10. Methods for Studying Lakes and Points to Note

For the recovery of a lake, research on biota and various analyses, evaluations and assessments of those results are necessary to provide fundamental data. This chapter briefly explains those methods.

10-1 Methods of Biological Study and Points to Note

10-1-1 Purposes

Certain types of microbes inhabit clean lakes, rivers and sea areas because they prefer a clean environment. On the other hand, if water becomes polluted, microbes with a preference for a polluted environment start to emerge. Species of inhabitants depend on the degree of water pollution. The relation between inhabitants and water pollution has been studied by many researchers, and concluded in the scheme of saprobic organisms that contain microbes. According to this scheme, water areas are classified into the following four saprobic levels: polysaprobic, α-mesosaprobic, β-mesosaprobic and oligosaprobic (saprobity). Also, each species of plankton has a suitable trophic level (trophity). Previous studies have found that trophic levels are linked to the frequency of plankton, and the levels are classified into eutrophic, mesotrophic and oligotrophic. A saprobic level defines the decomposition intensity of organic matter including dead organisms. A trophic level defines the intensity of the primary production of organic substances. Saprobity has been applied to the methods for assessing water pollution caused by human activities. Trophity has been used to analyze inland water in wildernesses that are free of human influences. However, considering the above definitions, both saprobity and trophity can be applied to every water area. Saprobity and trophity are symbolized as follows: os: oligosaprobic and oligotrophic, β-ms: β-mesosaprobic and mesotrophic, α-ms: α-mesosaprobic and mesotrophic, ps: polysaprobic and eutrophic. Because every species of organism including plankton requires a certain suitable water quality to grow, the study on biota is able to determine the saprobity and trophity in a water area. This approach has a great significance in helping people to understand water pollution as a current social problem, because pollution can be recognized not by difficult chemical analyses, but by friendly biological observations. Table 10-1-1 shows the types of bio-indicators.

Table 10-1-1 Characteristics of bioassey

(1) **ecological index：the one that conforms to the waste water biotic index system**

（for the overall evaluation such as water environment/the water quality）

a) On the basis of biotic characteristics, species composition, individual numbers

b) On the basis of characteristics of dominant species

c) On the basis of diversity of community

d) On the basis of the substance metabolism, nutrition characteristics, oxygen demand, etc. in one system

(2) **physio-biochemical index：the one that conforms to growth, activity, etc.**

（for the evaluation of materials in the water environment）

a) On the basis of the oxygen demand quantity of an individual creature or mixture community

b) On the basis of the reaction of the cell / organization of an individual creature

c) On the basis of the growth condition of a specific creature

d) On the basis of the change of specific bio-materials / life substance

10-1-2 Methods

Water quality analyses including biological studies usually proceed in the following steps: (1) confirming the objectives of the study, (2) brainstorming (discussion), (3) making a research plan, (4) doing preliminary research, (5) doing full research, (6) brainstorming (discussion), (7) concluding the results and (8) making reports. Researchers have to clearly understand what their goals are, and what and how far they will research. It is desirable to make a work schedule in advance.

Generally, the distribution of organisms is affected by the water flow and abiotic environment of a lake. Particularly, a horizontal distribution is easily affected by water flow, which is largely related to the shape of a lake basin, locations of inlets and outlets and the water amount. Therefore, in advance of any research, the following tasks are necessary: (1) organizing and checking the information of a targeted lake, (2) sorting out the traits of targeted biotic communities, (3) confirming study locations and study objectives and (4) observing the lake altogether and correcting the research schedule in advance. Timing for sampling depends on the objectives of the research and the targeted biotic communities. If the research is being conducted to record secular changes of a lake, sampling can be done once a year. But in that case, the sampling must be made within a specific period of a year. A season such as spring, summer, autumn and winter would typically be used to show a sampling period. However, in Japan located in the Temperate Zone, dominant species, biomass and biological activity can change drastically during a three-month period. The sampling period should be specified at least as a certain period of a month, such as the beginning, middle or end of the month. In other words, a research schedule has to be planned according to the study objectives and the life history of organisms. The average doubling time of phytoplankton in natural lakes is about 7 days. For a detailed research on annual changes relating to phytoplankton, sampling should be made every seven to ten days. For further research in a reproductive period, sampling should be made every two to three days. The sampling period and sampling interval of the biological research should not be determined automatically. For example, in research on a creature in a certain period such as a reproductive period, a hatching period and an eclosion period, the sampling period and interval should be determined by the targeted creature's biological activity. If the targeted creature has the possibility of daily rhythmic activity, the sampling time should be carefully chosen, and it is desirable to be fixed at a specific time in the day. In the research of a bacterium whose cells divide into new cells extremely rapidly compared to other organisms, sampling may have to be made every few hours and special treatments for samples are needed. In short, the sampling timing and interval are important for meeting the study objectives.

The sizes of plankton are diverse, ranging from a few microns to more than 1 mm. In other words, larger species are several thousand times the size of smaller species. In addition, a smaller species has a larger population. Therefore, there is no satisfying sampling method for capturing all these diverse sizes of plankton at once, other than the methods that collect plankton divided by their sizes. Surface water can be scooped up by a container. For sampling water at each vertical depth, conventional water samplers (Heyroth type water sampler, Kitahara type advanced water sampler, Van Dorn water sampler, Ekman water sampler, water column sampler, suction water sampler etc.) can be

used. If those water samplers are not available, researchers themselves should design a sampler. Surface water is often collected by a bucket, a plastic beaker or a sampling bottle directly. However, if the vertical temperature distribution in a surface layer is large, or a large number of phytoplankton gathers in the surface layer, direct sampling should be avoided. If the density of plankton is small, filtering by a quantitative plankton net is necessary. As for quantitative sampling, the Hensen type net, which consists of a filtering cloth with meshes measuring some 0.06-mm in diameter, is suitable. This sampling net meets the international standard No. 25 or the Japanese standard XX17. For sampling by a net at a targeted depth, the speed for pulling up the net should be about 1 m/s. At a higher speed than 1 m/s, the sample cannot be filtered sufficiently. If the speed is lower than 1 m/s, some zooplankton can escape from the net. The net can be pulled up from 1 to 2 m in eutrophic water at the maximum, and 5-10 m or more in oligotrophic water. However, different nets can become clogged in different conditions; therefore, sampling should be completed within those limits. A filtered volume of water can be calculated by multiplying the area of the net mouth by the length that the net was pulled up. Figure 10-1-1 and Figure 10-1-2 show sampling instruments.

The identification of phytoplankton and zooplankton is made by microscopic observation at a magnification from 100 to 800 times, except for the observation of the distribution pattern of striation on the cell wall of diatoms. The number of plankton can be counted by using an eyeglass with a magnification from 10 to 20 times and an object glass with a magnification from 10 to 40 times. Phytoplankton and zooplankton are counted at a magnification from 400 to 800 times and from 100 to 200 times, respectively. In addition, graduated slide glasses, cover glasses and graduated pipettes are necessary for the observation. The slide glass should be graduated every 0.5 mm. The cover glass should measure 24 mm by 36 mm, because the pipetting volume of the sample is 0.1 ml. The pipette should be accurate enough to measure 0.1 ml and should have a bore of 2 mm or more to capture larger zooplankton. Plankton counting trays are also commercialized for easy quantitative sampling. Because the amount of phytoplankton is usually shown

Fig. 10-1-1 Plankton net

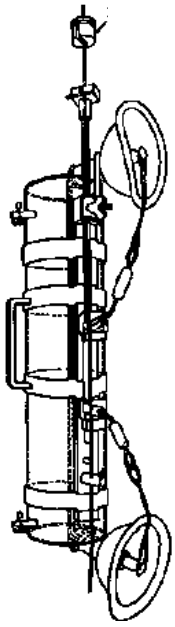

Fig. 10-1-2 Vandorn Water Sampler

by the number of cells, it should be counted by microscopic observation. In the case of phytoplankton (algae) that forms colonies, after calculating the average number of cells in a colony, the whole number of cells can be calculated by multiplying the number of colonies by the average number of cells. The amount of zooplankton is shown by the population. After counting, the amount of plankton should be converted to a population density in natural water. The population

density in natural water is calculated with the degree of the concentration of the sample and the volume of the observed sample. The amounts of phytoplankton and zooplankton are conventionally shown by the number of cells in 1 ml and the number of the population in 1 l, respectively. Figure 10-1-3 and Figure 10-1-4 show microscopes.

Fig. 10-1-3 Optical microscope

Fig. 10-1-4 Stereomicroscope

10-1-3 Points to Note

The classification and identification of plankton by microscopic observation are sometimes difficult, because they depend on morphological knowledge. Automatic identification by illustrations or photos of organisms should be avoided. It is important to first determine whether an object is a living organism or a nonliving object. Fibers of plants can be confused with fungi or cyanobacteria. Daily practices of microscopic observation from 1 to 2 hours are required to get accustomed to microorganisms and to obtain accurate results. Because there are quite various species of plankton, it is desirable to keep picture books and specialized books at hand. The identification of species is a fundamental task for both qualitative and quantitative analyses. Some species of zooplankton that move actively are particularly difficult to identify. Because the fixation of plankton causes the destruction or contraction of cells, physical controlling agents or anesthetics are usually used. 1% gum arabic solution or 10% methyl cellulose solution can be used as a physical controlling agent. These agents can significantly lower the swimming ability of plankton. However, it should be remembered that these agents also largely contort and change the shapes of plankton from their original swimming shapes in water. The moment just before water on a slide glass dries up is the best time for the observation of plankton in a natural condition. The more water on the slide glass is evaporated, the less actively plankton moves, and accurate observation of the plankton becomes possible. Photographs can capture every cell

organ clearly in such conditions. However, plankton is destructed when the water dries up completely.

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10-2 Analysis Method of Nitrogen and Phosphorus, and Points to Note

10-2-1 Purposes

Nitrogen and phosphorus are essential for microbes to propagate. On the other hand, these substances are related to the eutrophication of an enclosed water area and the biological treatment of wastewater. And those substances have significant effects on water organic pollution and the biological decomposition of organic matter. In a water area where there is a habitation of plants, which photosynthesize with photosynthetic pigments, i.e. aquatic plants and phytoplankton, the condition can be classified into eutrophic, mesotrophic or oligotrophic according to the concentrations of nitrogen and phosphorus. In ordinal lakes containing abundant minerals, the amount of nitrogen and phosphorus is usually insufficient and the propagation of aquatic plants and phytoplankton is restricted. Therefore, the concentrations of nitrogen and phosphorus compounds are usually used to classify the nutrient condition of a lake. Although a lot of classification methods are suggested, conditions are generally distinguished by the following criteria: for the concentration of total nitrogen (T-N), 0.2 mg/l or more is classified as eutrophic, 0.03 to 0.2 mg/l as mesotrophic and 0.03 mg/l or less as oligotrophic, and for the concentration of total phosphorus (T-P), 0.02 mg/l or more is classified as eutrophic, 0.003 to 0.02 mg/l as mesotrophic and 0.003 mg/l or less as oligotrophic. These nutrient conditions affect the propagation of phytoplankton, and consequently, change the water quality. Therefore, the color, transparency and pH of water, the concentration of dissolved oxygen, chlorophyll a, total nitrogen and total phosphorus are usually used as criteria to determine the nutrient level of a lake.

10-2-2 Methods

The industrial wastewater testing method JIS K0102 specifies the following analytical methods. Regarding the analyses of total nitrogen (T-N), (1) the aggregate method，(2) ultraviolet spectroscopy，(3) the hydrazinium sulfate reduction method，(4) the copper-cadmium column reduction method and (5) the thermal decomposition method are available. For the ammonium ion (NH_4-N), (1) indophenol blue spectroscopy，(2) the neutralization titration method，(3) the ion electrode method and (4) ion chromatography are used. Regarding the nitrite ion (NO_2-N), (1) naphthylethylenediamine spectroscopy and (2) ion chromatography are employed. For the nitrate ion (NO_3-N), (1) reducing distillation-indophenol blue spectroscopy，(2) the reducing distillation-neutralization titration method，(3) copper-cadmium column reduction-naphthylethylenediamine spectroscopy，(4) brucine spectroscopy and (5) ion chromatography are available. For organic nitrogen, (1) indophenol blue spectroscopy and (2) the neutralization titration method are available. To determine total phosphorus (T-P), (1) the potassium peroxodisulfate decomposition method，(2) the nitric acid-perchloric acid decomposition method and (3) the nitric acid-sulfuric acid decomposition method are used. For the phosphate ion (PO_4-P), (1) molybdenum blue (ascorbic acid reduction) spectroscopy and (2) molybdenum blue (tin (II) chloride reduction) spectroscopy are used. And the JIS K0102 also specifies the determination of hydrolysable phosphorus. Although auto analyzers are not included in the official methods, these are widely used because their analyses are highly consistent with those of the official methods. The auto analyzers are effective for analyzing many samples simultaneously and for minimizing measuring errors by analysts. Figure 10-2-1 shows an auto analyzer.

The following describes manual analyses of nutritive salts. These methods are helpful for a laboratory in which auto analyzers are not available, or which has been temporarily built near a field.

Fig. 10-2-1 NP Auto Analyser

•Measurement of Total Nitrogen

This section describes ultraviolet spectrometry as a colorimetric analysis of total nitrogen. First, an alkaline solution of potassium peroxodisulfate is added to the sample and heated at ca. 120℃. In the sample, nitrogen compounds change to nitrate ions, and organic substances are decomposed. After the pH of the sample is adjusted to 2-3, the absorbance is measured at 220 nm that is absorbed by the nitrate ions. This method is applied to samples with small

volume, those containing decomposable organic substances or those that do not contain enough bromide ions or chromium to affect the measurement. 5 to 50 μg of nitrogen is measurable. The repeatability is 3 to 10% (the coefficient of variation).

〔Reagents〕 (1) distilled water, (2) hydrochloric acid (1+16), (3) hydrochloric acid (1+500), (4) a mixed solution of sodium hydrate and potassium peroxodisulfate, (5) nitrogen standard solutions (0.1 mgN/ml, 20 μgN/ml)

〔Procedures〕 (1) 50 ml of the sample is separated into an analysis bottle. (2) After 10 ml of the sodium hydrate and potassium peroxodisulfate solution is added to the sample, the sample is immediately sealed and mixed. (3) The sample is heated at about 120℃ for 30 min in an autoclave. (4) The bottle is pulled out from the autoclave and cooled at room temperature. (5) 25 ml of the supernatant of the sample is separated into a 50-ml beaker. (6) The pH of the supernatant is adjusted to 2-3 by adding 5 ml of hydrochloric acid (1+16). (7) The supernatant is separated into an absorption cell and the absorbance is measured at 220 nm. (8) For a blank test, 50 ml of water is prepared in an analysis bottle, and its absorbance is measured in the same way. This absorbance is used to correct the absorbance of the sample. (9) The concentration of total nitrogen (mgN/l) in the sample is calculated with a calibration curve.

•Measurement of Ammonium Ion

Ammonia contained in the sample water is easily affected by microbes, and often changes, while the water is stored. Therefore, samples should be tested as soon as possible after sampling, or should be treated for frozen storage. It should also be remembered that in many cases, the atmosphere in a laboratory contains ammonia vapor and the sample can be contaminated by it. Analytical methods for ammonia in lake water are the Nessler Method, the pyridine-pyrazolone method, the oxidizing method to nitrous acid, the indophenol method, etc. The Nessler Method is easy to operate, but its sensitivity is low. The pyridine-pyrazolone method is complicated, and not suitable for routine testing. On the other hand, both the oxidizing method to nitrous acid and the indophenol method are highly sensitive and accurate. This section explains the generally used indophenol method. 5 to 100 μg of ammonium ion is measurable. The repeatability is 2 to 10% (the coefficient of variation).

〔Reagents〕 (1) distilled water, (2) a solution of sodium hydrate (200 g/l), (3) a solution of sodium phenoxide, (4) a solution of sodium hypochlorite (10 g/l of available chlorine), (5) ammonium-ion standard solutions (1 mgNH^{4+}/ml, 10 μgNH^{4+}/ml)

〔Procedure〕 (1) An appropriate volume of the sample (containing 5 to 100 μg of ammonium ion) is separated into a 50-ml graduated cylinder with a stopper. The total volume of the sample is adjusted to 25 ml by adding water. (2) After 10 ml of the solution of sodium phenoxide is added to the sample, the sample is sealed and shaken. (3) After 5 ml of the sodium hypochlorite solution is added to the sample, water is also poured into the cylinder until the surface of the sample reaches the marked line of 50 ml. Then, the cylinder is sealed and shaken. (4) The sample is permitted to stand at 20 to 25℃ for some 30 min. (5) A portion of the sample is separated into an absorption cell and the absorbance is measured at around 630 nm. (6) For a blank test, 25 ml of water is prepared, and its absorbance is measured in the same way. This absorbance is used to correct the absorbance of the sample. (7) The amount of ammonium ion is derived from the calibration curve and then the concentration of ammonium ion in the sample ($mgNH_4^+$/l) is calculated.

• **Measurement of Nitrite Ion**

This section explains naphthylethylenediamine spectroscopy, which is generally used as a colorimetric analysis of nitrous acid. This method calculates the concentration of the nitrite ion from the absorbance of red azo compounds that are produced in the following steps. After the sulfanilamide (4-aminobenzenesulfonamide) is added to the sample, the diazotization of sulfanilamide occurs by the nitrite ion contained in the sample. And, by adding N-1-naphthylethylenediamine (N-1-naphthylethylenediammonium dichloride), the azo compound is produced in the sample. 0.6 to 6 μg of nitrite ion is measurable. The repeatability is 3 to 10% (the coefficient of variation).

[Reagents] (1) distilled water, (2) a solution of 4-aminobenzenesulfonamide, (3) N-1-naphthylethylenediammonium dichloride, (4) nitrite-ion standard solutions (20 μg NO_2^-/ml, 2 μg NO_2^-/ml)

[Procedure] (1) An appropriate volume of the sample (containing 0.6 to 6 μg of nitrite ion) is separated into a 10-ml graduated cylinder with a stopper. Then, the total volume of the sample in the cylinder is adjusted to 10 ml by adding water. (2) 1 ml of the solution of 4-aminobenzenesulfonamide is added to the sample. After the sample is shaken, it is permitted to stand for about 5 min. Then, 1 ml of the solution of N-1-naphthylethylenediammonium dichloride is added to the sample. After the sample is shaken, the sample is allowed to stand for some 20 min at room temperature. (3) A part of the sample is separated into an absorption cell, and then absorbance is measured at around 540 nm. (4) For a blank test, 10 ml of water is prepared, and its absorbance is measured in the same way. The absorbance of the sample is corrected by the absorbance of the blank test. (5) The amount of nitrite ion is derived from the calibration curve and then its concentration of nitrite ion in the sample ($mgNO_2^-$/l) is calculated.

• **Measurement of Nitrate Ion**

This section describes brucine spectroscopy as a colorimetric analysis of nitrate ion. The amount of nitrate ion is determined by the absorbance of yellow compounds, considered as oxidation products, which are produced by the reaction of nitrate ions and brucine in a strong acidic condition with sulfuric acid. Nitrite ion in a sample causes the positive error of measurements. To avoid the interference, 4-amino benzene sulfonate (sulfanilic acid) is added to a solution of the color-producing brucine solution to decompose nitrite ion. 5 to 10 μg of nitrate ion is measurable. The repeatability is 3 to 10% (the coefficient of variation).

[Reagents] (1) distilled water, (2) sulfuric acid (20+3), (3) a solution of brucine and 4-amino benzene sulfonate, ④ nitrate-ion standard solutions 1 mg NO_3^-/ml, 0.1 mg NO_3^-/ml)

• **Measurement of Total Phosphorus**

This section describes the procedure of the potassium peroxodisulfate decomposition method combined with molybdenum blue spectroscopy as a colorimetric analysis of total phosphorus. Potassium peroxodisulfate is added to the sample. The sample is heated at about 120℃ and organic substances in the sample are decomposed. The concentration of total phosphorus is calculated by the quantity of phosphate ion in the sample determined by molybdenum blue spectroscopy of the solution. 1.25 to 25 μg of phosphorus is measurable. The repeatability is 2 to 10% (the coefficient of variation).

[Reagents] (1) distilled water, (2) a solution of ascorbic acid, (3) a solution of potassium peroxodisulfate, (4) a

solution of ammonium molybdate, (5) a mixed solution of ammonium molybdate and ascorbic acid, (6) phosphate-ion standard solutions (50 μgP/ml, 5 μgP/ml)

[Procedure]

(1) 50 ml of the sample is separated into an analysis bottle. (2) After 10 ml of the potassium peroxodisulfate solution (40 g/l) is added to the sample, the bottle is sealed and mixed. (3) The sample is heated at about 120℃ for 30 min in an autoclave for thermal decomposition. (4) The sample is pulled out from the autoclave and is cooled at room temperature. (5) 25 ml of the supernatant is separated into a test tube with a stopper. (6) After 2 ml of the mixed solution of ammonium molybdate and ascorbic acid is added to the supernatant and shaken, the mixture is allowed to stand for about 15 min at a temperature from 20 to 40℃. (7) A portion of the solution is separated into an absorption cell, and its absorbance is measured at 880 nm. (8) For a blank test, 25 ml of water is prepared in an analysis bottle, and its absorbance is measured as above. The absorbance of the sample is corrected by the absorbance of the blank test. (9) The concentration of total phosphorus (mgP/l) is calculated with a calibration curve.

- **Measurement of Phosphate Ion**

This section explains molybdenum blue spectroscopy as a colorimetric analysis of phosphate ion. 7-ammonium 6-molybdate and tartrate antimonate (III) potassium are added to the sample. In the sample, the phosphate ion reacts with these reagents and produces heteropoly compounds. The heteropoly compounds are reduced by L (+)-ascorbic acid, and produce molybdenum blue. The quantity of phosphate ion is determined with an absorbance of the molybdenum blue. 2.5 to 75 μg of the phosphate ion is measurable. The repeatability is 2 to10% (the coefficient of variation).

[Reagents] (1) distilled water, (2) a solution of ascorbic acid (72 g/l), (3) a solution of ammonium molybdate, (4) a mixed solution of ammonium molybdate and ascorbic acid, (5) phosphate-ion standard solutions (0.1 mg PO_4^{3-}/ml, 5 μg PO_4^{3-}/ml)

[Procedure] (1) An appropriate volume of the sample containing 2.5 to 75 μg of phosphate ion is separated into a 25-ml graduated cylinder with a stopper. Then, the total volume of the sample in the cylinder is adjusted to 25 ml by adding water. (2) 2 ml of the mixed solution of ammonium molybdate and ascorbic acid is added into the cylinder. After the sample is shaken, it is permitted to stand at a temperature from 20 to 40℃ for about 15 min. (3) A portion of the sample is separated into an absorption cell, and its absorbance is measured at 880 nm. (4) For a blank test, 25 ml of water is prepared, and its absorbance is measured in the same way. The absorbance of the sample is corrected by the absorbance of the blank test. (5) The concentration of phosphate ion in the sample (mg P/l) is calculated with a calibration curve.

10-2-3 Points to Note

Organic nitrogen compounds in water are decomposed to ammonium ion (NH_4-N). It also changes to nitrite ion (NO_2-N) by nitrifying bacteria such as Nitrosomonas and Nitrobactor, then becomes nitrate ion (NO_3-N). Generally, nitrogen compounds including ammonium ion are unstable and vulnerable to microbes. Therefore, analyses of nitrogen compounds should be made as soon as possible after sampling. If samples have to be stored for quantitative

analyses, the pH of the sample is adjusted from 2 to 3 by adding 1 ml of the reagent. If the sample is stored to determine nitrite ion, the reagent is chloroform. In other cases, the reagent is hydrochloric acid or sulfuric acid. Then, the sample is stored from 0 to 10℃ in the dark. For a few days' storage, the sample can be stored without any treatment from 0 to 10℃ in the dark.

Phosphorus compounds in water exist in the forms of phosphate（inorganic phosphate）, various forms of polyphosphoric acid and phospholipid. These forms always change by the effects of microbes in water. Because these phosphorus compounds are difficult to measure separately, those compounds are divided into groups according to their characteristics and measured to evaluate the water quality. A standard method also divides these compounds into three groups such as phosphate ion（PO_4-P）, hydrolyzable phosphorus and total phosphorus（T-P）, and determines each concentration. An analysis of the filtered sample determines the dissolved phosphorus, which can be distinguished from the concentration of suspended phosphorus compounds.

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10-3 AGP Method and Points to Note

10-3-1 Purposes

The effects of toxic substances and nutritive salts in water can be evaluated by the observation of microbe propagation inoculated into the water. This evaluation is called bioassay, which includes cultural tests of microorganisms or algae. The cultural test of algae examines the nutrient level by the maximal amount of inoculated algae in the sample water, which is cultivated in the best *in vitro* condition. This method is based on Liebig's law of the minimum（Figure 10-3-1）. According to the law, if more than one essential nutrient exists, the least amount of an essential nutrient restricts organisms' propagation. For details, firstly, algae

Fig.10-3-1 Concept of Liebig's minimum-law

are cultured until the propagation reaches a stable state at a certain illumination and temperature. Next, the dry weight of the algae is measured. The measurement shows the Algal Growth Potential (AGP). This method is usually called the AGP method. In this method, a culture solution is made of lake water, river water or wastewater that flows into a targeted lake. And algae are cultivated in the culture solution. The algae's propagating characteristics are used to evaluate the AGP of the sample water from various aspects. Therefore, this method is used not only for understanding the degree of eutrophication but also to predict and control the water quality. Typical applications of this method are: (1) the assessment of the degree of eutrophication, (2) the estimation of restricting nutritive salts, (3) the evaluation of the efficiency of water treatments such as denitrification and dephosphorylation, (4) the assessment of the effect of waste water on eutrophication, (5) the estimation of available nutritive salts for algae and (6) the detection of the restricting substances of algae's propagation.

10-3-2 Methods

A conical flask or L-shape culture tube of a volume from 300 to 1000 ml is used for the culture container. A certain species of algae is inoculated into 100 to 500 ml of the sample water in the container, and cultivated until its propagation reaches maximum at a stable temperature (20 or 25℃) and a stabile illumination. The period for reaching maximum propagation is usually 1 to 3 weeks, though it depends on species and cultural conditions. The illumination is 1,000 lx for cyanobacteria, 4,000 lx for other species. Shaken culture and stationary culture are prepared for the fresh water sample and sea water sample, respectively. For the stationary culture, the culture solution should be stirred once a day because algae tend to stick and propagate on the wall of a container. Inoculated algae are usually a dominant species in the targeted water area, such as *Selenastrum capricornutum*, *Microcystis aeruginosa*, *Acenedesmus quadricauda* and *Chlorella vulgaris* in fresh water; *Skeletonema costatum*, *Thalassiosira pseudonana* and *Dunaliella tertiolecta* in sea water. These species are generally inoculated because they are easily separated and grow stably, and the culture, measurement and comparison with other water areas are not difficult. *Selenastrum capricornutum* is used as a standard worldwide because: (1) it grows in various nutrient levels form oligotrophic to eutrophic, (2) its shape is relatively stable in various environments, (3) it is not prone to condensing and (4) its culture is easy and allows stable propagation.

The population of algae is counted by microscopic observation or the absorbance at 750 nm to confirm whether the propagation of algae reaches the maximum. Afterward, the culture solution is filtered by a membrane filter, and the dry weight of the algae in the solution is measured. Using a Coulter counter is the easiest way, because it can calculate the dry weight from an average cell volume and the number of particles. If such an instrument is not available, the dry weight can be calculated from the number of cells counted by microscopic observation. Alternatively, AGP can be calculated from the amount of chlorophyll and TOC, or total organic carbon. This method also detects propagation restricting substances. According to Liebig's law of the minimum, among all the nutritive substances that algae need, algae's propagation is restricted by one in the least amount in a system. When a propagation restricting substance is added to the system and cultured, the algae continues to grow until the amount of the added substance or other existing nutritive substances becomes a restricting factor for its propagation. In other

words, if the propagation is stimulated by adding a nutritive substance, the very substance can be determined as the propagation restricting factor. The substances to add are usually 0.05 to 0.1 mg/l of phosphorus (K_2HPO_4), 1.0 to 2.0 mg/l of nitrogen ($NaNO_3$), 1.0 to 2.0 mg/l of iron ($FeCl_3$) and 1.0 to 2.0 mg/l of chelate compound ($Na_2EDTA \cdot 2H_2O$). Various vitamins can also be added. To evaluate the effect of wastewater, 1 to 20% of wastewater is added. Figure 10-3-2 shows one example of an AGP test. In this test, to detect nutritive salts that restrict algal propagation, *Selenastrum capricornutum* was inoculated into sample water collected from Lake Kasumigaura. The water had already been treated for the thermal decomposition of contents. The results show that the propagation of *Selenastrum capricornutum* in Lake Kasumigaura is restricted by the shortage of nutritive salts, mainly nitrogen. This research indicates that inflow loads into Lake Kasumigaura have to be reduced, because *Selenastrum capricornutum* increases with the concentration of nitrogen.

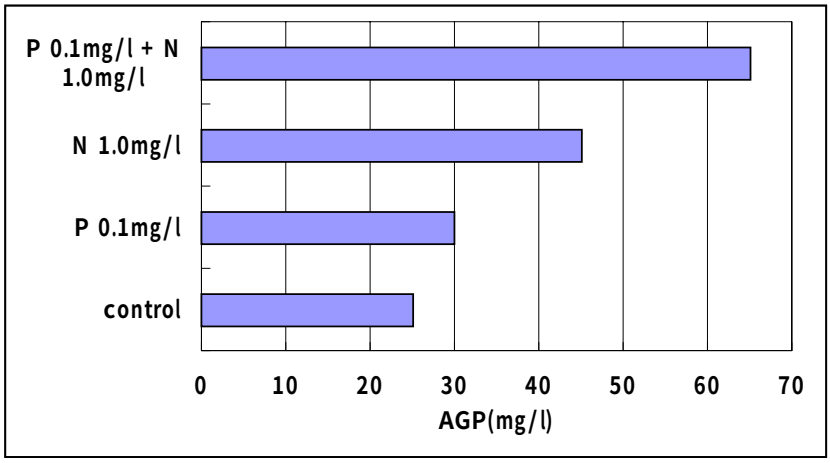

Fig. 10-3-2 Determination on limiting nutrient salt of *Selenastrum capricornutum* by AGP test

10-3-3 Points to Note

AGP is usually 1 mg/l or less, 1 to 10 mg/l and 20 to 30 mg/l or more in oligotrophic, mesotrophic and extremely eutrophic water, respectively. Of course, AGP varies depending on the species of inoculated algae, some of which do not grow in particular water. Therefore, it is also important to evaluate the water quality by the ease of propagation for a certain species. Sometimes, rather than AGP, the adaptability of various algal species should be studied by cultural tests.

As for pretreatment of the samples, samples are usually filtered or thermally decomposed, though both pretreatments are not appropriate. Because filtering removes all of the nutritive substances contained in the solid state, and nutritive substances do not elute sufficiently by the thermal decomposition. And the degree of elution is different between samples. Although the further improvement of pretreatment has been required, no better method has been developed yet. Therefore, it is desirable to test both samples, the filtered sample and the thermally decomposed sample, and to make a comparative study. As for seawater, because thermal decomposition often inhibits algae's growth, filtering is the only alternative. Regarding another pretreatment, preliminary examinations show that aerobic decomposition (aerobic treatment in the dark) from 1 to 2 months has high repeatability and a high elution rate. Although it takes a long time, this pretreatment might be suitable for the evaluation of restricting factors. Algal cultural tests are also used for the assessment of toxic substances in a sample. In this assessment, the specific growth rate of algae is measured. The cultural period is 2 to 4 days. The effect of a toxic substance is evaluated by EC50 (Median Effective

Concentration) as well as the evaluation using microzoon. In either case, the same cultural test should be made three times for one sample. To obtain statistical data, it should be made five or more times.

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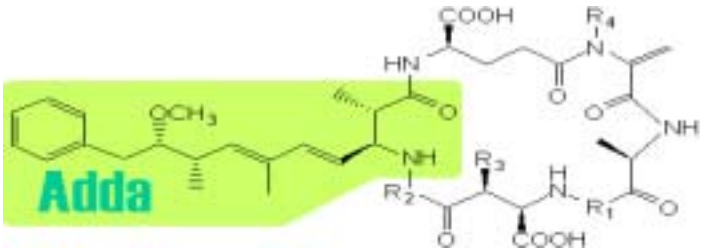
10-4 Analyses of Microcystin and Other Minor Chemicals, and Points to Note

10-4-1 Purposes

The algal-bloom phenomenon, which is an abnormal upsurge of cyanobacteria, is caused by the eutrophication of lakes that relates to the nitrogen and phosphorus in industrial, domestic and agricultural wastewater. Twelve genera of cyanobacteria have been found to produce toxins. However, the chemical structures and mechanisms for producing toxins have been found for only 8 of these genera such as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria* and *Umezokia*. Figure 10-4-1 shows the chemical structure of microcystin, and Table 10-4-1 shows its toxicological characteristics. Fresh-water lakes are used for water supplies and the water is drunk by livestock and wild animals, and is also used for fish culture. Therefore, the pollution of lakes by cyanobacteria toxins causes not only damage to the health of people and animals, but also economic losses. A toxin produced by cyanobacteria is a hepatotoxin or a neurotoxin. Microcystin, nodularin and cylindrospermopsin belong to the former, and anatoxin-a, anatoxin-as and aphantoxin belong to the latter. Among these toxic compounds, microcystin that exists worldwide attracts much attention due to its carcinogenicity and liver toxicity that is stronger than potassium cyanide. For instance, at a hospital in Brazil, a few dozens patients died from a treatment that used water containing microcystin. In view of these facts, the World Health Organization (WHO) set the standard for the concentration of microcystin in water at 0.1 μg/ml. In line with that decision, Japan also established the standard in 1999. Consequently, research on minute chemicals such as microcystin is essential for evaluating the safety of water.

Table 10-4-1 The toxicological characteristics of microcystin

	Compound	LD_{50} (μg/kg, mouse, i.p.)
L-Amino Acids (R_1, R_2)	Microcystin LR / LA	＜100
	Microcystin YR	＜100
	Microcystin RR	400～800



化合物	R_1	R_2	R_3	R_4	分子量
Microcystin LA	Leu	Ala	CH_3	CH_3	909
Microcystin LR	Leu	Arg	CH_3	CH_3	994
Microcystin YR	Tyr	Arg	CH_3	CH_3	1,044
Microcystin RR	Arg	Arg	CH_3	CH_3	1,037
3-Desmethylmicrocystin LR	Leu	Arg	H	CH_3	980
7-Desmethylmicrocystin LR	Leu	Arg	CH_3	H	980
3,7-Didesmethylmicrocystin LR	Leu	Arg	H	H	966

Fig 10-4-1　Chemical structure of microcystin

10-4-2 Methods

Because microcystin is a strong hepatotoxin, tests using mice have been conducted mainly to evaluate its toxicity. However, chemical analyses with accurate separation and reliable detection have always been required because microcystin has as many as 50 components and their toxicity is not exactly the same. Generally, analyses of organic compounds need steps such as extraction, cleaning, separation and determination. The combination of these steps should be made after each step is optimized.

The method using a high-speed liquid chromatography (HPLC, shown in Figure 10-4-2) combined with an ultraviolet detector and the bioassay using mice are established as the determination methods for microcystin. Also, extraction and refining methods for microcystin have been established. N-butanol is commonly used to extract microcystin. In some cases, 50% hydrous methanol and 5% aqueous solution of acetic acid are also used. An extracted microcystin in a solution is concentrated and dried. And the microcystin is dissolved into 5% acetic acid aqueous solution, and then absorbed by an ODS cartridge. The cartridge is cleaned by 20% methanol, and 80% methanol is used to elute the fraction of microcystin.

The fraction of microcystin is analyzed by HPLC using an ODS column or DEAE column. For the mobile phase of an ODS column, 50 mM phosphate buffer (pH3.0) / methanol (capacity ratio: 4/6) or 20 mM ammonium acetate / acetonitrile (capacity ratio: 25/75) is used. For the mobile phase of a DEAE column, 20 mM phosphate buffer (pH7.0) containing 0.3 M ammonium acetate is used. Microcystin is detected by the absorbance at 239 nm, which is absorbed by the conjugated diene in Adda (3-Amino-9-methoxy-2,6,8-trimethyl- 10-phenyldeca-4,6-dienoic Acid). In the cases of reversed phase chromatography, an elusion order of homologs of microcystin depends on the pH of the mobile phase. This is because the separation is affected by the reversed phase as well as by the cation exchange at silanol groups that remain in the silica of the ODS support. When the pH of a mobile phase is neutral, the elusion order is

microcystin-LA，-LR，-YR and -RR, though the separation of microcystin-LR and -YR is quite difficult. In the case of an acid mobile phase, retention times of all of the microcystin homologs except -RR are longer than other pH conditions.

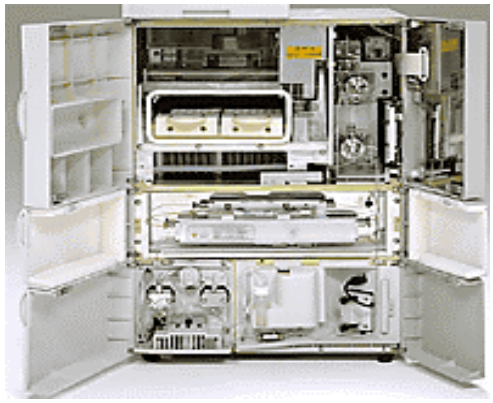


Fig. 10-4-2 High-speed liquid chromatography

Usually, microcystin is detected by UV absorption. However, because the molecular absorption coefficient of microcystin is only log4.4 at 239 nm, which shows the maximal absorption, further improvement has been required to realize a more sensitive analysis method. The HPLC/FABMS method provides high sensitivity. Also, there are some ongoing studies to develop other high-sensitive methods using the HPLC/fluorescence detector, or GC/MS. In these methods, the Adda group of microcystin is cut at the diene position by the oxidization caused by potassium permanganate/sodium periodate. Then, MMPB（2-methyl-3-methoxy-4-phenylbutylic acid） is produced. And the methyl ester of the MMPB is measured by GC/MS, or the MMPB labeled with the fluorescent pigment is measured by HPLC. Although this method is not able to detect the homologs of microcystin, it is able to determine a whole amount of microcystin in water and *Nodularia* toxins that also contain Adda. Because MPB and MMPB-d3 have been commercialized, a quantitative method using SIM（Selected Ion Monitor）with MMPB-d3 as an internal standard has been developed.

Regarding immunochemical analyses, there is ELISA (Enzyme-Linked Immuno Sorbent Assay) that uses the polyclonal antibodies and monoclonal antibodies of microcystin. Because microcystin itself does not have antigenicity due to its small molecular size, an artificial antigen has to be made. The antigen is microcystin-LR that is combined with a protein carrier such as BSA (bovine serum albumin) by using water-soluble carbodiimide. It is measured by Radio Immuno Assay (RIA), direct ELISA and indirect ELISA. For RIA, microcystin-LR labeled with tritium is reacted with antibodies. And after unreacted microcystin and microcystin-antibody compounds are separated by ammonium sulfate precipitation, the radioactivity of the supernatant or precipitated matter is measured by a scintillation counter to determine the microcystin that is combined with antibodies. By combining the microcystin with radioactive labels and the sample, it is possible to recognize the amount of microcystin in the sample.

Direct ELISA uses a plate coated with antibodies, and the microcystin labeled with horseradish peroxidase（HRP）and the sample are reacted with the antibodies on the plate. The microcystin in the sample is determined by the absorption

at 490 nm that is caused by an enzyme reaction, whose substrate is o-phenylenediamine. When microcystin exists in a sample, it causes a decrease in the amount of the HRP labeled microcystin combined with the antibodies on the plate. Therefore, microcystin in a sample is determined by the reduction of the absorbance.

Indirect ELISA uses a plate coated with the microcystin-LR combined with poly-L-lysine. Antibodies are reacted with the microcystin-LR on the plate. And the amount of the combined antibodies is determined with HPR labeled rabbit immune globulin. Analytical kits for ELISA are also commercialized. The detection limits of RIA, direct ELISA and indirect ELISA are 1.15 ng/ assay, 20 pg/ assay and 2.85 pg/ assay, respectively. Indirect ELISA is the most practical among these methods due to its simple procedure. The detection limit of commercial ELISA analytical kits is 50 pg/ml. Because these kits do not require complicated operation, many samples can be analyzed in a short time with high sensitivity. The monoclonal antibody used in these kits detects the structure of Adda that causes the toxicity of microcystin. Therefore, these kits can detect all of the toxic microcystin at once.

10-4-3 Points to Note

Sampling for the analyses of microcystin concentration should be done at some growing phases of algae, such as the period when the algae begin to emerge, one when they flourish and one after they have vanished. Although microcystin is a toxin contained by cells, it is excreted from cells as the cell bodies are decomposed. Therefore, microcystin sometimes remains in lake water after algae have vanished. Microcystin decreases after being photodegraded by sunlight, and the amount is lowered to below the detection limit within 1 to 2 weeks. Also, it is strongly biodegradable and is decomposed by bacteria and the metabolic products of microbes in water. Therefore, samples should be analyzed as soon as possible after sampling. It should also be remembered that microcystin reference standards for instrumental analyses and the reagents of commercial ELISA kits have an expiration date. They should be purchased while considering when the examination and analyses are to be made.

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10-5 Handy analyses and Points to Note

10-5-1 Purposes

There are various easy methods for analyzing water quality. Among them, described in this section is the pack test （shown in Figure 10-5-1） that is often used in environmental education classes and by nongovernmental

organizations.

10-5-2 Methods

A color-producing reagent is decompressed and sealed in a small plastic tube. The air in the tube is pushed out through a hole made by a pin, and the tube is immersed into the sample water. Then, a certain volume of the sample, which is imbibed into the tube, reacts with reagents and develops color. The degree of the color development is compared to a standard method (printed), which usually comprises 5 to 6 degrees of color development. The concentration is determined by the color of the standard method most similar to the color of the sample. Although this method provides fewer measurable objects and low sensitivity, it is helpful for field analyses to estimate approximate values, because of its simple and short time procedures that require almost no instruments. As mentioned above, this method is also suitable for the analyses of water quality by the general public who do not belong to research laboratories. The measurable objects are pH, NH_4-N, NO_2-N, NO_3-N, PO_4-P, COD, Cl^-, DO and others, though they depend on the makers. Test-paper typed analyses of the similar objects are also commercialized, and they are simpler than the pack test. Other types of commercialized handy kits, not in the form of the pack test, are available for the tests of viable cell count and coliform bacteria count. It is reported that these tests have a similar accuracy to the official methods.

Fig. 10-5-1Handy Analytical Kit (pack test)

10-5-3 Points to Note

It is necessary to check the degree of agreement between the handy tests and official tests such as the JIS method, by analyzing some sample water using both the handy and official methods at the same time. In this case, the distilled water containing only the objects to be measured is not sufficient as the sample water, but the target water containing the objects and other substances should be tested as well. As for the pack test, coexistent substances in the sample might affect the results. When special care for water sampling and analytical procedures is required as in the case of the analyses of minute toxins, the water sample, obtained from the same place and at the same time, should be analyzed by more than one person and the results of the analyses should be cross-checked. And, each analyst needs to present detailed records concerning the water sampling method, the storage conditions of reagents and chemicals and analytical procedure. Such presentation makes it possible to explain the differences found in the cross-checked results.

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10-6 Evaluation of Results and Points to Note

10-6-1 Purposes

Although the existing problems of water environment have not been sufficiently studied, various new problems continue to emerge. Consequently, further studies are necessary to promote this. Researchers should fully examine the results of field research and *in vitro* experiments, and should precisely understand what these results indicate.

Methods for evaluating ecosystem conditions in lakes are broadly divided into (1) chemical analyses such as water quality tests and (2) biological analyses including one using bio-indicators. Chemical analyses have less interpersonal errors, but the evaluation might be transient and lopsided at a specific time. On the other hand, although biological analyses require some experience, long-term environmental changes or the environmental history can be comprehensively evaluated. Chemical analyses can be quantitative, while biological analyses tend to be qualitative. There are various evaluation and analytical methods available. Statistics management methods, for instance, demand various analytical methods depending on purposes. Researchers have to choose proper evaluation methods considering their purposes. Improper methods yield only meaningless results. Researchers should evaluate their results keeping in mind what they want to study, and should not reach subjective conclusions on purpose.

10-6-2 Methods

To assess the nutrient level of a lake precisely, one ideal method is to measure as many factors as possible, and examine the many parameters that are determined by these factors. However, measuring many factors requires much effort, and the methods of compiling data also have many problems. Meanwhile, the evaluation using a single parameter might bring contradictory results. For instance, one factor indicates eutrophic and another indicates oligotrophic. The ideal indicators for evaluating nutrient levels require both the accuracy that is achieved by a composite parameter and the simplicity of single parameter. These requirements are met when a certain relation between parameters exists, and such a relation allows the creation of indicators with a single parameter. TSI（Carlson index） is a typical example of this. This index is also called the trophic state index, and can be calculated from 3 independent parameters, such as the concentration of chlorophyll a, the concentration of total phosphorus and transparency. The most appropriate index can be chosen according to the study objectives. And when all of the 3 parameters are used in measurements, it is easy to make a comparison between parameters by calculating each index. Thus, TSI facilitates the understanding of the characteristics of a lake. At the same time, it is possible to describe the state of eutrophication of a lake in detail because nutrient levels are shown by the sequent numbers from 0 to 100.

The N/P ratio is also used to evaluate a lake's condition. The N/P ratio refers to the ratio between T-N (mg/l) and T-P (mg/l) in the sample water. An N/P ratio of around 10 is unusually considered well-balanced. However, some reports

show that algae are likely to grow at an N/P ratio of about 13.5, and the ratio of somatic cell composition of sea algae is 7.2 (Redfield ratio). Therefore, a well-balanced N/P ratio might be wider than the above-mentioned figure. In ecosystems in lakes, nitrogen fixing cyanobacteria increase when the N/P ratio is low. If the N/P ratio is high, cyanobacteria and pico-plankton propagate. There has been some previous research in which cultivation was effected in lake water, where animals were removed and nitrogen and phosphorus were added, under the condition of an N/P ratio from 5 to 45. In the conditions of low N/P ratios, diatoms such as the genus *Nitzschia* and *Synedra*, and green algae such as the genus *Scenedesmus* grew dominantly, and in the conditions of high N/P ratios, cyanobacteria such as the genus *Synechococcus*, which is one species of pico-plankton, became dominant. It is also reported that a dominant species changed from nitrogen fixing *Cyanobacteria Anabaena* to the genus *Synechococcus* as the N/P ratio was artificially increased. There is also an assumption regarding Lake Kasumigaura that the increase of the N/P ratio from less than 10 to more than 20 caused the change of a dominant species from *Cyanobacteria Microcystis* to *Oscillatoria agardhii* from summer to autumn. Thus, the change of the N/P ratio causes the change of a dominant species of phytoplankton. Consequently, when an environmental condition is evaluated, it is essential to pay attention to the N/P ratio as well as the concentrations of nitrogen and phosphorus.

This section describes the evaluation of river environments from the perspective of biotic communities. Emphasis has been put on the effects of water pollution, and the environmental factors that have an effect on organisms have been evaluated by the species compositions of biotic communities. However, recently, the conservation and recovery of peculiar biotic communities and biodiversity including natural waterfronts and diverse flow paths are being considered more important than ever. Under these circumstances, the conventional methods are becoming insufficient for analyzing and evaluating river environments. And new methods are required to deal with various factors that have an effect on biotic communities. The evaluation of species diversity uses some indexes including the Shannon diversity index.

While conserving diverse species is a goal of environmental conservation and recovery, species diversity is also considered to show the degree of environmental stress on a biotic community. When the stress is large, the diversity becomes smaller, and a place with smaller diversity is regarded to have a problematic environment. However, there are some opinions that the species diversity index is not appropriate for environmental evaluation suggesting that it is vague that the index is meaningful. Concerning organisms in rivers, studies have been made since long ago about the change of species composition with water pollution. And many indexes showing water pollution were originated such as the Bech index. In these methods, species are divided into 2 to 5 groups according to their resistance to water pollution. Evaluation points of water quality are given to each group, and the number of species and the population of each group are counted, and then the water pollution index is calculated in an equation. Because those indexes are specialized in the evaluation of water quality, they have high correlation with various measured values of water quality. However, these indexes can only be used on limited occasions.

Multivariate analyses are expected to deal with various environmental factors and unlimited organisms. Multivariate

analyses are a generic name for the methods of compiling data that are composed of many variables, aiming to extract patterns or structures behind the data. These methods are generally used in questionnaire research, marketing research, client management and epidemiological survey. Multivariate analyses can also be applied to the data of species composition that contains many species, in other words, variables. And studies using these analytical methods have been promoted in such fields as vegetation science and synecology. There are two types of multivariate analyses: (1) classifying multivariate analyses and (2) grading multivariate analyses.

10-6-3 Points to Note

Water ecosystems in lakes and seas are composed of algae working as producers, bacteria as decomposers and animals as predators. A normal system is maintained and functioned by the harmonious coexistence of these creatures. However, water ecosystems have been destructed by the inflow of polluting substances produced by human activities. Therefore, it is necessary to maintain sound water ecosystems and coexist with them, from the perspective of the water ecosystem as well as that of the users of the system. For that purpose, the adverse effects on water ecosystems must be prevented including the increase of nitrogen concentration and the N/P ratio. And new energy-cost-effective technologies are required to remove nitrogen and phosphorus at the same time in the process of wastewater treatment.

People usually think that visually clear water is good. And the amounts of nitrogen and phosphorus are used to determine water quality that is related to the value of the Biological Oxygen Demand (BOD) and eutrophication. However, from the perspective of maintaining the lives of all living creatures in the environment including mankind, studies to examine the cytotoxicity and carcinogenicity of minute chemicals, and the endocrine disrupting chemicals in water are extremely important. These dangers cannot be estimated by visual cleanness or the BOD value; therefore, the simplistic determination of water quality is very risky. It is important to understand from which perspective the water quality evaluation is being made.

It is usually considered that unacceptable abnormal measurements are caused by mistakes. But these measurements might show the possibility of abnormal environmental conditions. Even if the abnormality cannot be explained at the time of the measurement, the explanation might become possible in future. Therefore, abnormal measurements should be recorded with notes, and should not be deleted from the record. When the mean value is calculated, it depends on the assay whether abnormal measurements are included or deleted. However, because it is not clear whether the applied assay is suitable for the tested environmental condition, both calculations including the abnormal measurements and excluding the abnormal measurements should be made, or some measurements can be selected by researchers.

Fantasy is to imagine causal relations without logic. Hypothesis is one of those causal relations that provide logic. The hypothesis for explaining a fact is not always one. A hypothesis that seems to support the results of a study might not be right. When only one hypothesis among various hypotheses is able to support the results, is the very hypothesis proved to be close to the truth.

Water ecosystems are places of recreation and relaxation for people. However, it is also humans who cause the pollution of the environment by discharging nitrogen, phosphorus and other polluting loads. This fact has to be recognized and tackled with the tasks for preserving and improving the water quality.

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