeding minute crustacean belonging to *Daphnia*, with a physical size of 0.6–1.2 mm, and it is usually parthenogenetic (only in females), but males appear with the deterioration of the environment, due to water pollution and other stresses leading to the formation of resistant eggs. They are also useful as live baits for ornamental fishes and toxicity testing of chemicals, and they have gained attention as a biomanipulation-introduced species, among other applications. In this section, focusing on the competitive relationship with *Aeolosoma hemprichi*, which is the top predator in the microcosm, *Moina macrocopa* is added at one, five, and ten times the introduction amount of *Aeolosoma hemprichi* individuals, and the structure of the ecosystem and its effect on function are studied.

In any of the addition systems, the producers *Chlorella* sp. and *Tolypothrix* sp. decreased immediately after the introduction of *Moina macrocopa* (Fig. 7.39). The cause was thought to be that predation pressure increased from the *Moina macrocopa* invasion and *Tolypothrix* sp. could not grow sufficiently long due to amputation by the swimming behavior of *Moina macrocopa*. In predatory taxa, *Lecane* sp. and *Cyclidium glaucoma* showed a decreasing tendency, and, in the 10-times addition system, extinction was confirmed on the 18th day immediately

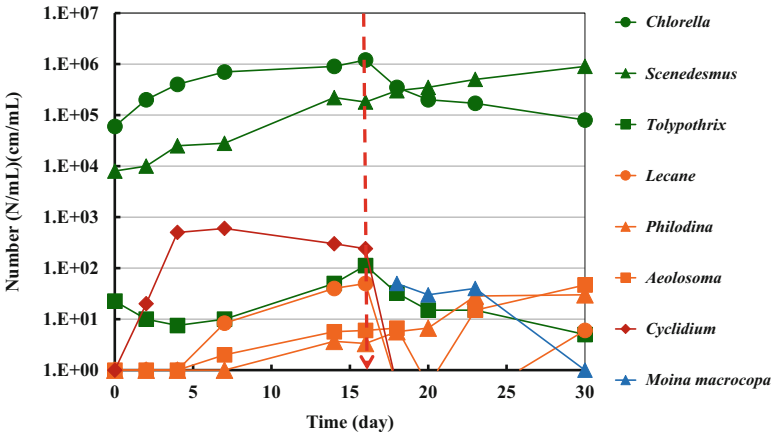

Fig. 7.39 Population dynamics of *Moina*-added microcosm N-system

after addition. This is thought to be indicative that the competitive relationship of consumers in the microcosm became severe due to the predation pressure of *Moina macrocopa*, and the differences in superiority or inferiority of predation ability were exhibited. In addition, the *Moina macrocopa* added as a foreign organism in all additive systems converged on the 30th day, by the time of measurement. In the 10-times addition system, assuming the invasion of a larger amount of alien species, the number of individuals of *Aeolosoma hemprichi* increased dramatically on the 30th day. This is because the existence of metabolites generated by the molting remnants of *Moina macrocopa* was a physical obstacle to filter-feeding zooplankton's predation behavior, and only *Aeolosoma hemprichi*, the detritus feeder, which has an opening-based feeding intake, advantageously predated green algae *Chlorella* sp.

From the P/R ratio, it can be seen that the consumption activity of dissolved oxygen (DO) increases as the added amount of *Moina macrocopa* increases, immediately after the start of continuous measurement in the 10-times addition system (Fig. 7.40). After the DO value attenuated for about 70 h, it stabilized. For this reason, introduction of the 10-times amount determined that the ecosystem was affected by the variation in the structural type of the structural parameter and the attenuation of the DO value of the functional parameter. With the top level of the food chain (highest predator) invaded in the microcosm, the maximum no-effect concentration was estimated to be between five and ten times that of the existing top-level predator. Furthermore, since the *Lecane* sp. is drastically decreased in the 5-times addition system from the structural parameter, the m-NOEC (microcosm maximum effect-free concentration) is considered to be at a relatively low concentration in the 5- to 10-times systems. In the case where a top-level predator (*Moina macrocopa*) was introduced into the microcosm, two predators were confirmed to go extinct in the 10-times addition system. There was a risk that the existing top-level predators would increase due to the invasion of alien species that could become higher predators.

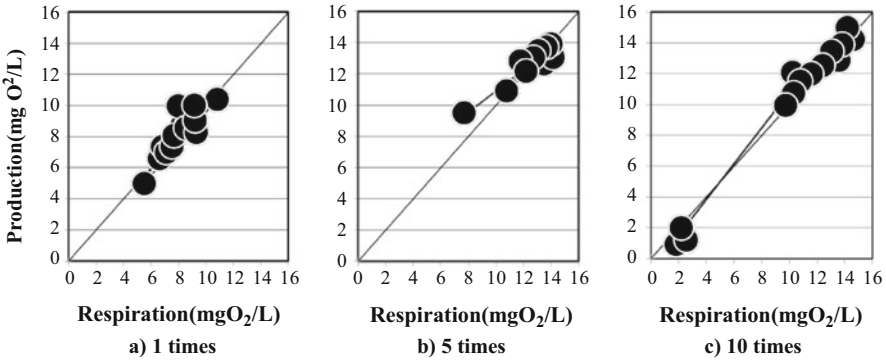

Fig. 7.40 Time course of P/R ratio in *Moina macrocopa*-added microcosm N-system. (**a**) One times. (**b**) Five times. (**c**) Ten times

7.14.6 Chlorella *sp. (Producer)*

Chlorella sp., one of the producers in the microcosm, was introduced into the microcosm as the introduced species for bottom-up biomanipulation. As structural parameters, no species became extinct within a culture period. The population of *Chlorella* sp. decreased in the 1-times quantity addition system from the time of addition to the 23rd day and increased afterward on the 30th day. On the other hand, the population of *Chlorella* sp. in the 10-times quantity addition system continued decreasing slightly from the time of addition to the 30th day and decreased approximately 78% more from the time of addition in the 30th day; this remarkable decrease in the population was confirmed. In addition, on the 30th day, the population of *Lecane* sp., *Philodina erythrophthalma*, and *Aeolosoma hemprichi*, which were the predators, were shown to decrease as *Chlorella* sp. was added. Whereas, the populations of *Cyclidium glaucoma* increased in comparison with the control system, and it followed that the quantity of existence increased. It is thought that the feeding niche of *Cyclidium glaucoma* broadened by the decrease in the population of three species above it in the food chain, which shared prey with *Cyclidium glaucoma*. The producers compete through the acquisition of the nutrient salts in the microcosm, which can also conjugate. Therefore, it is thought that the population increase in *Tolypothrix* sp. occurred because of the surplus nutrient salt, which was produced by a population decrease in *Scenedesmus quadricauda* and *Chlorella* sp.

As a functional parameter, in the case of *Chlorella* sp. in the 1-times quantity addition system, activity increased in comparison with the control system. This is regarded as an effect through the improvement of the photosynthetic efficiency from the increase in population. However, the DO just after the addition was in a state of high activity in the 10-times addition system, but the amplitude of the wave pattern decreased with the progression of the time, and an activity drop was shown. As for

10-times quantity addition system, the light inside of the flask is not at full intensity, and it is thought that the photosynthesis efficiency is low and counters the population growth of phytoplankton. This is a photosynthesis inhibition phenomenon, similar to algae in the natural ecosystem, and contributes to the extinction of fish from poor oxygenation in lakes and marshes. It is thought that the decrease in the population of *Chlorella* sp., which is a chlorophycean, is caused by a drop in their photosynthesis efficiency. However, it becomes a P/R ratio = 1 within each system, and it is thought that the ecosystem function in a 10-times addition system is low, but the system is maintained. From this, the 10-times quantity addition system was able to reproduce the decrease in activity from algae in a natural aquatic ecosystem, but it is thought that there is not recognized any influence such as the ecosystem collapses.

7.14.7 Scenedesmus quadricauda *(Producer)*

Scenedesmus quadricauda, one of the producers in the microcosm, was introduced into the microcosm as the introduced species for bottom-up biomanipulation. As a structural parameter, no species became extinct within a culture period. The population of *Scenedesmus quadricauda* decreased in the 1-times quantity addition system from the time of addition to the 23rd day, and it increased afterward on the 30th day. In contrast, the population of *Scenedesmus quadricauda* in the 10-times quantity addition system continued decreasing slightly from the time of addition to the 30th day.

As a functional parameter, in the case of *Scenedesmus quadricauda* in a 1-times quantity addition system, activity increased compared with the control system. This is regarded as an effect through the improvement of the photosynthetic efficiency from the increase in population. However, the DO just after the addition was in state of high in activity in the 10-times addition system, but the amplitude of the wave pattern decreased as time progressed, and an activity drop was shown. However, it becomes a P/R ratio = 1 within each system, and it is thought that the ecosystem function that a 10-times quantity addition system has is low, but the system is maintained. From this, the 10-times quantity addition system was able to reproduce the decrease in activity from algae in a natural aquatic ecosystem, but it is thought that there is no influence.

7.14.8 Tolypothrix *sp. (Producer)*

Tolypothrix sp., one of the producers in the microcosm, was introduced into the microcosm as the introduced species for bottom-up biomanipulation. As a structural parameter, each constituent organism class in the 1-times quantity addition system remained in the system. *Tolypothrix* sp. maintained a population in the 10-times quantity addition system until the 30th day, but *Cyclidium glaucoma* was not observed after the 18th day. *Aeolosoma hemprichi*, *Philodina erythrophthalma*,

and *Cyclidium glaucoma* multiplied in the 1-times quantity addition system just after addition, compared to N_{30}, but *Cyclidium glaucoma* perished in the 10-times quantity addition system, and *Aeolosoma hemprichi*, *Philodina erythrophthalma*, and *Lecane* sp. remarkably increased from 4 times to 12 times in abundance. It is thought that the factor leading to the population increase in *Aeolosoma hemprichi*, *Philodina erythrophthalma*, and *Lecane* sp. was that it became easier to prey on microorganisms when *Cyclidium glaucoma* perished. Furthermore, it is thought that three kinds of predators multiplied in form to fill the niche of *Cyclidium glaucoma* in the microcosm as a result of the 10-times quantity added of *Tolypothrix* sp. because they have the characteristic of easily becoming an algal floc since *Tolypothrix* sp. is a *conferva*, and *Aeolosoma hemprichi* and *Philodina erythrophthalma* multiply in a cohesion body as a living space. In addition, it was thought that the extinction of *Cyclidium glaucoma* was caused by the fact that an algal floc of *Tolypothrix* sp. inhibited swimming. From the comparison of $B_{16–30}$, because the abundance of the predators, except *Cyclidium glaucoma*, increased with an increase in the quantity of addition, it was revealed that the addition of *Tolypothrix* sp. had an influence on the predator.

As a functional parameter, from the time course of DO and P/R ratio, it appears that the amplitude (activity) increases with an increase in the quantity of addition. *Tolypothrix* sp., a blue-green algae, photosynthesizes in the microcosm, but, when comparing the 10-times quantity addition system with the 1-times quantity addition system, the amplitude (activity) of the 10-times quantity addition was approximately two times that of the 1-times quantity addition, and it was shown that the addition quantity and activity did not necessarily agree. In addition, it is thought that the transition of the system occurred regardless of an active increase, although *Cyclidium glaucoma* perished in the 10-times quantity addition system, as for the P/R ratio because it is stable in $P/R = 1$.

7.14.9 Bacillus cereus *MC (Decomposer)*

The competitive relationship among the four species of bacteria that are the lowest species in the microcosmic food chain was studied. *Bacillus cereus* MC, one of the indigenous bacteria in the microcosm, was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Bacillus cereus* MC slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Bacillus cereus* MC has been shown to be a suitable food source for microcosm heterotrophs. By comparison, in N_{30}, no significant difference was observed between the system with *Bacillus cereus* added and the system without it (control system).

7.14.10 Pseudomonas putida *MC (Decomposer)*

Pseudomonas putida MC, one of the indigenous bacteria in the microcosm, was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Pseudomonas putida* MC slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Pseudomonas putida* MC has been shown to be a suitable food source for microcosm heterotrophs. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas putida* added and the system without it (control system).

7.14.11 *Bacterial Mixed Culture*

A mixed culture of four species of indigenous bacteria in the microcosm, *Bacillus cereus*, *Pseudomonas putida*, *Acinetobacter* sp., and coryneform bacteria, was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. As a result, the total number of bacteria CFU slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. The bacterial mixed culture has been shown to be a suitable food source for microcosm heterotrophs. By comparison, in N_{30}, no significant difference was observed between the system with the bacterial mixed culture added and the system without it (control system).

7.14.12 Pseudomonas putida *prS2000 (Alien Species)*

Pseudomonas putida prS2000 as alien species was added at 1, 10, and 100 times the abundance of indigenous bacteria in the microcosm, and its influence on the structure and function of the ecosystem was examined. *Pseudomonas putida* prS2000 slowly decreased at 1-, 10-, or 100-times inoculation concentrations in viable counts using the selective media method after microcosm inoculation, and the abundance of microorganisms (in particular, its primary consumer, the protozoan ciliate *Cyclidium glaucoma*) in the microcosm slowly increased. *Pseudomonas putida* prS2000 has been shown to be a suitable food source for microcosm heterotrophs. By comparison, in N_{30}, no significant difference was observed between the system with *Pseudomonas putida* added and the system without it (control system).

7.14.13 Plasmid pBR325(Gene Biomanipulation)

As a form of bio-augmentation, *Escherichia coli* HB101/pBR325 decreased immediately after being added in the microcosm by counting CFU on the selective medium plate, and, at the same time, the bacteriophagous ciliate, *Cyclidium glaucoma*, the primary predator, increased. It was indicated that the predator-prey interaction between the introduced bacteria and the primary predatory protozoa was influenced strongly. Furthermore, bacteriophagous metazoans, the rotifers *Lecane* sp. and *Philodina erythrophthalma*, and the oligochaete, *Aeolosoma hemprichi*, also increased slowly. From these outcomes, the proliferation and decay of the introduced *Escherichia coli* HB101/pBR325 were considered to be controlled by the predation of indigenous microorganisms.

As gene biomanipulation, in the case of the direct addition of the pBR325 plasmid alone, there were no observed increases in heterotrophs in the microcosm, but the CFU on the selective medium plate increased. This means the introduced non-transmissible plasmid pBR325 was taken up by indigenous bacteria and phenotypically expressed its gene information, i.e., the non-transmissible plasmid can remain by horizontal transferring between introduced bacteria (host strain) and indigenous bacteria under the biological interactions in the microcosm (Fig. 7.41). From these outcomes, *Escherichia coli* HB101/pBR325, the introduced bacteria, decreased immediately through the control of the heterotroph's predation effect, but the non-transmissible plasmid pBR325 can remain through bacterial horizontal transferring. Moreover, nearly the same behavior of the introduced *Escherichia coli*

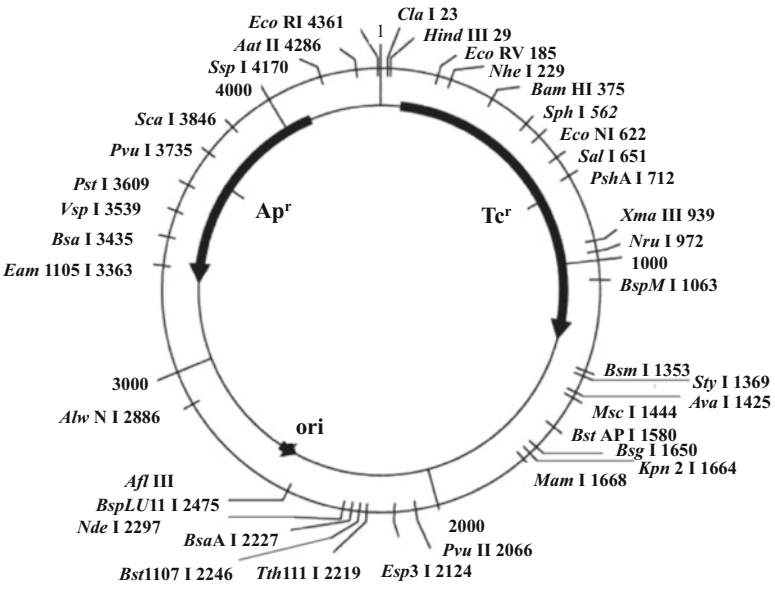

Fig. 7.41 Gene map of plasmid pBR325

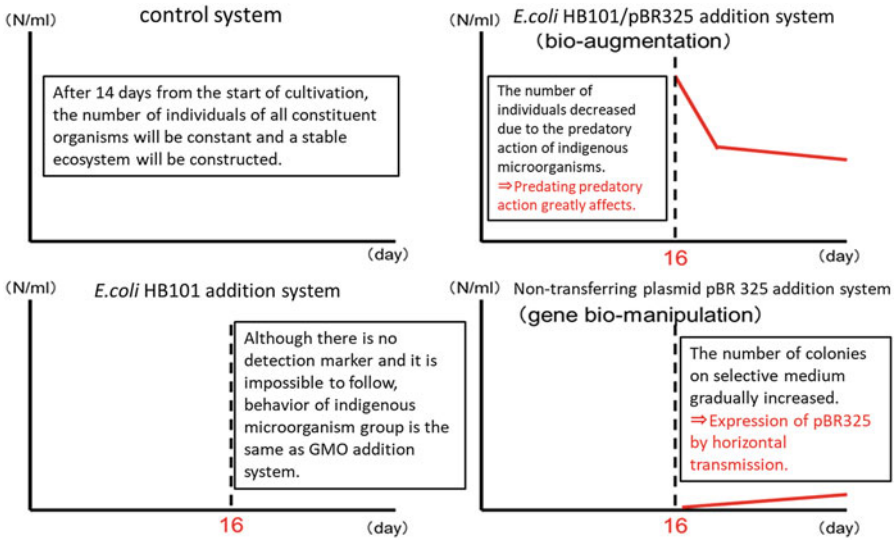

Fig. 7.42 Gene expression of non-transmissible plasmid pBR325 in microcosm N-system

HB101/pBR325 as a GEM was observed in a natural lake model ecosystem, that is, a naturally derived microcosm, which was made from natural lake water, including natural microorganisms and nutrients (Fig. 7.42).

From the asymptote, the microcosm N-system was shown to have the full stability of the system with respect to the population density of the original constituent microbial aspects, even if they are inoculated as constituent microbes from the microcosm N-system. In contrast, when it is inoculated with a foreign microbe, which was not a constituent microbe, it was shown that the influence on a system from highly advanced consumers was greater than in that from a lower-level prey introduction. It was also shown that the ecosystem impact statement, which includes the microbial interactions such as prey-predator interaction is able to be evaluated.

7.15 Climate Change

The microcosm N-system includes material circulation, energy flow, and microbial interactions, which are the foundations of natural ecosystems. It is possible to set the culture conditions of the microcosm to reflect the influence of global climate change, using the microcosm to diagnose the influence of climate change on Earth's ecosystems. In this section, the effects of climate change on ecosystems are investigated using the microcosm N-system.

7.15.1 Global Warming

Global warming is one of the most important environmental problems because of the seriousness of its effects on ecosystem structure, including upon energy flow, material circulation, and biological interactions. The Intergovernmental Panel on Climate Change (IPCC) reported in 2007 that 40% of all wildlife species on Earth will become extinct with a 4 °C increase in global atmospheric temperatures. However, information about the mechanisms underlying the relationship between global warming and the succession of biological species in aquatic environments has been insufficient to establish countermeasures, either academically or administratively. For this reason, experimental data are of extreme importance and must be obtained rapidly and in great abundance. We investigated the effects of temperature on microbial community function and structure using the experimental flask-sized microcosm.

A flask-sized microcosm was cultured at 25 °C under 2400 lux (12 h. L/12 h. D) and without stirring for use as the control system. To assess the influence of temperature, 30 °C, 35 °C, and 40 °C were used as the high-temperature conditions of global warming, and 10 °C and 20 °C were used as the low temperature conditions of global cooling. The vessel for cultivation was a 300 mL Erlenmeyer flask, and 200 mL of Taub's basal medium was poured; the concentration of the polypeptone as a substrate was adjusted to 100 mg/L. Plankton observation using an optical microscope was conducted from the start of culturing, on days 0, 2, 4, 7, 14, 16, 18, 20, 23, and 30. The kind of change observed in a flask under each temperature condition was used as the structural parameter. Additionally, the DO concentration was measured consecutively from the 16th day of the culture period onward to evaluate the functioning of the ecosystems by using the P/R ratio as the functional parameter.

In high-temperature conditions (35 °C and 40 °C), zooplankton consumers disappeared, whereas all microbial species maintained coexistence at 30 °C. The no-effect temperature in the microcosm was evaluated as less than 35 °C from the viewpoint of ecosystem structure. With increases in temperature, the activity of the system increased, and the P/R ratio was stable. However, the DO amplitude damped with the extinction of the microcosm at 35 °C. The maximum environmental temperature to have no effect on the microcosm, judging from the functional parameter, was evaluated to be less than 35 °C. At the lowest temperature (10 °C), the consumer population of the microcosm decreased, the activity of the ecosystem decreased, and the rate of succession slowed. It was shown that the influence of high temperatures was particularly strong with respect to the consumers, and the entire system eventually collapsed (Figs. 7.43 and 7.44).

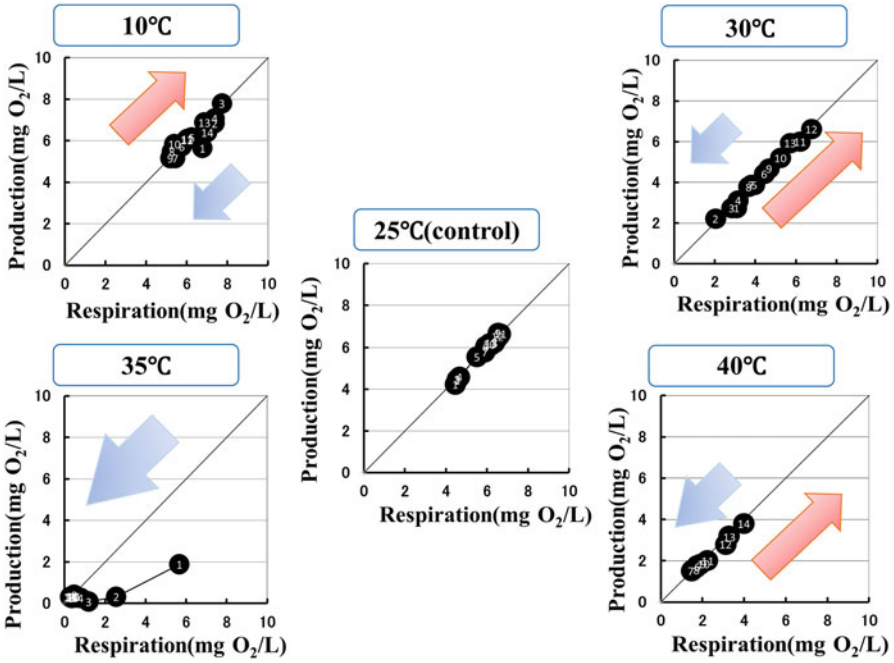

Fig. 7.43 Time course of P/R ratio in each culture temperature
→: activity of microcosm system increased in time course
→: activity of microcosm system decreased in time course
*The activity in each temperature was increased initially and then decreased at 10 °C with zooplankton decrease, decreased initially and then increased at 30 °C with no damage of microbiota, decreased at 35 °C with zooplankton disappearance, and decreased initially and then increased at 40 °C with zooplankton disappearance

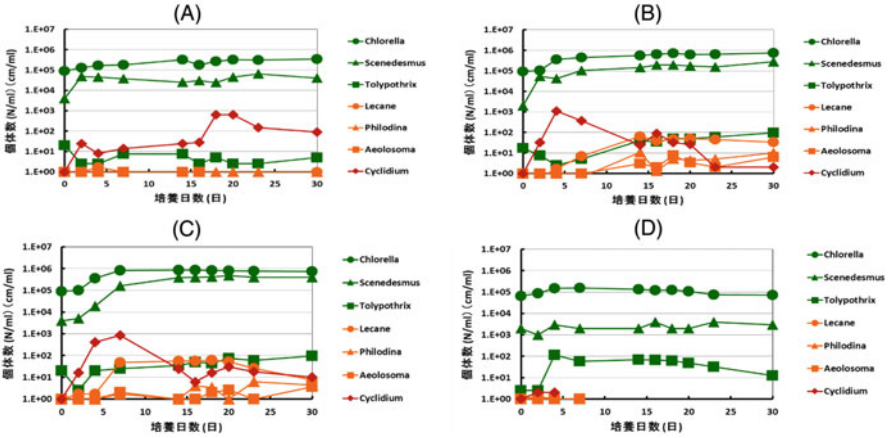

Fig. 7.44 Time course of population in each culture temperature. (**a**) 10 °C (low temp.). (**b**) 25 °C (normal temp.). (**c**) 30 °C (high temp.). (**d**) 40 °C (high temp)

7.15.2 Acid Rain

To assess the effect of acid rain on ecosystems, an HCl addition investigation was conducted by Dr. Sugiura. The microcosm was cultured with the addition of HCl at concentrations of 10 μmol, 20 μmol, 35 μmol, 75 μmol, 100 μmol, and 200 μmol at the start of cultivation (day 0) to investigate the effects of acid rain on ecosystems. The pH values were 5.40, 4.92, 4.45, 3.78, 3.60, and 3.15. Except for the 10–35 μmol of HCl addition systems, the time series of the P/R ratio exhibited different behaviors between the addition and control systems. Some species of microorganisms in the microcosm went extinct with 100 μmol of added HCl. The difference in the time series of the P/R ratio from the control system increased according to the increase in the amount of HCl added until the 75 μmol concentration was reached. Changes in the P/R ratio over time without the extinction of microorganisms was considered to be caused by changes in the biological interactions and balance with the addition of HCl. The fact that microorganisms in the microcosm N-system could resist changes in pH is considered to result from its biodiversity and suggests that it is useful as a model of natural ecosystems (Sugiura 1993).

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