Methods for Studying Blue-green Algae in Ricefields: Distributional Ecology, Sampling Strategies, and Estimation of Abundance

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ABSTRACT

Many methods for studying blue-green algae (BGA) in wetland ricefields have been adopted, modified, designed, and tested during the last 5 yr at the International Rice Research Institute. This paper is the first of a series that summarizes these methods. The introduction reviews the agronomical role of BGA in wetland soils. The second part of the paper presents the distribution laws of BGA populations and their N_2-fixing activities, the design of sampling strategies in field studies, and the expected accuracy of measurements. The third part of the paper presents qualitative and quantitative methods for evaluating BGA in fields, soil samples, or inocula. It emphasizes on biomass estimation by harvest, indirect visual measurements, or index of abundance; and indirect counts of BGA populations by plating soil suspension-dilutions on solid media. All methods are adaptable for eukaryotic microalgae. Some are adaptable for filamentous and macrophytic algae.

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Blue-green algae (BGA) constitute the largest, most diverse, and most widely distributed group of prokaryotes that performs oxygenic photosynthesis. Several genera can fix atmospheric nitrogen (N_2), and thus contribute to maintaining the fertility of natural and cultivated ecosystems, especially wetland ricefields (Roger and Kulasooriya 1980).

Moderate but constant yields have been obtained for decades when rice was grown without N fertilizer. Soil N content generally remained stable, indicating that exported N is replaced. Biological N_2 fixation (BNF) is the major replacement mechanism (Roger and Watanabe 1986). BNF is vital to subsistence rice farming when N fertilizer is not available, and is potentially important in managing nutrients to reduce agrochemical use with modern rice technologies.

BGA are the only N_2-fixing microorganisms that generate their own photosynthate from CO_2 and water. This trophic independence has led many researchers to investigate their agronomic potential. Since De (1939) attributed the natural fertility of tropical ricefields to BGA, hundreds of papers have been published on their use as biofertilizer (Roger and Kulasooriya 1980, Roger 1991). Test tube-grown BGA have been extensively studied. Taxonomy, morphology, micromorphology, physiology, enzymology, and genetics are well documented. On the other hand, BGA ecology is imperfectly understood, and most agronomic studies are restricted to comparing rice yield in inoculated and noninoculated plots. This might have led to erroneous estimates of BGA inoculation potential. Roger (1991) concluded that:

■ BGA are ubiquitous in ricefields,
■ BGA may yield about 20-30 kg N/ha per crop,
■ factors that limit BGA growth (P deficiency, grazing, N fertilizer inhibition) usually hamper indigenous strains and inhibit the establishment of inoculated nonindigenous strains,
■ foreign strains inoculated in wetland soils rarely become established, and
■ agricultural practices encouraging BGA growth should focus on alleviating limiting factors and enhancing the growth of indigenous strains rather than on inoculating foreign strains.

"Super N_2-fixing strains" and N-excreting strains have been identified. Biotechnology offers new tools to select or create strains with features favorable for agronomic use, but virtually nothing is known about the environmental conditions and inoculum attributes that permit introduced strains to colonize new environments.

The major research objectives for BGA in ricefields are understanding their ecology; and studying the fate of experimentally inoculated strains and quantifying their populations, biomasses, and activities to explain the mechanisms involved in yield increase.

Field methods include the following:
■ qualitative and quantitative evaluations of BGA in a field, soil sample, or inoculum;
■ evaluations of N_2 fixation and photosynthesis by BGA in situ; and
■ establishment of adequately designed field studies that employ strategies and methods of sampling that consider BGA distributional ecology and ecosystem characteristics.

This paper is the first of a series to present methods that have been adopted, modified, designed, or tested at the International Rice Research Institute.

We provide the reader with a survey of possible methods and with information on the factors that affect the measurements. We point out the limitations of these methods to help researchers avoid their misuse and adapt the methods to specific situations or requirements. Experiments are reported and summarized in the text.

The second part of the paper considers the distribution laws of BGA populations and their activities in wetland soils, the implications for designing sampling strategies in field studies, and the accuracy of data in relation to sampling methods and density.

The third part of the paper deals with methods for qualitative and quantitative evaluations of BGA in fields, soil samples, or inocula.

DISTRIBUTIONAL ECOLOGY OF BLUE-GREEN ALGAE AND SAMPLING STRATEGIES

The validity and accuracy of quantitative measurements of BGA abundance and activities in situ depend on how representative the sample(s) are of the whole population. This depends on the heterogeneity of the organisms' distribution and the method and density of sampling or subsampling. Therefore, before designing a sampling method, the distribution law of the organisms or the activity in the type of environment to be studied must be characterized. This should determine the sampling strategy and the density of sampling needed to obtain a given representativeness of the samples.

To characterize the BGA distribution law, this section uses sets of quantitative data that include BGA counts, bio-

Table 1. Statistical symbols and abbreviations.

m   :   arithmetic mean of n values of a variable (x)
m_a :   arithmetic mean of n_a values of a variable (x_a)
n   :   number of data (n_a : number of data for the variable x_a)
s^2 :   estimated variance of the mean (m) of n values of (x)
        when needed we use
        s_x^2 : estimated variance of the mean (m) of n values of (x)
        s_y^2 : estimated variance of the mean of n transformed values (y)
        of (x) with y = f(x) (most often y = $\log_{10}$ (x + 1))
s   :   estimated standard error of the mean (m) of n values of (x)
        similarly we use, when needed, s_x and s_y
t   :   Student-Fischer t variable
CV: coefficient of variation (%) = 100 s/m
CI :   confidence interval is expressed as

$$m \pm \frac{ts_x}{\sqrt{n}}$$

L_u and L_l: upper and lower limits of the confidence interval
P   :   accuracy = CI/m
P_e :   accuracy defined as half of the difference between the upper
        and lower confidence limits,

$$P_e = \frac{1}{2} \frac{L_u - L_l}{m}$$

p   :   probability level of statistical tests

mass measurements, and N_2-fixing activity estimates by acetylene-reducing activity (ARA). Methods used for BGA counts are described on pages 12-17; methods for biomass measurements are described on pages 10-12. The ARA method is described and discussed in depth in the second paper of this series. Acetylene is a competitive inhibitor for nitrogenase, which transforms acetylene into ethylene. Estimates of N_2 fixation may be derived from measurements of ethylene produced when incubating N_2-fixing organisms in an enclosure with about 10% acetylene added to the atmosphere.

Table 1 lists the statistical symbols and abbreviations used in this paper.

Distributional Ecology of Populations and their N_2-fixing Activity

The classical method of determining the distribution law of a variable is to establish a histogram of replicated measurements. In biological sciences, this method usually requires many simultaneous measurements, which is rarely compatible with the techniques used by soil microbiologists.

The study of the correlation between means and variances of several groups of replicated measurements performed at different times and locations using the same technique provides an indirect and more convenient method for microbiologists. When a variable is normally distributed, the means (m) and the variances (s^2) are independent. A correlation between mean and variances indicates a non-normal distribution.

The non-normal distribution of organisms in aquatic or terrestrial environments is characterized by a linear correlation between means and variances of groups of replicated measurements. Plotted on a log-log graph, the points (m; s^2) exhibit a linear arrangement. The arrangement, known as the

Taylor's power law (Taylor 1961), demonstrates that the variance (s^2) of a population is proportional to a fraction power of the arithmetic mean (m):

$$s^2 = am^b \quad \text{or} \quad \log s^2 = \log a + b \log m.$$

The intercept (a) and the slope (b) of the regression curve, on a log-log scale, are population parameters. The slope (b) is an index of the distribution law of the variable and determines the transformation that normalizes the distribution as follows:

- A slope (b) close to 1 characterizes Poisson distributions which can be normalized by transforming the data by

$$y = x^{1/2}.$$

- A slope between 1 and 2 characterizes negative binomial distributions which can be normalized by transforming the data by

$$y = x^{1-(b/2)} \quad \text{or} \quad y = \log_{10} (x + x_0)$$

where x_0 is a constant that can be determined by a graph method.

- A slope of 2 characterizes log-normal distributions (the logarithms of the values are normally distributed) which can be normalized by transforming the data by

$$y = \log_{10}x \quad (\text{or } y = \log_{10}(x+1))$$

if there are x values equal to 0).

Distributions with (m; s^2)-regression curve slopes between 1.7 and 2.4 can be considered as log-normal (Roger et al 1981). The standard deviation is close to the mean, and the CV is close to 100%.

The study of the correlation between means and variances of replicated field measurements of BGA populations (enumerations or biomass estimates) and photodependent ARA has shown that, in most cases, the variables have an aggregative distribution that approximates a log-normal pattern. This distribution is observed for single-locus samples collected in the same plot, and for single-locus and composite samples collected in replicated plots.
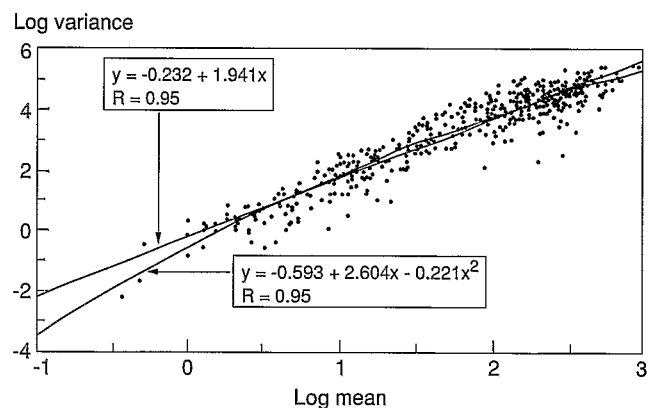
Log variance

1. Correlation between means and variances of 442 groups of acetylene-reducing activity (ARA) measurements in 5 replicated 4- × 4-m plots.

Table 2. Estimates of the standard error of the mean of the logarithms (s_y) of replicated measurements under various experimental conditions.

Variable	Groups of data (no.)	Replicates (no.)	Sub-samples[a] (no.)	Slope of the m/s² regression	S_y values			
					Min	Max	Median	SD
BGA number in 16-m² plots	36	4	10	1.9	0.04	0.90	0.16	0.22
BGA number in 16-m² plots	32	4	10	1.9	0.04	0.56	0.12	0.11
Algae number at 40 spots in a 0.25-ha ricefield	22	40	1	1.9	0.21	0.98	0.46	0.19
BGA biomass in 16-m² plots	67	4-5	16	1.5	0.06	0.89	0.26	0.17
ARA in 16-m² plots	28	10	5	2.0	0.19	1.08	0.42	0.26
ARA in 1-m² microplots	80	5	8	1.9	0.05	1.10	0.25	0.20
ARA in 16-m² plots	196	5	8	1.9	0.03	0.99	0.29	0.19
ARA in 16-m² plots	48	5	8	1.7	0.02	0.98	0.38	0.24
ARA in 16-m² plots	120	5	8	1.8	0.05	1.00	0.44	0.23
ARA in 16-m² plots	24	4	16	1.9	0.09	1.33	0.43	0.26
ARA at 35 spots in a 16-m² plot	2	35	1	-	0.47	0.63	0.55	-
Total (all measurements)	635				0.02	1.33	0.32	-

[a]Number of single-location samples combined in a composite sample.

For example, Figure 1 correlates the means and variances of field measurements of the in situ N_2-fixing activity of BGA estimated by their photodependent ARA in 5 replicated 4- x 4-m plots. The slope of the linear regression is close to 2 and indicates a log-normal distribution. Measurements used composite samples of 8 soil cores, each 2 cm in diameter. Cores were incubated under fluorescent light (30 klux) for 1 h at 28-30 °C. A binomial regression did not improve the correlation coefficient (0.95), thus indicating that a linear regression is a suitable model. Similar regressions with a slope near 2 were found for enumerations of BGA and microalgae within a plot and in replicated plots (Table 2).

Some microbial populations and microbial activities, such as BNF, follow log-normal distributions in soils and plant rhizosphere (Roger et al 1977, Roger et al 1978, Loper et al 1984) whereas some microbial populations in water may follow Poisson and negative binomial distributions (El-Shaarawi et al 1981). The most common explanation of log-normal distributions is that small variations of normally distributed physicochemical factors in the environment induce exponential responses of the biological variables (Frontier 1973).

In most cases, the log-normal distribution is an acceptable model for BGA counts and for biomass and ARA measurements. Exceptions include experimental plots with a bloom and BGA counts of plowed soils. In experimental plots with a bloom, biomass measurements of replicated plots exhibit distributions with slopes of the (m.s²) regression between 1 and 2. This is because the small plot size limits algal growth, decreasing the variability among plots (Fig. 2).

Because spores are more evenly distributed, CVs lower than 100% may occur in BGA enumerations after plowing.

Sampling Strategy and Measurement Accuracy

For normal distributions, the confidence interval (CI) and parametric statistical variables (e.g., Student-Fischer's t-test) must be calculated using the logarithms of the original data.

A higher sampling density (number of subsamples per unit area) and more replicates are needed to obtain the same accuracy with log-normal distributions than with normal distributions.
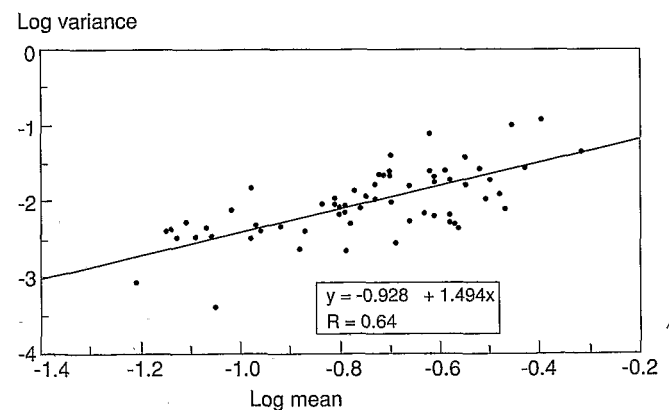
The accuracy (P) of the mean of n measurements, or the representativeness of a sample composed of n subsamples, can be defined as the fraction of the mean that is added to or subtracted from the mean to obtain its upper (L_u) and lower (L_l) confidence limits. In a normal distribution, the CI is expressed as:

$$m \pm \frac{ts_x}{\sqrt{n}} \quad L_u = m - \frac{ts_x}{\sqrt{n}} \quad L_l = m + \frac{ts_x}{\sqrt{n}}$$

where t is the Student-Fischer t-variable and s_x is the variance of n data.

The accuracy (P) is calculated as

$$P = \frac{ts_x}{\sqrt{n}} \left(\frac{1}{m}\right) \quad \text{or} \quad P = \frac{1}{2} \left(\frac{L_u - L_l}{m}\right)$$

Log variance

$$y = -0.928 + 1.494x$$
$$R = 0.64$$

Log mean

2. Correlation between means and variances of 64 groups of biomass measurements of BGA in 5 replicated 4- × 4-m plots with bloom.

For a log-normal distribution, once the CI of the logarithms of the data has been determined, it is possible to return to the original data and calculate the accuracy of a mean (or the representativeness of a composite sample). As the CI of the original data is dissymmetrical, only the equivalent accuracy (P_e)

$$P_e = \frac{1}{2}\left(\frac{L_u - L_l}{m}\right)$$

can be used (Roger and Reynaud 1978). The calculation of the accuracy (P_e) takes into account the number of replicates and the standard deviation (s_y) of the logarithms of the data.

$$P = \frac{1}{2}\left(10^{\frac{ts_y}{\sqrt{n}}} - 10^{-\frac{ts_y}{\sqrt{n}}}\right)$$

Figure 3 graphically represents this function for s_y^2 values ranging from 0.05 to 1.0. The graph can be used to forecast the accuracy of replicated measurements when n is known and s_y was estimated from previous measurements. It can also be used to determine the number of replicates needed for a given accuracy or the number of subsamples needed for a given representativeness of a composite sample.
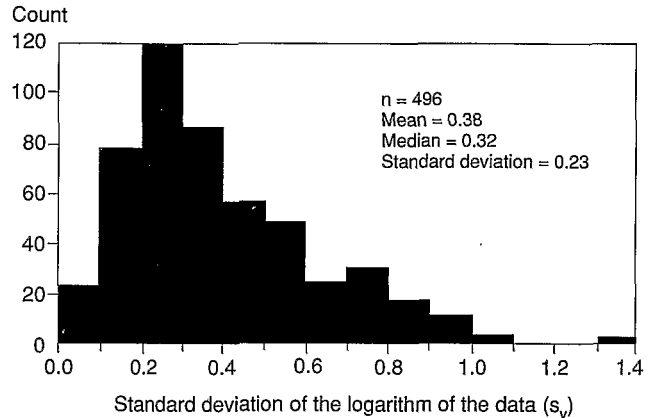
The value of s_y varies with the variable measured, the method of measurement, and the density of populations. Table 2 presents estimates of s_y values obtained under various conditions. Figure 4 is a histogram of almost 500 s_y estimates obtained from ARA measurements from 4-5 replicated plots. The dissymmetrical distribution of the s_y values indicates that the median rather than the mean should be used in general calculations involving estimates of s_y.

Number of replicated plots

Figure 3 and s_y values (Table 2 and Fig. 4) indicate that field measurements of BGA are not accurate. The number of replicates needed for 0.1 or even 0.2 accuracy is usually too high to be performed. The median of 653 s_y estimates is 0.317.

4. Histogram of 496 estimates of the standard error of the logarithms (s_y) of ARA measurements in 4 or 5 replicated plots. (s_y values are from ARA measurements in 4 or 5 replicated plots, using composite samples of 8-16 cores, 2 cm in diameter.)
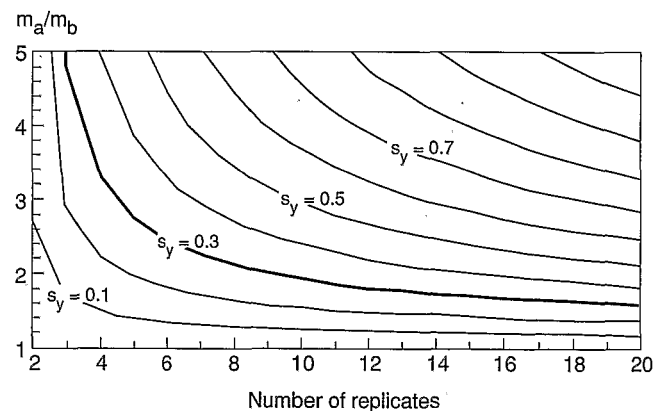
The curve for $s_y = 0.3$ in Figure 3 indicates that, on an average, measurements in 10 replicated plots provide an accuracy of 0.50 (the CI is equal to the mean).

However, in field experiments, the main concern is not the accuracy of the mean, but the number of replicates needed to establish a significant difference between two means—m_a and m_b. Assuming that $n_a = n_b$ and $s_{ya} = s_{yb}$, the ratio m_a/m_b required to establish a significant difference between m_a and m_b $(p = 0.05)$ is given by:

$$\frac{m_a}{m_b} = 10^{\sqrt{\frac{2S_y^2}{n}}\, t_{(2n-2)}}$$

where n is the number of replicates for each measurement, s_y^2 is the variance of the transformed data, and $t_{(2n-2)}$ is the Student-Fischer t-variable at (2n-2) degrees of freedom.

Figure 5 presents the calculation of m_a/m_b for 2<n<20 and 0<s_y<1. The values of m_a/m_b for the median value of s_y^2 (0.3) show that, on an average, five replicated plots will show statistically significant differences between values with a

3. Accuracy (P_e) of a mean of log-normally distributed data as a function of the number of replicates (n) and the standard error of the logarithms of the data (s_y).

5. Ratio (m_a/m_b) between two means of n replicated field ARA measurements ensuring a significant difference between m_a and m_b. (s_y = standard error of the logarithms of the data; p = 0.05.)

Count
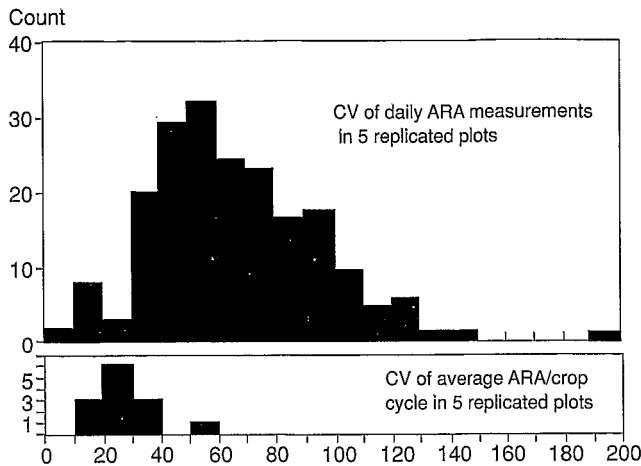
6. Histograms comparing the coefficients of variation (CVs) of average ARA values during a rice crop cycle with those of daily values.

ratio of about 3. Ten replicates permit separation of values with a ratio of 2. Field experiments on rice usually have 3-4 replicates. This is adequate for yield measurements (which are normally distributed) but may be low for BGA studies. An accuracy of 3.0 allows differentiation of 2 values with a ratio >5, and is sufficient for enumerations of BGA populations that vary from a few to more than 10^5 colony-forming units (CFU)/cm^2. The same accuracy is low for ARA measurements that vary between 50 and 800 μmol of acetylene/m^2 per h when N_2 fixation is of agronomic importance.

The variability among plots is usually high (CV = 100%) and the accuracy of the mean of interplot measurements will depend mostly on the number of replicates. However, values integrated along the whole crop cycle are usually not log-normally distributed and their variability is lower than that of daily measurements. This is because dynamics of populations or activities in replicated plots may exhibit relatively similar patterns but different durations of the lag phase. Therefore, measurements performed on the same day may vary markedly among plots, whereas integrated activity during the crop cycle is less variable. Figure 6 compares the CVs of replicated daily measurements of ARA with those of the average ARA values during the crop cycle. During the crop, 15 measurements were performed in 60 plots with 12 treatments. The average CV was 67% for daily measurements and 28% for ARA over a crop cycle.

Number of samples per plot
Figure 3 and s_y values from Table 2 show that, on an average ($s_y = 3$), 55 single measurements must be performed per plot to obtain an accuracy greater than 20% (P<0.2). This value is too large to allow routine measurement and indicates that the number of samples collected within a plot will usually be a compromise between accuracy and methodological limitations. Values in Figure 3 agree with the report by Roger and Reynaud (1978) that the mean value of *Anabaena* counts in 40

soil core samples taken at regular intervals in a 0.25-ha ricefield had a CI of +32% and -27% of the mean, or a precision of about 0.3.
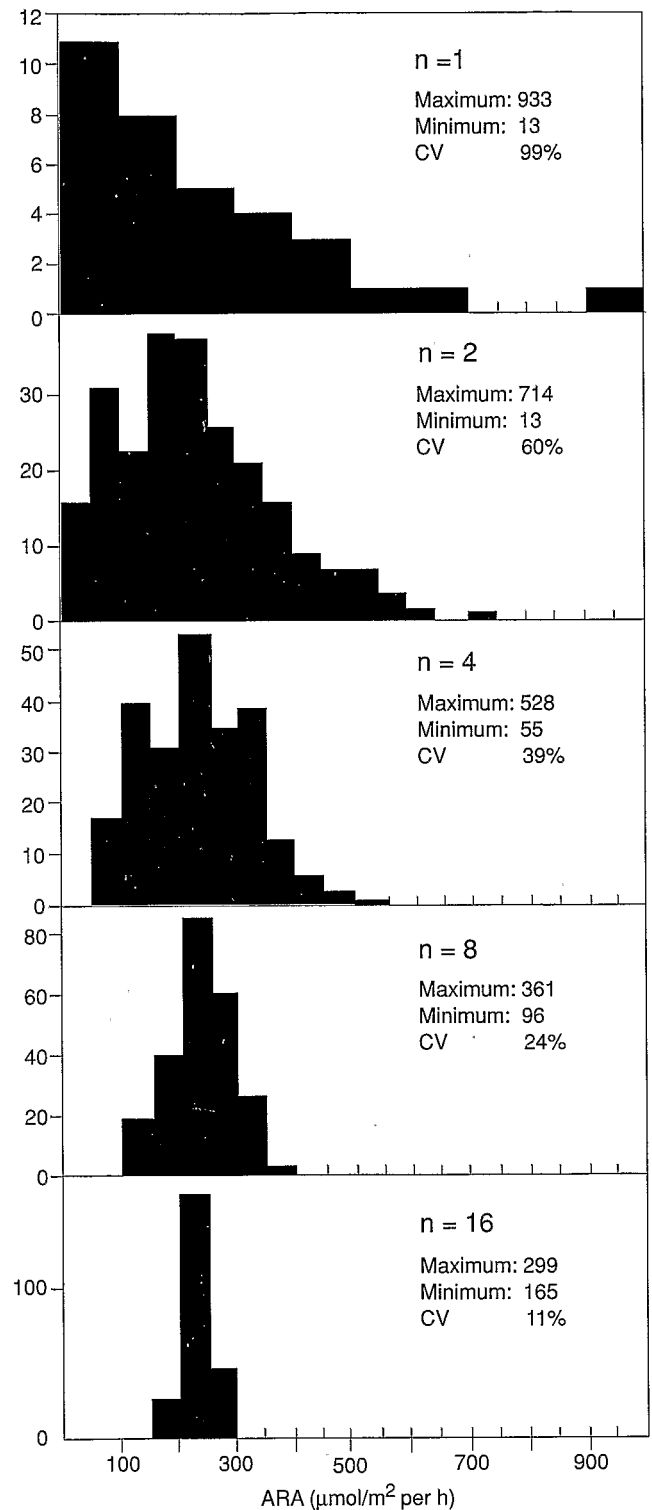
7. Histograms of 230 ARA estimates in a 4- × 4-m experimental plot calculated by random selection of 2, 4, 8, and 16 values measured on a single core. The first histogram presents the 35 measurements on a single core. CV = coefficient of variation, n = no. of replicates.

Experimental	Sampled area	Confidence interval
177 cm^2 x 16 = 2832 cm^2		+ 43.3%
		- 30.2%
(5 cm^2 x 6) x 16 = 480 cm^2		+ 46.3%
		- 31.6%

8. Comparison of single-locus and composite samplings for ARA measurements (Traore et al 1981). (Upper and lower parts of the confidence interval expressed as % of the mean of 16 values.)

The most efficient method of decreasing the measurement variability within a plot is to replace individual samples by composite samples. This changes the log-normal distribution of the data to a normal distribution if the number of subsamples is high enough (Fig. 7).

As a rule, the measurement accuracy is improved more by increasing the number of subsamples than by increasing their size. Traore et al (1981) compared the utilization of large single samples with that of small composite samples for ARA measurements (Fig. 8). Photodependent ARA of a soil colonized by BGA was measured using either 16 plastic cylinders, 15 cm in diameter or 16 glass bottles containing 6 core samples, 2.5 cm in diameter. Traore et al found that employing composite samples permitted a sixfold decrease of the total soil quantity used without decreasing the measurements' accuracy.

However, six subsamples were not enough to ensure normalization of the data. Figure 9 presents the average, maximum, minimum, standard deviation, and CV of 230 estimates of ARA in a single plot. The values were calculated by random selection of 2-20 values among 35 values measured on 35 single soil cores collected in a 16-m^2 plot. Figure 9 shows that the 100% CV observed with the log-normal distribution of single locus measurements rapidly decreases

ARA (μmol C$_2$H$_2$/m^2 per h) and CV (% x 10)

Number of core samples
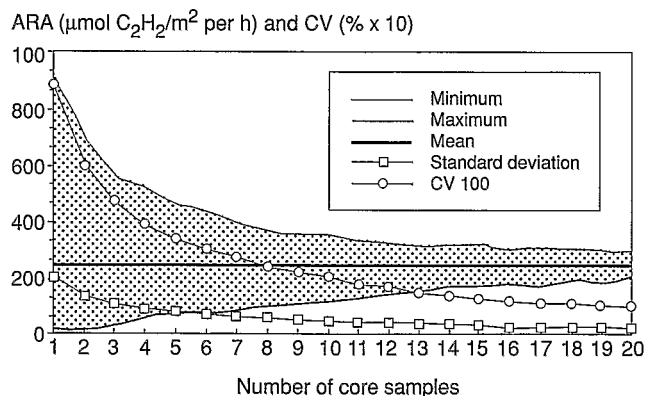
9. Mean, maxima, minima, standard deviations, and coefficients of variation of 230 estimates of ARA in a 16-m^2 plot calculated by random selection of 2-20 values among 25 single-locus measurements within the plot.

with the dissymmetry of the upper and lower values when the number of subsamples increases.

This indicates that good accuracy of replicated measurements on single-locus samples, or good representativeness of a single measurement on a composite sample, can be obtained within a plot by increasing the number of subsamples. Therefore, the accuracy of the mean of measurements in replicated plots will depend more on the number of plots than on the accuracy of individual measurements.

When the variability among plots is high, values in each plot need not be determined with high accuracy. Therefore, when determining the optimal number of subsamples to collect per plot, consider the accuracy or representativeness for the lower limit and the number of replicated plots for the upper limit. The maximum acceptable size of the composite sample and the minimum size of the subsamples also must be taken into account.

For example, consider an experimental design with 5 replicated plots per treatment, where ARA is measured on a composite sample of 8 cores, 1.7 cm in diameter. The average representativeness of composite samples is about 0.6 as determined in Figure 3 for n = 8 and s$_y$ = 0.3 (median of s$_y$ values in Figure 4). A 1.0 average accuracy can be expected for the mean of the measurements in the 5 replicated plots using the median of s$_y$ values in Table 2 (s$_y$ = 0.3, n = 5 in Figure 3). Thus, there is little advantage in collecting more subsamples per plot unless higher accuracy is needed for comparison with other intraplot measurements.

Sampling Procedures

As phototrophic organisms, ricefield BGA usually locate in floodwaters and at the soil-water interface. Spores are found from the surface through deeper soil layers.

The easiest method of gathering floodwater and soil in one sampling operation is to collect core samples that ascend to the desired depth. A small core diameter (2-3 cm) allows easy collection and permits use of many subsamples, which increases the reproducibility of the composite sample measurements. After collection, cores can be processed as a whole or separated into floodwater, surface, and deep soil.

Many authors have expressed BGA abundance in rice soils as CFU/liter of water, CFU/g of dry soil, or both. When accurate information on the sampling method is not provided, extrapolated values per field area are unreliable. Core sampling allows the data to be expressed per area (BGA or ARA/cm^2) and allows extrapolations and comparisons by field area.

While collecting cores, the operator must minimize disturbance of the sample and of the ecosystem. Muddy core floodwaters reduce light availability and affect ARA measurements. Figure 10 depicts the method for collecting core samples. Insert sampling tubes (1.7 cm diam, 10 cm long) into the soil surface by gentle alternate twisting until the bottom end of the tube is the desired distance from the soil surface. To avoid muddying the waters, the operator should stand on a

1 and 2: Push the tube down with gentle alternate twisting.
3 : Place a stopper in the top of the tube.
4: Rotate the tube to enlarge the hole.
5: Remove the tube.
6: Place a stopper in the bottom.
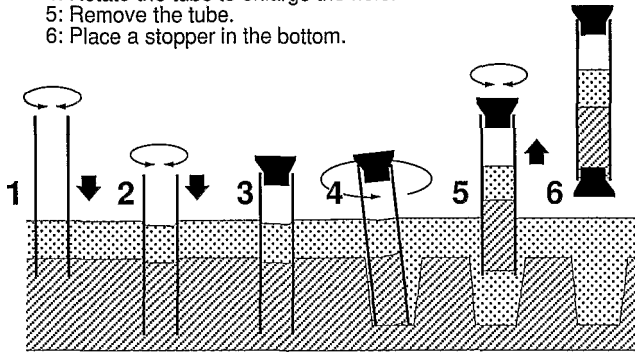
**10.** Core sampling in wetland soils.

dike or wooden board placed across the plot while inserting the tubes. To remove each tube with minimum disturbance of the ecosystem, place a rubber stopper in the top of the tube, rotate the tube to enlarge the hole in the soil, and, if necessary, support the soil with a finger while removing the tube. Place a rubber stopper in the bottom of the tube and remove the one on top.

Blue-green algae are usually irregularly distributed within a plot. Figure 11 presents common types of BGA distribution. Because of the uneven distribution, random sampling is definitely the worst sampling method to use. Sampling strategy must be designed after careful examination of the plot and must be adapted to the specific situation.

When no algal growth is visible, take samples at regular intervals on a transect through the plot.

If few colonies are floating on the water surface, collect some for identification, but do not collect them in the cores when sampling for ARA measurements or counts.

When patches of algal mat float on or partly cover the water surface, visually evaluate the percentage of area covered by the mat and take the same percentage of cores within the mat.
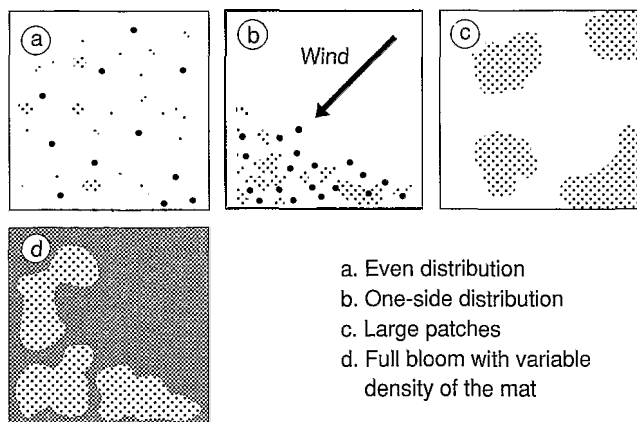
a. Even distribution
b. One-side distribution
c. Large patches
d. Full bloom with variable density of the mat

11. Major types of BGA distribution in ricefields.

Dominant directional wind pushes the floating mat in one direction and leads to a gradient of algae and algal propagules in the plot. Sample along a transect that passes through the center of the plot and is oriented toward the dominant wind direction.

Process fresh samples as soon as possible. Algal populations can change rapidly, especially if algal grazers (snails, ostracods, etc.) are collected in the tube.

In dry soils, when core sampling is usually not possible, collect subsamples by delineating areas with a core borer and removing the upper 0.5 cm layer of soil with a knife blade. Dry soil samples are usually used only for enumerating algae and the final size of the composite sample is not a limiting factor. Therefore, a larger core borer (3-5 cm diam) can be used, which facilitates sampling.

Collect samples of dry algal crusts for chemical analysis and/or counts by delineating 100-cm^2 areas and removing the crusts with a knife blade. For comparison, collect samples of the soil underneath the crust. Composite sampling is recommended because of the variability in thickness and specific composition of algal crusts in the fields.

CHARACTERIZATION AND QUANTIFICATION OF BGA POPULATIONS

Each method used to characterize and quantify algal populations in wetland soils has advantages and disadvantages. Only the most suitable methods are discussed in detail. Table 3 summarizes the methods, the environmental situations, and the variables for which they are suitable.

Qualitative Study of the BGA Flora

Many ecological studies of BGA in ricefields have been records and identifications of species (Roger and Kulasooriya 1980). The studies often employed the liquid culture method, which involves inoculating a small quantity of soil in an erlenmeyer flask. Algae are sampled and identified at intervals. Because of medium selectivity and competition between strains, this easily employed method does not ensure that all species present in the soil will grow in the flask, resulting in underestimation of BGA occurrence and diversity. Early studies reported few N_2-fixing BGA in ricefields because their findings were derived through the liquid culture method (Watanabe 1959; Watanabe and Yamamoto 1971; Venkataraman 1975).

Quantitative studies during the last decade showed that soils under rice cultivation consistently contain N_2-fixing BGA, frequently at high densities (Roger et al 1987). Plating soil suspension-dilutions on agar medium (see p. 13) permits a more complete record of strains present in a soil and allows easier isolation and identification than does the liquid culture method. It also allows quantitative estimation of the populations. We do not recommend the liquid culture method, or qualitative studies in general, because more useful information can be obtained from quantitative studies with little additional work.

Table 3. Comparison of the suitability of the different methods for characterizing/quantifying populations of BGA.

Method	Record of species		Biomass estimation	
	Qualitative	Quantitative	Relative	Absolute
Liquid culture (page 9)	NR Underestimates no. of species. Provides limited information	NA	NA	NA
Biomass harvest (page 10)	R* Microscopic observations on harvested material	NA	R* For moderate to dense algal growth	R* For moderate to dense algal growth
Square counts (page 11)	NA	NA	R* For sparse algal growth	R* For sparse algal growth. Requires standardization
Index of abundance (page 11)	NA	NA	R	R Requires standardization
Pigment measurement (page 12)	NA	NA	NR Index of abundance or O_2 provides same information with less work	NA
Direct counts (page 12)	NR Does not allow strain isolation	NR Time-consuming	NR	NR Requires standardizastion
MPN (page 13)	NR Only the most abundant species is recorded	NR Underestimates algal populations	NR Underestimates algal populations	NR
Plating (page 13)	R Allows isolation of major strain	R Allows isolation of major strain	NR Other methods provide same information with less work	NR Requires standardization
Dissolved oxygen	NA	NA	R	NR Requires 24 h measurement and standardization

NR = Not recommended, R* = recommended for specific cases or with limitations, R = recommended, NA = not applicable.

BGA abundance in soil can be determined by:
- direct and indirect biomass estimates,
- measurement of pigments,
- direct observation and counts,
- indirect counts utilizing the serial inoculation of soil suspension-dilutions in tubes of media and calculation of the most probable number (MPN) from a record of the tubes showing algal growth, and
- serial plating of soil suspension-dilutions on a solidified medium in petri dishes, and counting colonies after 3-4 wk of growth.
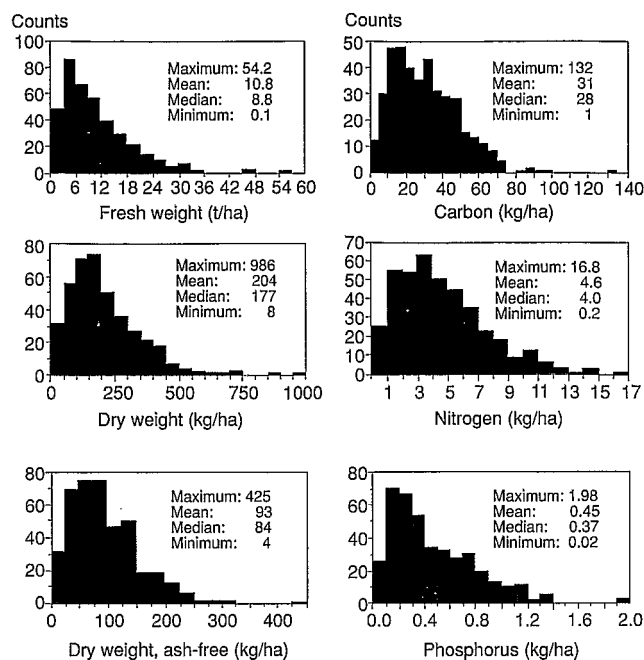
Estimation of Algal Biomass

Direct measurements

Collecting algae in a representative plot area is the easiest and most efficient way to estimate algal biomass when a bloom develops. Sampling precautions should be taken to ensure reliable measurements. A composite sample of the algal biomass should be collected when most algae are floating— avoid sampling in the early morning.

Depending on the algal distribution, collect subsamples at regularly distributed sites, or along a transect, or at chosen sites (see p. 9). In a 4-×4-m plot, we usually collect algae from 16 squares delineated by 4 rice hills. A plastic frame (i.e., a bottomless plastic tray) facilitates sample collection. To collect floating BGA, we use a metallic screen 15 cm in diameter attached to a 2-m handle. Combine and mix the 16 subsamples before measuring.

Collect epipelic algae (and macrophytic algae) by hand. The biomass of epipelic algae is often lower than that of floating algae. It is best not to try collecting epipelic algae when their biomass is low because it cannot be harvested without simultaneously collecting large quantities of soil which interfere with the measurements.

Direct measurements of field-grown BGA biomass exhibit a wide range of dry matter (0.2-14%) and ash (31-71%) content (Roger et al 1986). The nitrogen in 1 t of fresh weight (fw) of BGA averages 1.2 kg but may vary from 0.1 to 4 kg. To provide information on the agronomic significance of the biomass as fertilizer, measurements should include determi-

Counts, Fresh weight (t/ha) — Maximum: 54.2, Mean: 10.8, Median: 8.8, Minimum: 0.1

Counts, Carbon (kg/ha) — Maximum: 132, Mean: 31, Median: 28, Minimum: 1

Dry weight (kg/ha) — Maximum: 986, Mean: 204, Median: 177, Minimum: 8

Nitrogen (kg/ha) — Maximum: 16.8, Mean: 4.6, Median: 4.0, Minimum: 0.2

Dry weight, ash-free (kg/ha) — Maximum: 425, Mean: 93, Median: 84, Minimum: 4

Phosphorus (kg/ha) — Maximum: 1.98, Mean: 0.45, Median: 0.37, Minimum: 0.02

12. Distribution of 400 estimates of biomass of N_2-fixing BGA in plots where their growth was visible to the naked eye.

nation of fw, dry weight (dw), N, and possibly ash, C, and P contents. A bloom of agronomic significance has a biomass greater than 10 t fw/ha, and is visible to the naked eye. Figure 12 presents data on 400 quantitative estimates of BGA biomasses raised in experimental plots where growth was visible. Mucilaginous strains (*Nostoc*, *Gloeotrichia*, and *Aphanothece*) dominated the biomasses. An average bloom, corresponding to about 5 kg N/ha, had about 11 t fw/ha. The largest bloom recorded covered floodwater with a thick layer of large colonies and had 54 t fresh biomass/ha, corresponding to 425 kg dw of ash-free algal material, or about 17 kg N/ha.

Visual estimates

Square count method. Rice plants in transplanted plots provide a grid that can facilitate the visual record of algal growth. In 4-× 4-m plots, one may rapidly and systematically examine the floodwater surface and record the number of squares delineated by four rice hills where BGA are present. Figure 13 shows the correlation between the percentage of squares with floating mucilaginous colonies of *Nostoc* and *Aphanothece* and the BGA biomass. This method of algal biomass estimation requires case-to-case standardization because the regression between percent of squares with algae and algal biomass depends upon the type of algae and algal growth. But the square count method is less tedious and much faster than some direct measurements.

The operator's skill determines the accuracy of visual records. Coefficients of variation of counts by 4 independent untrained operators in 16 plots ranged from 10 to 50% and averaged 24%, while CVs of counts by 3 trained operators ranged from 2 to 33% and averaged 11% (Table 4).

$$y = 72.782 + 27.964x \quad R^2 = 0.714$$

BGA (kg fresh weight/ha) vs % of squares with BGA colonies
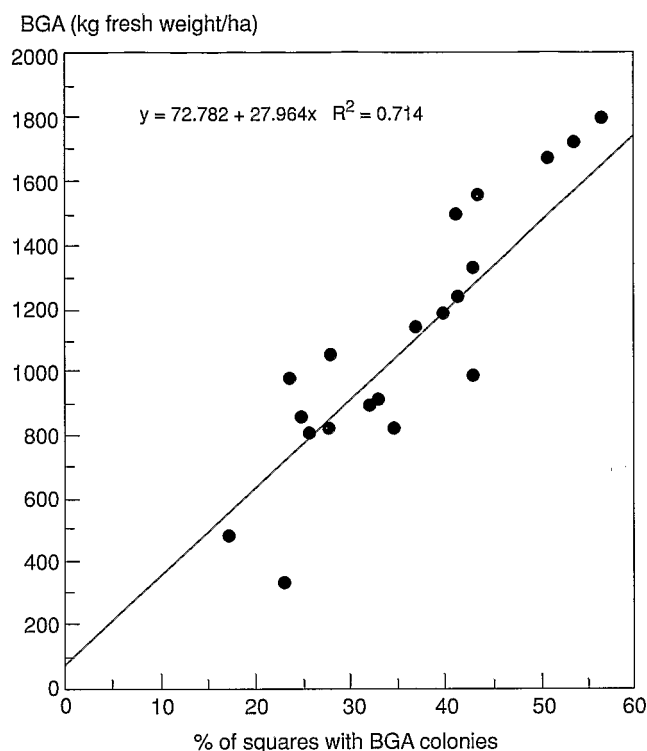
13. Correlation between algal biomass and percentage of squares delineated by four rice hills with floating BGA.

We recommend the square count method when algae are not abundant. When colonies or patches are numerous or when a bloom develops, the index of abundance is faster than the square count method.

Index of abundance. Different indices of abundance can be used, but simple scores (0-5) are subjective, provide limited information and, in particular, do not allow quantitative estimation of BGA biomass.

We use a semiquantitative index based on how much of the floodwater surface area is covered with algae (Table 5). This index allows a rough estimate of the biomass when standardized previously from harvesting algae. The record accuracy depends on the operator's skills and may vary significantly between operators, as shown in Figure 14. Coefficients of variation of estimates of BGA abundance in 65 plots by 5 independent, newly trained operators ranged from 11 to 71% and averaged 34%. In a later trial, the operators' accuracy improved to an average 23% CV (Table 4).

Table 4. Coefficients of variation (%) of visual estimates of algal abundance in 4-× 4-m plots performed by inexperienced (A) and experienced (B) operators.

	Square counts		Index of abundance	
	A	B	A	B
Number of operators	4	3	5	5
Number of plots (n)	16	16	65	65
Maximum CV for a plot	50	33	71	60
Minimum CV for a plot	10	2	11	0
Average CV	24	11	34	23

Table 5. Indices of abundance for blue-green algae (BGA) growth in wetland ricefields.

Status of BGA growth	Code	Index	Index %
No BGA visible	0	0	0
BGA present with a few colonies	+	1	5
BGA present	++	2	10
BGA present	+++	3	15
BGA present	++++	4	20
BGA covers 1/4 of the plot	1/4	5	25
BGA covers 1/3 of the plot	1/3	6	33
BGA covers 1/2 of the plot	1/2	7	50
BGA covers 2/3 of the plot	2/3	8	67
BGA covers 3/4 of the plot	3/4	9	75
BGA covers the whole plot	All	10	100

% squares with BGA (mean of 5)

15. Correlation between square counts and records of index of BGA abundance by five operators (a) and by individual operators (b and c).
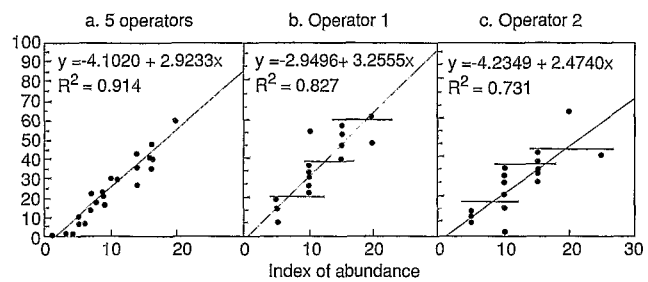
Figure 15 correlates square counts and indices of abundance in plots where algal biomass was lower than 15 t fw/ha. The average of both records by five operators correlated well (Fig. 15a). An experienced operator (Fig. 15b) satisfactorily partitioned the counts and showed little overlapping among indices, while an inexperienced operator (Fig. 15c) counted less squares with BGA and showed marked overlapping among indices.

Visual methods are less accurate than direct measurements and are difficult to use if the rice canopy is dense. However, when carefully applied, visual methods are useful to test the effects of treatments on the standing algae crop. Figure 16 exemplifies how the index of abundance may be used during a crop cycle to estimate the effect of various levels and methods of N fertilizer on N_2-fixing BGA. Because algal biomass varies widely between replicated plots, visual methods provide the same information as do direct measurements, especially during the first 30-50 d of the crop cycle, and require much less work.

Pigment measurement

Pigment analysis, especially the determination of chlorophyll *a*, is used frequently in limnological studies. Vollenweider (1969) and Parsons et al (1984) detail the methods for extracting pigment and determining chlorophyll *a* by spectrophotometry. Results from soils are affected when acetone extracts

colored organic substances such as humic acids and chlorophyll degradation products (e.g., phaeophytins and chlorophyllides). Therefore, the pigment measurement method should only be used to compare the effects of different treatments on the total algal biomass in the same soil. Because the same information can be obtained more easily by measuring dissolved oxygen, we do not recommend pigment measurement.

Estimation of Algal Abundance

Direct counts

Direct examination and counting are most frequently used in hydrobiological studies. Algae from a water sample are concentrated, fixed, counted, and identified using an inverted microscope. Direct counts have also been performed on soil suspensions. Vollenweider (1969) describes the techniques employed before the introduction of epifluorescent microscopy. Use of fluorescent microscopy and acridine orange stain facilitates identification of living algal cells or filaments (Tchan 1952, 1953; Brock 1978). Algae are recognized by the autofluorescence of their photosynthetic pigments. Acridine orange stains the cell walls and causes nuclear material to fluoresce, allowing determination of cell size. Improvements to Tchan's method include the use of gelatinized soil suspensions (diluted 10-30 times) smeared on glass slides, and use of a special haemocytometer (Drew and Anderson 1977).

BGA biomass (kg fw/ha)
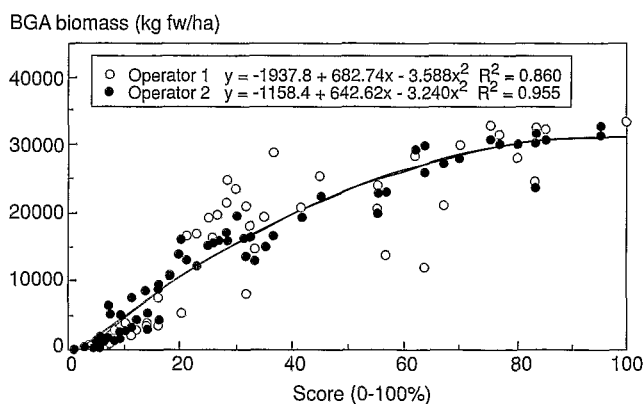
14. Correlation between direct measurements of BGA biomass in 16-m² plots and an index of abundance recorded by an inexperienced (o) and a trained (●) operator.

BGA index of abundance
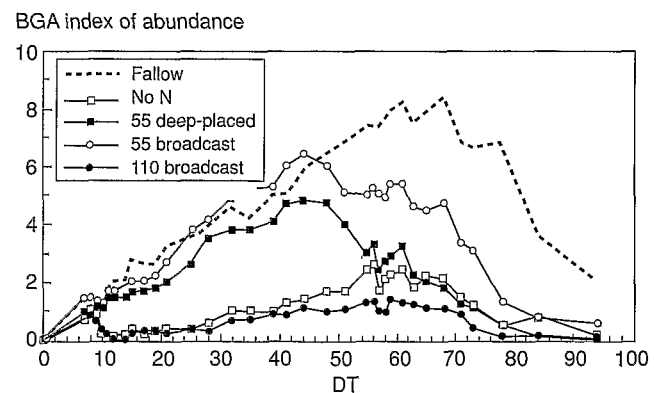
16. Effect of different levels and methods of applying N fertilizer on BGA growth as estimated by index of abundance. (0:nil, 10:full coverage of the floodwater.)

The major limitations of the direct count method are that
- it is tedious and time-consuming,
- soil particles and aggregates in the suspension hinder counting, and
- algal filaments break, making identification difficult.

The method is slow except when the algal population is dense.

At the optimum magnification (10×40) for direct algal counts with a haemocytometer (Martinez et al 1975), one field is 0.7 mm in diameter or 0.038 mm³. Assuming that:
- a 10^{-1} soil dilution is used;
- three slides per soil sample are used;
- at most, one transect of a cover slip (25 mm) is scanned for each slide, which corresponds to 35 fields;
- at least 20 algal units are counted per slide to ensure satisfactory accuracy; and
- examination time averages 10 s/field,

the minimum algal density that can be determined in 18 min $(35 \times 3 \times 10 = 1050$ s) is $60 \times 10^3 \times 10 / (35 \times 3 \times 0.038) = 1.5 \times 10^5$ CFU/cm² or 1.5×10^5 CFU/g of soil depending on the sampling method. This density is higher than the average for N_2-fixing BGA in rice soils (Roger et al 1987).

Direct counting is a long process. It is often inconvenient for strain determination, does not allow isolation of the strains, and is often inappropriate for simultaneous studies of many soil samples.

Most probable number

The most probable number (MPN) method is most frequently used to enumerate BGA in soils because it requires much less work than the plating method. However, the MPN method often underestimates algal abundance. Using both plate count and MPN methods, we compared counts of N_2-fixing BGA in soils and inocula grown on six commonly used media (Table 6). On three media, MPN counts were significantly lower than plating counts. Lower MPN values are partly due to less favorable conditions for algal growth in tubes, where CO_2 diffusion in the medium and light availability are less than those in petri dishes.

Strains growing as a mat in test tubes are difficult to identify. The MPN method provides little information on the qualitative composition of the algal flora. The species re-

Table 6. Comparison of plating and most probable methods for enumerating N_2-fixing BGA on six media.

Medium	Plating[a] (CFU[b]/g soil)	MPN[a] (no./g soil)
Allen and Arnon (1955)	2.8×10^4 a	2.2×10^4 a
Gerloff et al (1950)	2.4×10^4 a	5.4×10^3 b
Gorham et al (1964) (ASM)	2.9×10^4 a	1.6×10^4 a
Kratz and Myers (1955)	1.6×10^4 a	2.4×10^3 b
Stanier et al (1971) (BG 11)	2.5×10^4 a	5.4×10^4 a
Van Baalen (1965) (Dm)	2.3×10^4 a	2.4×10^3 b

[a]One soil sample enumerated in triplicate by plating and MPN. [b]CFU = colony-forming unit. Values in the same row or column followed by the same letter are not significantly different (p < 0.05).

corded in the last positive dilution will have the most CFU/ml. This species is often not dominant in terms of biomass, but is a small-celled species, such as a unicellular BGA or *Pseudanabaena*.

Therefore, we do not recommend the MPN method for algal count in soils.

Plating method

Plating is the most useful method for counting algae in submerged and dry soils. Roger and Reynaud (1978) and Reynaud and Laloe (1985) discuss its use in ricefields.

Description. We have developed the following standardized procedure:

1. Collect a composite sample of surface soil with the corresponding floodwater if the soil is submerged.

The composite sample is composed of at least 10 core subsamples (each 1.7 cm diam), including the top 0.5 cm of soil and, if the soil is submerged, the corresponding floodwater. Dry samples are ground to less than 1 mm.

2. Prepare a 10^{-1} dilution based on surface area.

Use core samples to prepare the suspension dilutions. Adjust the volume of the first soil suspension-dilution with distilled water to a value ten times the cm² value of the total surface area of the collected core samples. This provides a 10^{-1} dilution per surface area. For example, if 10 core samples 2 cm in diameter are collected, the total area sampled is $1 \times 1 \times 3.14 \times 10 = 31.4$ cm². The first dilution is adjusted to a total volume of 314 ml. One ml of this 10^{-1} suspension-dilution is equivalent to 10^{-1} cm² of soil.

When sampling on dw (for dry soils, soil-based inocula, etc.), prepare the 10^{-1} dilution by suspending 10 g soil in 90 ml distilled water.

Stir the first dilution at 400 rpm for 30 min to disrupt algal clumps. Dilute serially. Stir subsequent dilutions (10^{-2} to 10^{-6}) for 1 min before subsampling. Plate dilutions from 10^{-2} to 10^{-6} using three replicates/dilution.

3. Prepare and plate subsequent dilutions (10^{-2} to 10^{-6}) on petri dishes.

The total algal flora is evaluated on 1% solidified BG-11$_0$ medium (Stanier et al 1971) containing mineral nitrogen. Do not use agar concentrations higher than 1% as they inhibit growth of some BGA, especially the unicellular N_2-fixing BGA (Van Baalen 1965). Use the same medium, depleted of $NaNO_3$, to enumerate N_2-fixing BGA. Dry plates for 2 d before plating and then use, or store in sealed plastic bags.

4. Incubate for 3 wk under standardized conditions.

Incubate petri dishes at laboratory temperature (22-30 °C). Allen and Stanier (1968) suggested cultivation at 35 °C for selective BGA enrichment, but Rippka et al (1981) showed that the optimum growth temperature for some BGA is below 30 °C. Incubate under continuous light (about 800 lx) from cold white fluorescent lamps. To save shelf space, pile the three replicates of the same dilution in an inverted position 2 d after plating, and interchange them every 2-3 d to ensure similar illumination of the replicates. Counts may be per-

formed after 2 wk incubation, however 3 wk are needed to deplete N traces from the so-called "nitrogen-free" medium, and to bleach non-N_2-fixing strains. This facilitates counting of N_2-fixing BGA.
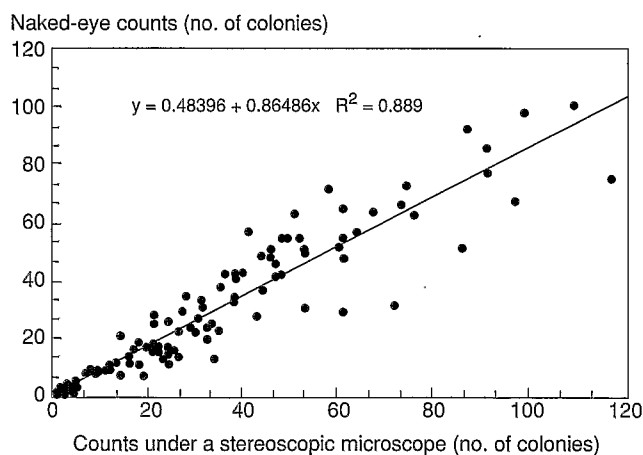
    5. Count under a stereoscopic microscope and identify under an optical microscope.

Observations and counts are usually done at two consecutive dilutions—10^{-3} and 10^{-4}, or 10^{-4} and 10^{-5}. At the higher dilution, colonies are less numerous and can be easily observed and picked up for identification with an ordinary microscope. The lower dilution is primarily used to obtain quantitative data. Place the petri dish on a transparent square screen with 1-cm mesh and count under a stereoscopic microscope. An operator who is familiar with major colony types can count with the naked eye, especially if colonies are large and well-individualized. However, counts taken with the naked eye tend to underestimate values by omitting small colonies. Counts under the stereomicroscope may overestimate values if some colonies are counted twice, or may underestimate the number of large and diffuse colonies. Thus, counts using the two methods may vary (Fig. 17).

    6. Interpret and express results.

Because of competition on petri plates, counts at two consecutive dilutions have a ratio of about 6 rather than the expected ratio of 10 (see next section). To avoid a marked effect of competition, we usually use data from dishes with 3-30 colonies. The value 30 allows significant colony counts but is low enough to avoid marked competition. This number may be increased when colony sizes are small, or decreased when colony sizes are large.

Depending on whether core or bulk sampling methods are used, counts are expressed as CFU/cm^2 or CFU/g dw of soil. Expressing the results per area is preferable to results per dw of soil, as the former permits extrapolations per hectare. Data in CFU/g dw of soil do not permit extrapolations to area due to the vertical variation of BGA propagule density in the soil. However, when extrapolations must be done from surface soils sampled by dw, results can be roughly converted to

Naked-eye counts (no. of colonies)

17. Comparison of naked-eye counts of BGA colonies on individual petri dishes with counts under a stereoscopic microscope.

Table 7. Correspondence between fresh weight, dry weight, soil area, and fresh soil volume in the 0-0.5 cm upper layer of 8 soils.

Soil	Fresh weight (g/cm³)	Fresh weight (g/cm²)	Dry weight (g/cm³)		Dry weight (g/cm²)	
			Air-dried	Oven-dried	Air-dried	Oven-dried
Maahas	1.55	0.77	0.74	0.71	0.37	0.36
Tiaong	1.57	0.79	0.70	0.67	0.35	0.33
Maligaya	1.79	0.89	0.87	0.84	0.43	0.42
Luisiana	1.68	0.84	0.83	0.80	0.42	0.40
Calauan	1.51	0.75	0.59	0.55	0.29	0.28
Saline alkali	1.48	0.74	0.35	0.34	0.18	0.17
Acid sulphate	1.55	0.77	0.52	0.51	0.26	0.25
Alkali	1.30	0.65	0.20	0.19	0.10	0.10
Av	1.55	0.78	0.60	0.58	0.30	0.29
SD	0.14	0.07	0.23	0.23	0.12	0.11
CI	0.12	0.06	0.20	0.19	0.10	0.09
CI (%)	7.6	7.6	32.6	32.7	32.6	32.7
CV	9.1	9.1	39.0	39.2	39.0	39.2

area by assuming that 1 cm^2 of soil is approximately equivalent to 0.3 ± 0.1 g dw, as determined from 8 soils (Table 7).

Reproducibility and accuracy. To test the reproducibility of the method (intraplot variability), we collected 30 composite samples (each consisting of 10 cores) from a 16-m^2 plot. Samples were plated and total algae and N_2-fixing BGA were counted. Total algae values ranged from 4.3×10^5 to 11.7×10^5 CFU (mean: 7.6×10^5, median: 7.3×10^5) (Fig. 18a). Values for the N_2-fixing BGA ranged from 11.3×10^3 to 45.0×10^3 (mean: 26.6×10^3, median: 27.0×10^3) (Fig. 18b).

Standard errors were 32 and 26% of the mean, respectively. This indicates that the representativeness (1.96 s expressed as a percent of the mean) of a single enumeration is about 60% and that 2 single enumerations cannot be considered significantly different if the ratio between the higher and the lower number is less than 4. Given an average value of 0.3 for s_y (Fig. 4), measurements in 5 replicated plots will show significant differences between 2 means with a ratio of 3.

The normality test had low significance (p = 0.19). However, because means and medians were nearly equal, and because of the general shape of the histograms, we assumed a normal distribution of replicated measurements on composite samples. The irregular shape of the histograms was partly due to a general bias observed with the plating method (see next section).

Plating methods are tedious, time-consuming, and inaccurate. Thus, counts are not frequently replicated and a composite sample collected from replicated plots is preferred. Our results show that, when used with care, the plating method does not require many replicates to show significant differences or changes among populations.

Factors influencing counts. The following factors may influence counts:

  ■ Medium. Using an artificial medium may result in some strain selection. We found that in terms of colonies recorded, there was no significant difference

Count
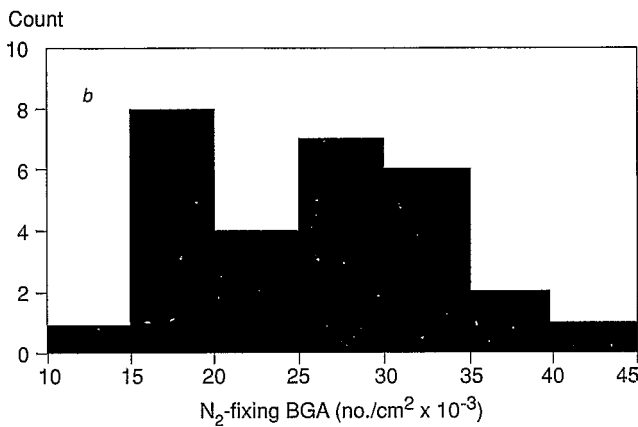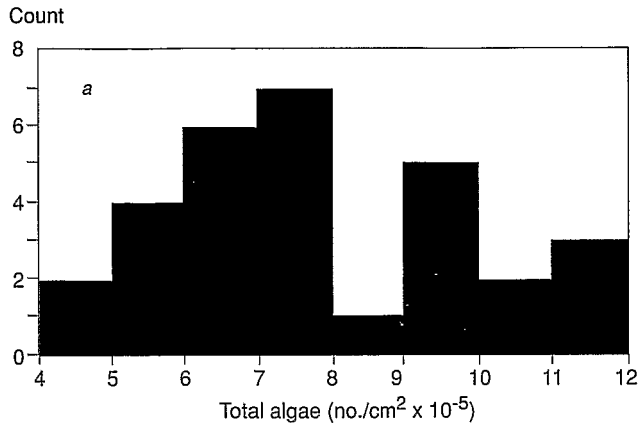
18. Histograms of 30 replicated enumerations of total algae (a) and N_2-fixing BGA (b) in a 16-m^2 experimental plot.

among five classical media (Table 6). However, colonies were larger on Allen and Arnon (1955), BG-11_0, and Dm media.

Cycloheximide, added at 20 ppm to a N-containing medium, inhibits the growth of eukaryotes but not prokaryotes, and therefore permits selective enumeration of BGA (Reynaud and Roger 1977).

Table 8. Effect of grinding and stirring the first dilution on BGA enumeration in dry soil-based inocula.

|	Level of significance[a]	
	Stirring	Grinding
Multistrain soil-based inocula		
Nostoc spp.	ns	ns
Aulosira sp.	ns	2%
Unicellular BGA	ns	ns
Total BGA	ns	ns
Monostrain soil-based inocula		
Nostoc sp. 1	ns	ns
Nostoc sp. 2	ns	5%
Anabaena sp.	ns	2%
Scytonema sp.	ns	ns
Tolypothrix sp.	ns	ns

[a]Results are the level of significance of variance analysis for 9 treatments (2 factors at 3 levels). Values are the mean of triplicate counts at two consecutive dilutions and were transformed into log for analysis. ns = not significant.
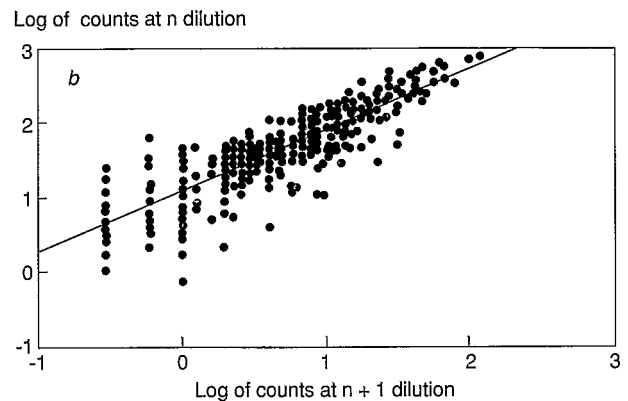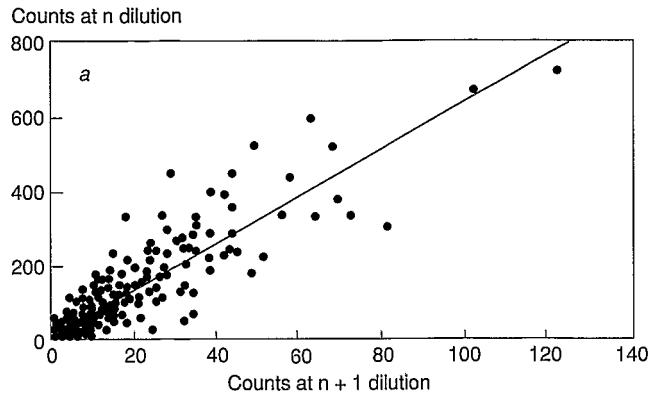

19. Correlation between counts (a) and logarithms of BGA counts (b) in 589 soil samples at two consecutive dilutions (n and n+1).

■ Grinding and stirring. Counts depend on the reliability of the dilution method. Unicellular strains that aggregate into packs (Humphries and Widjaja 1979) and filamentous forms such as *Oscillatoria* and *Lyngbia* are difficult to separate into cells. Fragmentation of moniliform filaments, which are easily separated from *Anabaena* and *Nostoc*, may give inflated counts.

We tested the effect of grinding and stirring on counts during dry sample preparation. Six soil-based inocula were ground and sieved at 2.0, 1.0, 0.4, or 0.25 mm and then stirred for 15, 30, or 60 min when
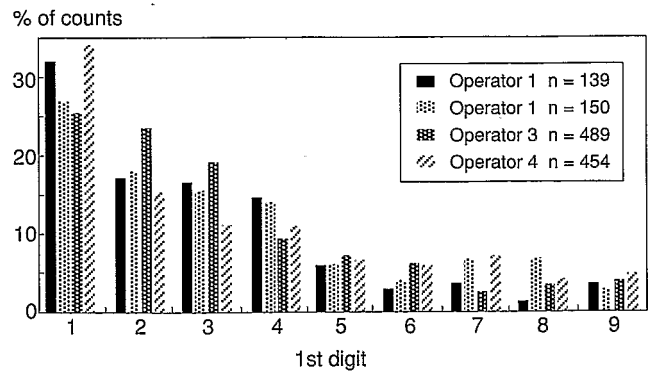
20. Four operators' relative frequencies of the first digit (1-9) of n counts of BGA in soil samples.
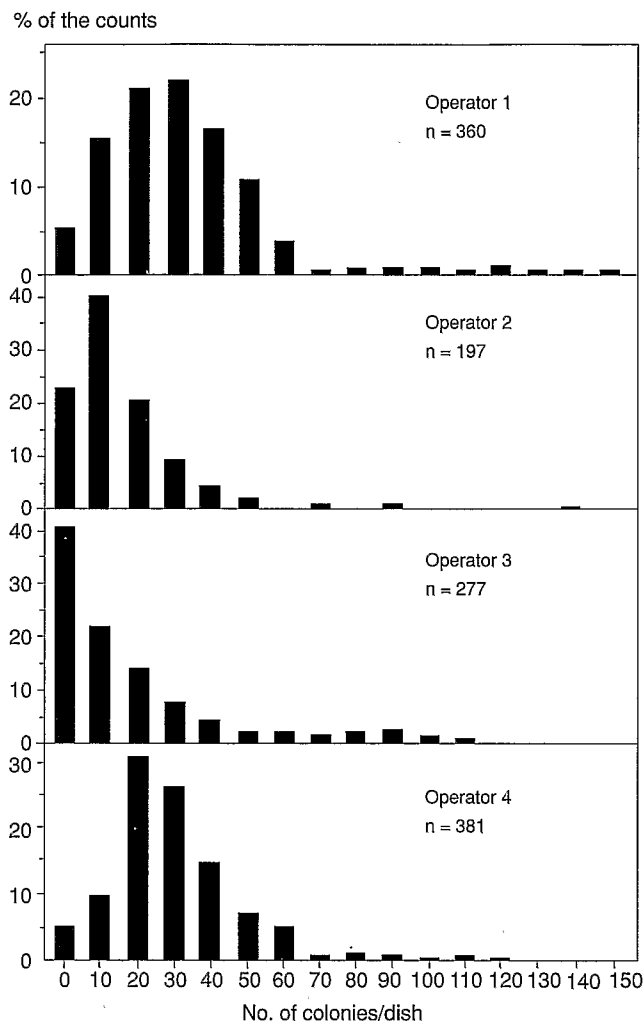
% of the counts

21. Histograms of the average number of colonies counted on triplicate petri dishes by four independent operators.

preparing the 10^{-1} dilution. Stirring for more than 15 min did not increase the count (Table 8). Grinding to a particle size less than 0.25 mm significantly decreased the count in 3 samples. Thus, drastic grinding must be avoided.

■ Competition on petri dishes. The competition among colonies on petri dishes was studied from triplicate counts at two consecutive dilutions from 589 soil samples. Figure 19 presents the linear regression for the original values and for the logarithms of counts. The ratio between counts at two consecutive dilutions, which theoretically should equal 10, is about 6. This probably results from competition among the usually large BGA colonies on petri dishes.

■ Bias in the relative frequencies of the first digit. The relative frequencies of the first digit (1-9) of large numbers of counts by four operators correlated negatively with the value of the digit (Fig. 20). Lower digits occurred much more frequently than higher ones, although an even distribution was expected. This bias

is caused by the range and distribution of the colonies counted per dish, and by the lower-than-theoretical value (10) of the ratio between counts at two consecutive dilutions.

We studied the relative distribution of the BGA colonies counted per dish by four operators (Fig. 21). They usually counted 5-50 colonies per dish, and the relative distribution of counts varied significantly with the operator. Operators usually did not count dishes with more than 50 colonies. Because dishes with more than 50 colonies are crowded and difficult to count, and because competition causes underestimation of the populations, operators counted at the next dilution. At the next dilution, plates have six times less colonies instead of the theoretical 10 times less. This replaces expected values ranging from 6 to 9.9 with values ranging from 10 to 16, leads to many data with a first digit of 1 (Fig. 22), and explains the bias observed in the distribution of the first digit.

Advantages and disadvantages. Plating soil suspension-dilutions has one major advantage—it is the only method that permits simultaneous enumeration, identification, and isolation of the algae present.

Three major limitations are as follows:

■ The plating method uses an artificial medium which may result in some strain selection and may not ensure the development of all species present. Relative growth frequencies may be altered. Gupta (1966) compared direct counts and soil culture. He pointed out that, while many species were observed both in situ and in soil cultures, some BGA (*Gloeotrichia* and *Aphanothece*) were recorded only in situ and others (e.g., *Fischerella*) only in soil cultures.

■ The method does not distinguish actively growing cells or filaments from spores or propagules dormant in the soil.
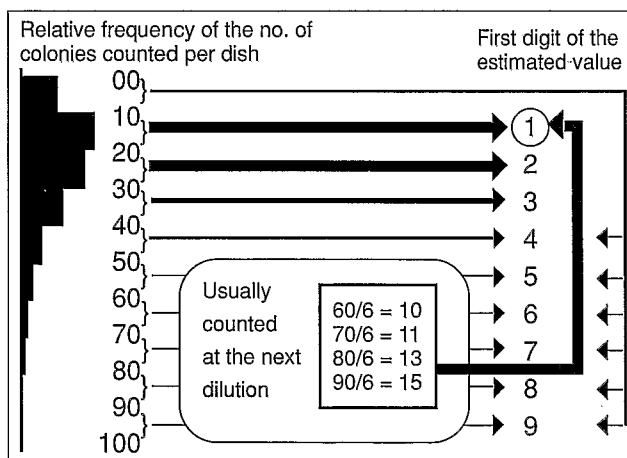
22. Schematic representation of the origin of the bias observed in the distribution of the first digit of BGA plate counts.

■ Because of competition among too many colonies, a bias is introduced in the numerical values, and strains present at densities lower than 1% of CFU are usually not recorded. Thus the method is suitable only for quantitative estimation of the major strains present.

Because of the limitations, plate count results should not be presented with decimals. Results should indicate the average colonies counted and the dilution. For example, if an average 43.7 colonies are counted at the 10^{-4} dilution, results should be presented as 44×10^4 CFU/cm^2 and not as 4.4×10^5.

Plating techniques can be improved by determining the mean volume of each "count unit" (cell, filament, or colony, depending on species) by directly examining the first dilution and multiplying the results by the corresponding "volume unit" (Roger and Reynaud 1977). However, this indirect method lacks accuracy.

CONCLUSION

The study of the distributional ecology of BGA in wetland soils shows that their populations, biomass, and N$_2$-fixing activity usually have an aggregative distribution that approximates a log-normal pattern. This distribution is observed for single-locus samples collected in the same plot and for single-locus and composite samples collected in replicated plots.

Because of the log-normal distribution of BGA,

■ the CI and parametric statistical variables (e.g., Student-Fischer t-variable) must be calculated using the logarithms of the original data, and

■ a higher sampling density and more replicates are needed to obtain a given accuracy than with normal distributions.

The composite sample method markedly decreases measurement variability within a plot and increases reliability of data. However, variability among plots is unlikely to decrease. Therefore, the accuracy of the mean of measurements in replicated plots depends more on the number of plots than on the accuracy of individual measurements. The analysis of large sets of field measurements shows that the number of replicated plots needed for a highly accurate measurement (i.e., a CI less than 20% of the mean) cannot be performed reasonably in a field experiment. Usually, measurements in 10 replicated plots provide 0.50 accuracy for which the CI is equal to the mean. Normally, statistically significant differences between values with a ratio of about 3 can be established from 5 replicated plots. Ten replicates separate values with a ratio of 2. Most field experiments on rice have 3-4 replicates. This is adequate for yield estimates—which are normally distributed—but may be low for BGA studies.

There is no universal method for determining BGA abundance. Choice of the method(s) depends on the state of the field, the purpose of the measurement, and, in many instances, manpower availability. Table 3 compares the methods' suitability for different variables and environmental situations.

In soils with no visible algal growth, we recommend the plating method, using a composite sample collected on area basis. To compare different soils, collect only the first 0.5 cm of soil. Deeper soil samples may be collected for estimating dormant spores or propagules in the soil. The plating method can also be used in fields with visible algal growth, especially to study the fate of an inoculated strain. For a quantitative estimate of the algal biomass, direct biomass measurements combined with visual records of abundance are much more accurate, informative, and less tedious than plating.

Measuring biomass from cell number and size is time-consuming and liable to subjective errors in volume determinations. An algal biomass of agronomical significance is visible to the naked eye and, in most cases, can be estimated by direct measurements.

In flooded ricefields, when algal growth is moderate and the rice canopy is not too dense, the most convenient way to estimate BGA biomass is the square count method combined with direct measurements using a quantitative composite algae sample. When algae are abundant or a bloom develops, direct biomass measurements are best combined with the record of an index of abundance, preferably performed by several operators. These methods should be combined with the collection of a composite sample to identify dominant strains and determine fw, dw, and ash, N, C, and P contents.

Among available methods for quantitative estimation of algal populations, only plating soil suspension-dilutions permits simultaneous enumeration, identification, and isolation of most algae present. However, the method does not distinguish actively growing cells or filaments from spores or propagules dormant in the soil, and uses an artificial medium which may result in some strain selection. Because of competition among colonies on petri dishes, quantitative estimates are biased and strains present at densities lower than 1% of CFU are usually not recorded. Therefore, the plating method is suitable only for quantitative estimation of the major strains present.

Biomass harvest and visual estimate methods for determining algal abundance can also be used for eukaryotic algae. The plating method can be adapted for eukaryotic microalgae by using suitable culture media and conditions of incubation.

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