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Characterization, quantification, isolation and conservation of N_2-fixing BGA from rice soils

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Abstract

This paper summarizes results concerning 1) the development of a methodology to estimate quantitatively and qualitatively populations of BGA in rice soils, 2) the application of these method to the study of 102 samples of wetland rice soils from several rice growing countries, and 3) the conservation of strains isolated during these studies.

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1. Introduction

At IRRI, our interest in BGA is in the ability of a number of genera to fix atmospheric nitrogen, which has implications in the maintenance of the fertility of natural and cultivated ecosystems.
The goal of the BGA program at IRRI is to understand the ecology of BGA in rice fields in order to develop cultural practices maximizing their nitrogen input to the ecosystem.

This paper summarizes results concerning 1) the development of a methodology to estimate quantitatively and qualitatively populations of BGA in rice soils, 2) the application of these methods to samples of wetland rice soils from several rice growing countries, and 3) the conservation of strains isolated during these studies.

2. Development of a methodology for quantitative and qualitative characterization of N_2-fixing BGA populations in soils

With regard to the applied aspect of the program, the methods to be developed had 1) to permit an estimation of the density of N_2-fixing BGA in soils with a reasonable accuracy (at least one order of magnitude), 2) to provide enough information on the nature of the dominant strains, and 3) to allow the study of several samples simultaneously, within a reasonable period of time, particularly in field experiments where several treatments and replicates are used.

21. Characterization of the strains

211. Limitations of traditional and modern taxonomy of BGA for ecological and agronomical studies

According to its classical definition as algae, BGA have traditionally been placed under the International Code of Botanical Nomenclature. In 1977, Stanier and Cohen-Bazire, emphasized their prokaryotic nature and stated that "the only logical treatment of the cyanobacteria (BGA) was to place them in the super kingdom Prokaryotae as a division of bacteria" under the rules of the Bacteriological Code. At present there is no general

agreement for placing BGA under a given code and most probably their future taxonomic treatment will take into account botanical and bacteriological criteria.

2111. Traditional taxonomy . Traditional classifications were based almost entirely upon morphological features, namely : 1) growth form : unicellular, colonial, filamentous, 2) compactness and shape of the colonies, 3) shape of the filaments, 4) sheath : presence, absence, shape, 5) differentiation of heterocysts and akinetes, 6) size and shape of cells, 7) polarity of filaments, 8) branching and nature of true branches.

The classifications based on morphological characters frequently are of little help in identifying BGA which exhibit polymorphism in response to changes in environmental conditions and with the age.

The divergence of opinion which has resulted from the use of morphological criteria is enormous and has led to a superfluity of taxa which renders traditional taxonomy inadequate for ecological studies, the determination of a strain requiring time consuming investigations in contradictory taxonomical keys and little chance of succes. As an example more than 350 species of Anabaena have been described on morphological basis whereas morphological criteria for this genus are the size, the shape and the relative position of 3 kind of cells (vegetative cells, akinetes and heterocysts) !!

2112. New trends in BGA taxonomy. The latest trends in BGA classification are directed towards evolving a system based on morphological, dynamic, physiological, biochemical and genetic characters. Possible non-morphological characters for taxonomic treatment of BGA are : Dynamic characters, physiological properties, fatty acid composition, pigment composition, isozymes, genome size, deoxyribonucleic acid base composition, and sequence-specific deoxyribonucleases. However achievements in this field are still inadequate to evolve a new taxonomy.

212. Simplified classification of N2-fixing BGA used at IRRI

Strains are grouped into broad taxa according to morphological criteria directly observed on the material growing on Petri dishes. The ability to form mucilaginous colonies with defined shape, which is associated with resistance to grazing (Grant *et al.*,1985), is taken as a major character.

Table 1 . Definition of the taxa of N2-fixing BGA[a] and most frequent growth behavior in wetland soils

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"Unicellular" group : Unicellular strains growing on BG-11 medium without nitrogen (*Aphanothece*, *Gloeothece*, ...). In situ, forms floating mucilaginous macrocolonies.

" *Anabaena* " group : Heterocystous strains with a thin sheath, without branching, do not form mucilaginous colonies of definite shape (*Anabaena*, *Nodularia*, *Cylindrospermum*, *Anabaenopsis* etc.). In situ, forms a fragile film at the surface of the floodwater.

" *Nostoc* " group : Heterocystous strains with a thick sheath, without branching, forming mucilaginous colonies of definite shape (*Nostoc*). In situ, forms floating mucilaginous macrocolonies.

" *Aulosira* " group : Heterocystous strains with a thick sheath, usually without branching, do not form diffuse colonies on agar medium (*Aulosira*). In situ, forms a resistant papyraceous film at the surface of the floodwater.

" *Scytonema* " group : Heterocystous strains with false branching, without polarity, forming velvet like patches on agar medium (*Scytonema*). In situ, grows adpressed on soil or epiphytically. Currently we did not observe a growth visible to the naked eye in a rice field.

" *Calothrix* " group : Heterocystous strains with false branching, with polarity, forming velvet like patches on agar medium (*Calothrix*, *Tolypothrix*, *Hassalia*, ...).In situ, grows adpressed on soil or epiphytically.

" *Gloeotrichia* " group : Heterocystous strains, with polarity, forming mucilaginous colonies of definite shape (*Gloeotrichia*, *Rivularia* , ...).In situ, forms floating mucilaginous macrocolonies.

" *Fischerella* " group : Heterocystous strains with true branching (*Fischerella* , *Westiellopsis* , *Stigonema* , ...). In situ, grows adpressed on soil or epiphytically. Currently we did not observe a growth visible to the naked eye in a rice field.

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a : All features refer to strains grown from soil or water samples dilutions plated on agarized BG-11 medium without nitrogen.

Taxa with this ability are the unicellular (*Aphanothece, Gloeothece* ...), *Nostoc,* and *Gloeotrichia* groups. This grouping has no taxonomical pretension but is a convenient tool for ecological studies because it permits a rapid identification and correspond to relatively well defined growth behavior *in situ* (Table 1).

22. Method for estimating BGA abundance in rice soils

221. Soil sampling

Soil algae are characterized by a very uneven distribution of a contagious type that approximates a log-normal distribution. The number n of soil subsamples to be collected to obtain a given representativeness of a composite sample is calculated as :

$$n = \frac{4t^2 s_y^2}{\left[\log \frac{1+p_e}{1-p_e}\right]^2}$$

where t is the t value of Student Fischer, s_y^2 is the variance of the logarithm of the data (the average value of Sy determined from 32 groups of replicated measurements was about 0 .25), and Pe is the representativeness of the composite sample (a measured value of X corresponds to a range of X ± pX with $0 < p < 1$)(Roger and Reynaud, 1978).

In a 16 m^2 experimental plot we usually collect 10 core subsamples, which corresponds to an average representativeness of about 0.45. Only the first 0.5 upper centimeter of soil and, in flooded conditions, the corresponding floodwater of the core are collected.

222. Choice of a method of enumeration

2221. Methods for quantitative estimation of BGA in soils

BGA abundance in soil might be determined by 1) direct observation and count, 2) indirect methods using soil suspension-dilutions of soil inoculated in selective media.

Direct examination and counting has been often used in hydrobiological

studies where algae from a water sample are concentrated, fixed, counted and determined using an inverted microscope. In the case of soil algae, instead of concentrating the sample, a homogenized soil dilution of soil has to be used which lead to three negative features: 1) algae are diluted and the method becomes tedious and time consuming, 2) numerous soil particles in the suspension are a hindrance to counts, 3) algal filaments are more or less broken into pieces and their determination difficult. Therefore the method is less convenient and is rarely used.

Indirect methods utilize either 1) 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, or 2) serial plating of soil suspension-dilutions techniques on agarized medium in petri dishes.

The MPN method has been most frequently used for enumerating BGA in soils because it requires much less work than the plating method in both inoculation and counting. However, identification of the strains is difficult and the method provides very little information on the qualitative composition of the algal flora.

2222. Comparison MPN/Plating

Six of the most common media used for growing BGA have been compared for enumerating N2-fixing BGA in soils and inocula, using plate counts and most probable number (MPN) method in test tubes (Table 2).

Table 2 : Comparison between plating and most probable method for enumerating N2-fixing BGA on six media.

Medium	Plating[a] (CFU/g soil)	MPN[a] (CFU/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.
Values in the same row or column followed by the same letter are not significantly different ($p < 0.05$).

With three of the media, counts by the MPN method were significantly lower than those on plates, indicating that the MPN method, which has been used in many ecological studies, might lead to an underestimation of BGA populations.

2223. Choice of the medium

Blue-green algae are aerobic photolithotrophic microorganisms. The only growth factor required by BGA is vitamin B12 which is stimulatory or essential for a few species, mostly marine. Consequently a mineral medium is recommended.

Many culture media have been proposed for blue-green algae, however, BGA growth does not generally require sophisticated ones. Most of the media have the following characteristics:

o A slight alkaline reaction is obtained through K_2HPO_4 or carbonate.

o Nitrogen, if used, is in the form of Ca or $NaNO_3$.

o Fe and Mg are always present.

o The concentration of the different elements largely varies among the different media .

o A micronutrient solution is added.

At present, 5 media and 3 modifications of one of these are listed in the ATCC catalogue (1982) for the growth of BGA.

Table 3 : Enumeration of N2-fixing BGA on different media.

Medium	(% of average)
Allen and Arnon (1955)	108 ± 35 a
Gerloff et al. (1950)	107 ± 40 a
Gorham et al (1964)(ASM)	119 ± 52 a
Kratz and Myers(1955)	64 ± 32 b
Stanier et al (1971)(BG 11)	102 ± 34 a
Van Baalen (1965)(Dm)	95 ± 44 ab

average ± standard deviation of 15 counts obtained from 5 soil samples and 10 dried inocula and expressed as % of the average of the counts on the six media.
Values followed by the same letter are not significantly different ($p < 0.05$) by

Kolmogornov-Smirnov test.

Six of the most common media used for growing BGA have been compared for enumerating N2-fixing BGA in soils and inocula by plate counts. Counts were lower on Kratz and Myers medium and did not significantly differ among other five media but there were significant differences in the size of the colonies. Allen and Arnon, B-11, and Dm media permitted a faster growth of BGA (Table 3).

In our experience, the BG-11 medium (Stanier et al., 1971) and its modification BG-11o without N salt (used for N2-fixing strains) has proven to be the most useful in the enumeration, isolation and culture of BGA from rice fields. It supports moderate to excellent growth of most of the strains. Poor growth was observed only with an *Anabaena* strain isolated from a very alkaline soil (pH 9.5). BG-11o medium is also not very efficient for growing *Gloeotrichia* which morphology is markedly affected (loss of tapering and radial disposition of the filaments in a mucilaginous colony).

223. Standardization of the plating method for rice soils

2231. Description of the method

The standardized method comprises the following steps:

Collection and preparation of the sample The composite sample comprises at least 10 core subsamples (2 cm in diameter) including the top 0.5 centimeter of soil and, if the soil is submerged, the corresponding floodwater. Dry samples are ground at less than 1 mm.

Preparation of the dilutions: To estimate algal populations on area basis, the volume of the first soil suspension-dilution is adjusted with distilled water to a value equal to ten times the value in cm^2 of the surface corresponding to ten core samples. This provides a 10^{-1} dilution on surface basis. When sampling is done on a dry weight basis (for dry soils, soil based inocula..), the 10^{-1} dilution is prepared by suspending 10 g of soil in 90 ml of distilled water. The first suspension is stirred at 400 rpm for 30 min to disrupt algal clumps, and then serially diluted. Subsequent dilutions (10^{-2} to 10^{-6}) are stirred for 1 minute before subsampling.

Plating: Dilutions from 10^{-2} to 10^{-6} are plated in triplicate using three replicates per dilution. The total algal flora is evaluated on 1% agarized BG-11 medium (Stanier *et al*,1971) containing mineral N. (Agar content higher than 1% has an inhibitory effect on some BGA growth, especially unicellular fixing BGA). The same medium depleted of NaNO3 is used for enumerating N2-fixing BGA.

Incubation : Petri dishes are incubated for three weeks at laboratory temperature (22-30 ºC) under continuous light (about 800 lux) provided by cold white fluorescent lamps. The three replicates of a the same dilution are piled and permuted every 2-3 days to ensure similar illumination of the replicates. Counts could be performed after two weeks of incubation, however three weeks are necessary to completely deplete the so-called "nitrogen free" medium from traces of nitrogen and to permit a bleaching of non-nitrogen fixing strains which renders counts of N2-fixing BGA easier.
Readings Counts are performed by observing, under a stereoscopic microscope, the petri dish placed on a transparent grid. Identification of the various types of colonies is made under an ordinary microscope.
Expression of the results Depending on the method of sampling, counts are expressed as number of colony forming units (CFU) / cm^2 of soil or number of (CFU) / g of soil. The expression of the results per area basis is preferable over dry weight basis because it permits extrapolations to hectare basis. Data on dry weight basis do not because of the vertical variation of the density of BGA propagules in the soil.

2232. Accuracy and reproducibility

The reproducibility of the method (intraplot variability) was tested by plating 30 composite samples collected from a 16 m^2 plot and enumerating total algae and N2-fixing BGA.

Fig1: Histogram of 30 replicated enumerations of total algae (10^5 colony forming units/cm^2) in a 16 m^2 experimental plot.

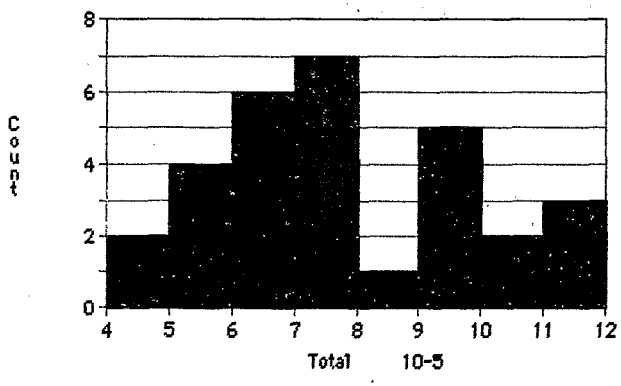
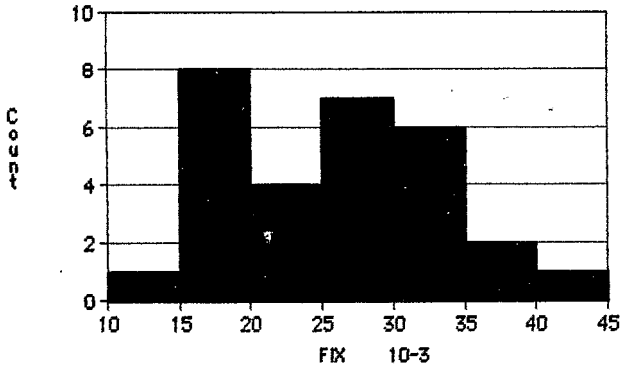


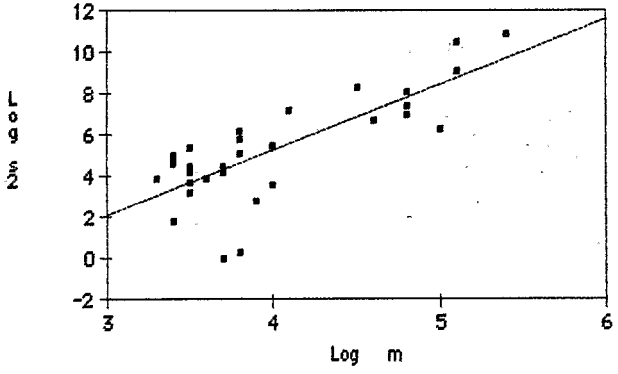
Fig 2: Histogram of 30 replicated enumerations of N2-fixing BGA (10^3colony forming units/cm^2) in a 16 m^2 experimental plot.



Values for total algae ranged from 4.3 x 10^5 to 11.7 x 10^5 (mean: 7.6 ; median:7.3)(Fig 1). Values for the N2-fixing BGA ranged from 11.3 x 10^3 to 45.0 x 10^3 (mean: 26.6 ; median:27.0) (Fig 2).

The test of normality had a low level of significance (p = 0.19) but the very small difference between means and medians and the general shape of the histograms allowed in first approximation to assimilate the distribution to a normal one. Standard errors were respectively 32% and 26% of the mean. This indicates that the accuracy of a single enumeration is about 60% and that two single enumerations are not significantly different if the ratio between the higher and the lower is less than 4.

Figure 3: Correlation between means (m) and variances(s^2) of 32 groups of BGA enumerations in 4 replicated plots (Log scale).



The interplot variability of the counts was studied from 28 groups of measurements in 4 replicated 16 m^2 plots. The distribution of the data was characterized by a correlation between the logarithms of mean and variances with a slope of the regression curve of 1.9, indicating a log normal distribution of the data (Fig 3).

Coefficients of variation (standard error expressed as a percentage of the mean) ranged from a few percent to around 100%. Higher coefficients were observed with growing populations of BGA; lower ones were observed with dry soils or where the relative contribution of spores present in the soil was high. Spores in soil are less unevenly distributed after plowing than growing populations. The number of replicates needed for a given accuracy can be calculated from the equation

$$\frac{X_2}{X_1} = 10^{\frac{2t s_y}{\sqrt{n}}}$$

where X1 and X2 are two closest, significantly different, mean values of n replicates, t is the variable of Student Fischer, s_y is the standard error of the data transformed by $y = \log (x + 1)$.(Roger and Reynaud 1978)

Considering an average value of 0.25 for s_y (see paragraph 211), on the average 15 replicates are needed to obtain significant differences between two means whose ratio is 2. Six replicates are needed for a ratio of 5. Two replicates are needed for a ratio of 10. Plating methods are considered, rightly, as tedious and time consuming. (As an example, the collection of the data used for drawing the conclusions presented in this paragraph, has needed the plating of more than 4,500 Petri dishes). For this reason, counts are frequently not replicated. Our results show that, when used with care, the plating method does not require very large numbers of replicates to show significant differences or changes among BGA populations.

2133. Bias

Effect of grinding and stirring. The accuracy of the counts depends on the reliability of the particular dilution method. Filamentous forms like *Oscillatoria* and *Lyngbia* are difficult to separate into individual cells, whereas moniliform filaments such as *Anabaena* and *Nostoc*, which are easily separated, may give inflated figures of abundance.

Table 4 :Effect of sample grinding (2mm, 0.4 mm, 0.25 mm) and time of stirring of the first dilution (15 min, 30 min, 60 min) on the enumeration of BGA in dry soil-based inocula of BGA

	Level of significance	
	Stirring	grinding
Multistrain soil-based unoculum		
Nostoc spp.	0.06	0.40
Aulosira sp.	0.26	0.02*
Unicellular	0.11	0.12
Total BGA	0.08	0.90
Monostrain soil-based unocula		
Nostoc sp.	0.50	0.77
Anabaena sp.	0.39	0.02*
Nostoc SL	0.97	0.04*
Scytonema sp.	0.53	0.91
Tolypothrix sp	0.28	0.82

[a]. Results are presented as the level of significance of variance analysis for 9 treatments resulting from the combinations of the two factors at three levels. Individual values are the average of triplicate counts at two consecutive dilutions and were transformed into log for analysis.

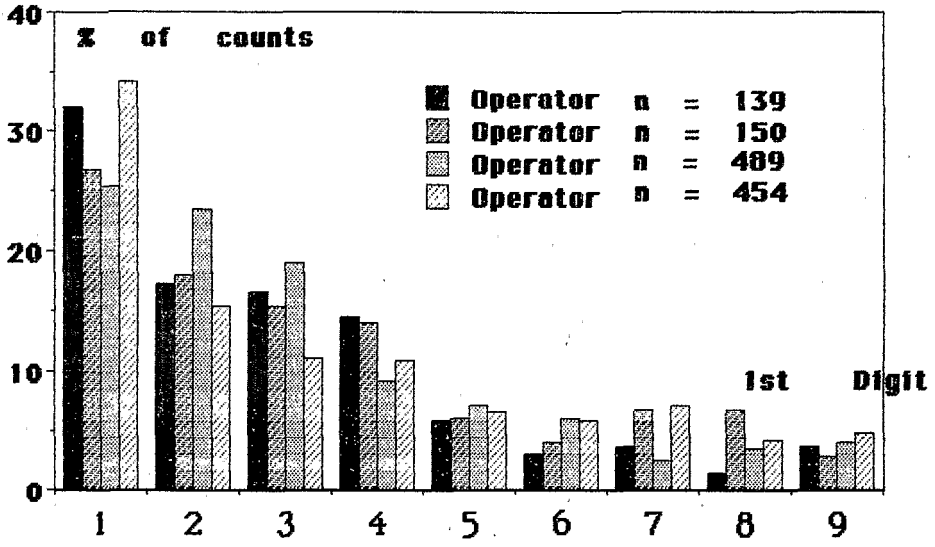
The effect of the preparation of dry samples was tested using six soil based inocula which were ground and sieved at 2.0, 1.0, 0.4, or 0.25 mm and then stirred for 15, 30 or 60 minutes when preparing the 10^{-1} dilution.

There was no effect of time of stirring on most of the samples. However, in the multistrain sample, the observed increase of counts for one of the strains (Nostoc sp.) with the time of stirring had a level of significance of 0.06 (Table 4).

Grinding had a significant effect in three of the samples for which grinding at <0.25 mm significantly decreased the counts.

When considering all samples as replicates by expressing the counts as % of the average value for each sample, the level of significance of F was 0.10 for stirring and 0.06 for grinding, showing that attention must be paid to the method of preparation of the dried samples and a too drastic grinding should be avoided.

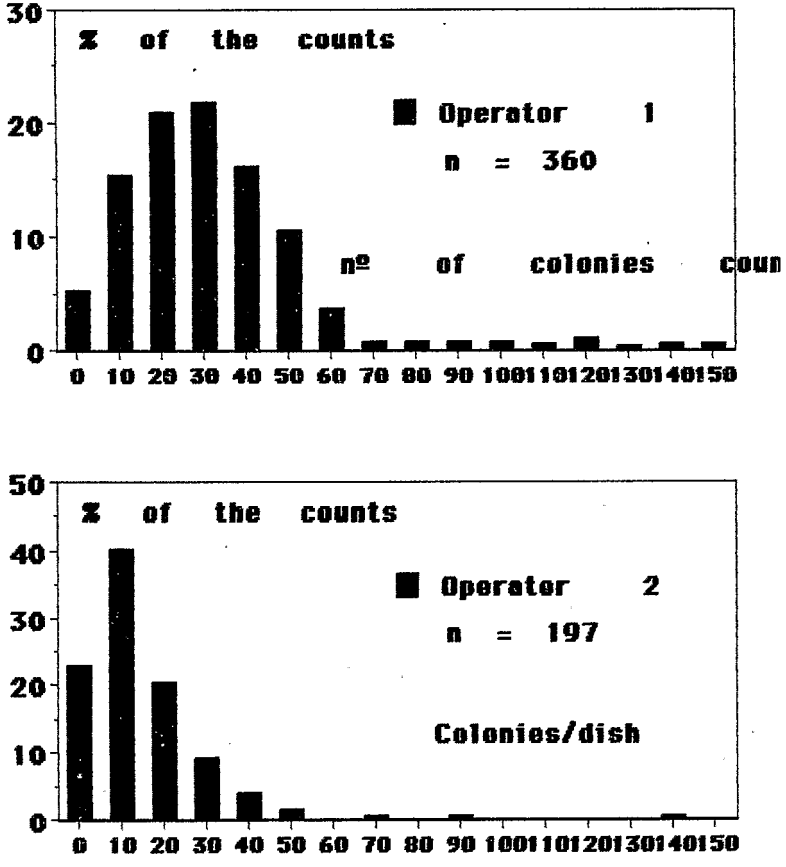
Figure 4 : Relative frequencies of the first digit (1 to 9) of n counts of BGA in soil samples by four different operators.

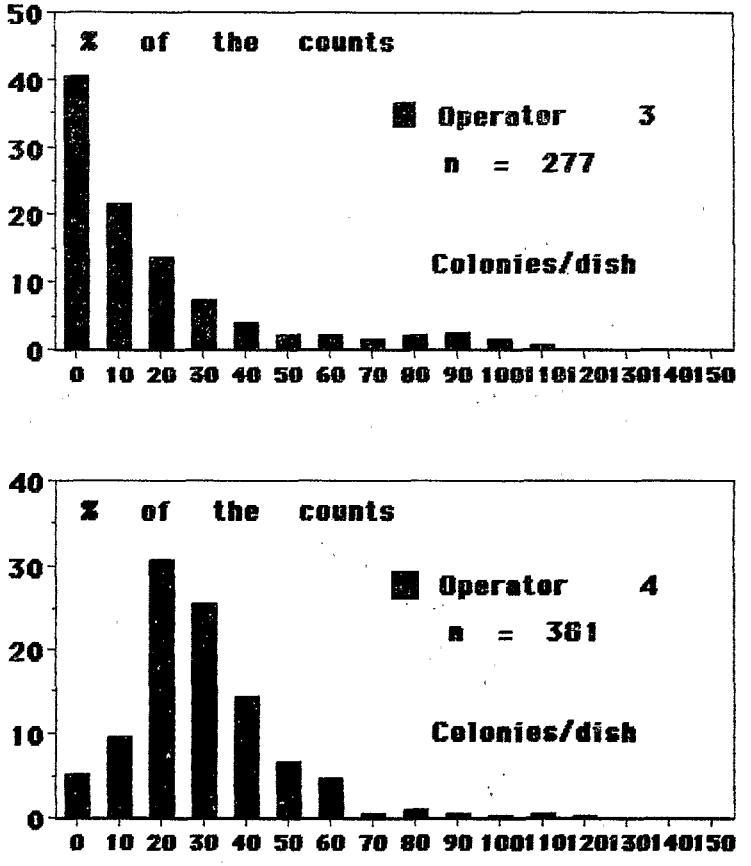


Biased distribution of the first digit of counts.Tthe study of the relative frequencies of the first digit (1 to 9) among large numbers of counts by various operators showed a negative correlation between the frequencies and the value of the digit (Figure 4). Lower digits were much more frequent than higher ones whereas an even distribution was expected. This bias, results 1) from the range and distribution of the number of colonies counted per dish and 2) from the fact that the ratio between counts at two consecutive dilutions is lower than the theoretical value of 10.

The study of the relative distribution of the number of BGA colonies counted per dish by four operators (Figure 5, abcd) shows that 1) between 5 and 50 colonies are usually counted per dish, and 2) the relative distribution of counts significantly varies with the operator.

Figures 5, abcd : Relative frequencies of the number of colonies of BGA counted per petri dish by four different operators.





The reason why operators most frequently do not count dishes where more than 50 colonies are present and perform it at the next dilution is 1) because counts are tedious and time consuming 2) dishes with so many colonies are usually very crowded and counts are difficult 3) and underestimation of the populations is expected because of competition.

Figure 6a : Correlation between counts of BGA in 589 soil samples at two consecutive dilutions (n and n+1)

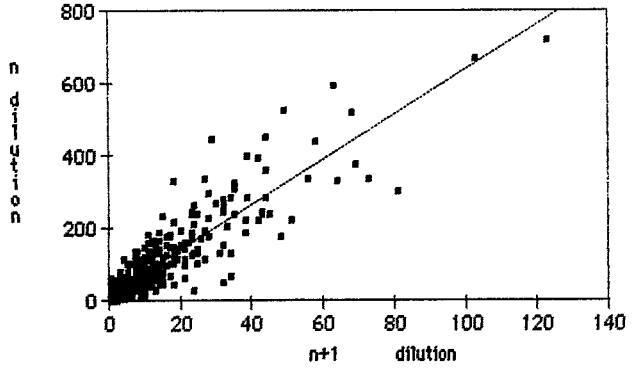
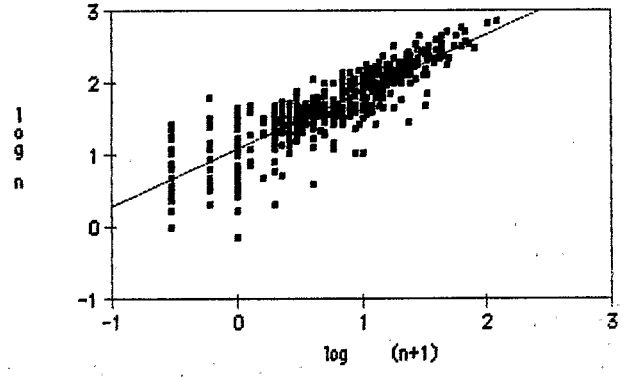


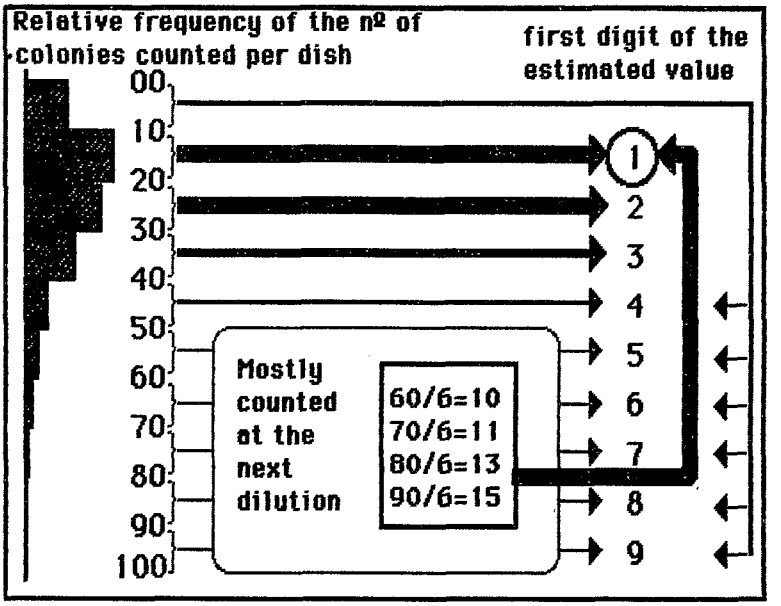
Figure 6b : Correlation between the logarithms of counts of BGA in 589 soil samples at two consecutive dilutions (n and n+1)



Ratio between counts at two consecutive dilutions. Figures 6a and 6b present the linear regression for 1) the original values and 2) the logarithms of counts at two consecutive dilutions prepared from 589 soil samples. The regressions showed that the ratio between counts at two consecutive dilutions, which should be theoretically equal to 10, was about 6. This results most probably from a competition of the usually large colonies of BGA on petri dishes.

Relating the ratio of 6 between two consecutive dilutions and the fact that dishes with more than 50 colonies are usually not counted explains the bias observed in the distribution of the first digit of the counts.

Figure 7: Schematic representation of the origin of the bias observed in the distribution of the first digit of plate counts of BGA



Counts on dishes having 50 to 100 colonies are replaced by counts at the next dilution, on plates having six times less colonies instead of the theoretical 10 times less. This replaces expected values ranging from 6 to 9.9 by values ranging from 10 to 16 and lead to a large number of data whose fist digit is1(Figure 7).

Another important aspect of the competition among too many colonies on a dish is that strains present at densities lower than 1% of the total CFU are usually not recorded. For a more complete inventory of the algal flora, it might be advantageous to complement quantitative measurements by the plating method with a qualitative study using the enrichment culture method.

2134. Conclusion

Plating of soil suspension-dilutions is the only method that permits simultaneous enumeration, identification and isolation of algae present in a soil sample. However, the interpretation of the results of plate counts must carefully consider the limitations of the method.

The main disadvantage of the plating method is that it may not ensure the development on the plates of all species present in the soil. As pointed out by Gupta (1966) who compared direct counts and soil culture , while many species were observed both *in situ* and in soil cultures, certain BGA (*Gloeotrichia* and *Aphanothece*) were observed only *in situ* and others (like *Fischerella*) grew only in soil cultures. Even if they grow, their relative frequencies may change.

Also 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 too many colonies, 1) a bias is introduced in the numerical values, and 2) strains present at densities lower than 1% of the total CFU are usually not recorded.

The method is, therefore, suitable only for quantitative estimation of the major strains present in a soil.

3. Occurrence of BGA in rice soils

The plating method was applied to the study BGA populations in102 rice soils from 5 countries, with regard to their major chemical properties.

31. Characteristics of the soil sample collection

Composite samples of wet surface soil composed of the top 0.5 cm layers of 10 core subsamples were collected with plastic tubes, 10 cm in length and 3 cm in diameter. Sampling points were located at 0.5 m intervals along a transect through the field. Dry soils, for which core sampling was usually not possible, were collected by delineating areas with the tube and removing the upper 0.5 cm layer of the soil with a knife blade. Samples were kept in plastic containers and processed within 10 days.

Among the 102 soil samples studied, 64 were collected from five major islands of the Philippines (north, central and south Luzon, Palawan, Samar, Bohol, and Mindanao), 31 came from four states in India (Uttar Pradesh, Karnataka, Tamil Nadu, and Andhra Pradesh), 6 from four states of Malaysia, and 1 from Portugal.

The soil samples correspond to a wide range of chemical properties (Table 5) . When compared with 410 soils of tropical Asia (Kawaguchi and Kyuma, 1977) their range of pH and average pH value were observed to be very similar ; their C and N contents were higher, mainly because a few peat soils were included in the sampling. Even after removing the values corresponding to the peat soils, average values for C (1.82%) and N (0.18%) were still slightly higher than those reported by Kawaguchi and Kyuma (1.4% C ; 0.13% N).

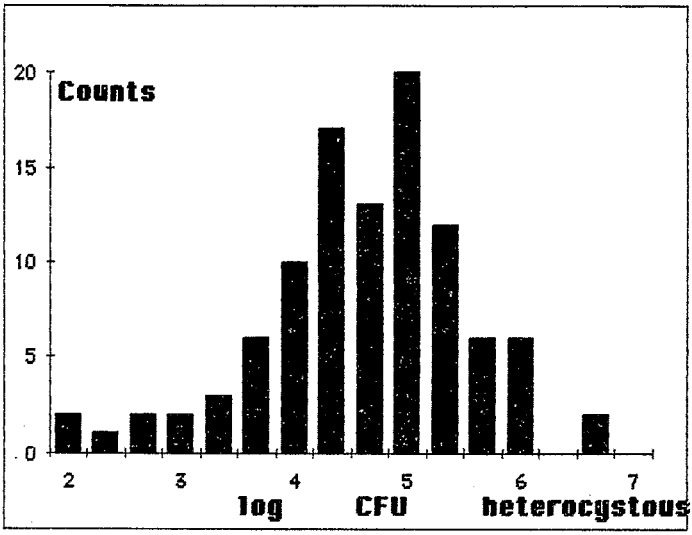
Data for wet and dry soils have inherent biases. Dry soils, which comprise a large percentage of samples from India, have a higher average pH (6.8) and available P content (31.6 ppm) than wet soils which were mostly collected in the Philippines(pH: 6.0 ; available P : 11.8 ppm). Kawaguchi and Kyuma (1977) also reported higher pH and available P content in Indian soils compared with Philippine soils. The high average available P value observed in dry soils is partly due to the inclusion of 3 samples from BGA multiplication plots which received a very high level of P fertilizer. Nevertheless when these three values are not considered, average available P still remains higher in dry soils (21.6 ppm) than in wet soils (11.8 ppm) and the values agree with those reported by Kawaguchi and Kyuma for India (21.9 ppm) and the Philippines (13.4 ppm).

Table 5 . Chemical properties and algal populations of the soils.

		C (%)	N (%)	C/N	P (ppm)	CEC meq/100g	pH	Tot.[a] (log CFU/cm^2)	Hcys[b] (log CFU/cm^2)
Nº of samples		77	78	77	72	59	98	60	102
Minimum		0.2	0.03	6.3	0.0	6.0	3.8	4.00	2.00
Maximum		28.8	2.61	20.0	267	105	8.8	7.73	6.90
Average	W[c]	3.19	0.26	10.5	11.8	36.4	6.0	6.01	4.48
	D	2.44	0.27	10.8	31.6	37.0	6.8	5.77	5.10
	T	2.92	0.26	10.6	19.5	36.6	6.3	5.59	4.75
Median	W	1.9	0.19	10.0	8.7	35.3	5.8	6.18	4.63
	D	1.5	0.15	10.5	12.0	36.0	7.0	5.73	5.11
	T	1.8	0.18	10.1	10.0	36.0	6.4	5.97	4.80
C.V.(%)		162	137	23	195	48	17	14	19
Normality[d]		**	**	ns	**	ns	ns	ns	ns

[a] : **Tot.**= total algal population ;
[b] : **Hcyst.**= heterocystous BGA ;
[c] : **W** = wet soils, **D** = dry soils, **T** = all soils ;
[d] : ** = significantly different from a normal distribution at $p < 0.01$; **ns** = not significant

Figure 8 . Histogram of the counts of heterocystous BGA (log CFU /cm^2) in 102 soil samples from rice fields.



32. Abundance of N2-fixing BGA in rice soils

Total algal populations ranged from 1.0 x 10^4 to 5.3 x 10^7 CFU/cm^2 and averaged 3.5 x 10^6 CFU/cm^2 . N2-fixing strains were present in all samples studied. Heterocystous BGA comprised, on an average, 9% of the total algal population, ranging from 1.0 x 10^2 to 8.0 x 10^6 CFU/cm^2 (average 3.2 x 10^5 ; median 6.4 x 10^4) (Fig 8). Heterocystous BGA occurred at densities higher than 10^3, 10^4, and 10^5 CFU/cm^2 in 95%, 85%, and 45% of the samples, respectively.

Quantitative surveys during the last decade in rice fields of several countries recorded heterocystous BGA with densities ranging from a few dozen to 10^7 CFU/g dw (Table 6). The average value that we observed was 3.2 x 10^5 CFU/cm^2, about four times higher than that of the data collected in the literature (2.5 x 10^5 CFU/g dw or 8.3 x 10^4 CFU/cm^2). This is partly because most data recorded in the literature were obtained by using the most probable number (MPN) method of enumeration. one which yields lower values than the plating method utilized in this study.

Table 6 . Density of N2-fixing BGA in rice soils (CFU/g d.w.)

COUNTRY	Samples Nº	% with BGA	Minimum	Maximum	Average	Median	Method	Reference
THAILAND	100	n.i.[a]	n.i.	n.i.	$8.6\ 10^3$	n.i	MPN[b]	ARARAGI & TANGCHAM, 1979
BANGLADESH	6	100	2.0×10^3	3.0×10^4	1.6×10^4	1.0×10^4	MPN	BHUIYA et al.,1981
SENEGAL	15	100	7.9×10^1	1.6×10^6	5.4×10^5	7.9×10^3	MPN	GARCIA et al.,1973
IRAQ	7	100	n.i.	n.i.	9.8×10^1	n.i.	MPN	HAMDI et al.,1978
PHILIPPINES	61	100	3.0×10^2	3.0×10^6	2.7×10^5	1.5×10^5	Plating	IRRI,1985
S.E. ASIA	25	100	1.0×10^3	1.0×10^7	1.0×10^6	1.0×10^5	MPN	KOBAYASHI et al.,1967
THAILAND	40	100	1.0×10^1	1.0×10^5	n.i.	8.0×10^3	MPN	MATSUGUCHI et al.,1975
INDIA	16	100	5.7×10^4	4.4×10^6	9.4×10^5	5.0×10^5	Plating	ROGER et al., 1987
INDIA	10	100	2.2×10^3	2.2×10^5	7.8×10^4	7.2×10^4	MPN	SAHA & MANDAL 1979
CAMBODIA	n.i.	n.i.	1.0×10^5	1.0×10^6	n.i.	n.i.	MPN	SUZUKI & KAWAI,1971
Pooled data	**230**		$\mathbf{1.0 \times 10^1}$	$\mathbf{1.0 \times 10^7}$	$\mathbf{2.5 \times 10^5}$	$\mathbf{10^4}$		

a n.i.= not indicated b MPN = Most probable number

Moreover, these data were frequently taken from samples corresponding to a thicker layer of soil (1 to 15 upper cm) than the one we used (0.5 cm), leading to a dilution of the more abundant algae in the uppermost portion of the soil. The average value of these pooled data (observed and collected) is 1.5×10^5 CFU/g dw, and the median is about

2.0 x 10^4 CFU/g dw. Present quantitative data show that N2- fixing BGA are more frequent in rice soils than it was estimated in earlier qualitative studies (Watanabe, 1959 ; Watanabe and Yamamoto, 1971 ; Venkataraman, 1975).

33. Dominant N2-fixing BGA in rice soils

Nostoc group was the most frequently recorded (Table 7), comprising, on an average, 62% of the CFU, followed by unicellular BGA (18%), *Anabaena* (8%), and *Calothrix* (7%). Other groups comprised less than 5% of the CFU.

The high incidence of *Nostoc* in the counts may partly be the result of half the samples being dry soils. As reported by Roger and Reynaud (1976) on Senegal rice fields, desiccation results in some selection of spore forming BGA. *Nostoc* incidence was higher in dry soils (80%) than in wet soils (47%). *Nostoc* was recorded in 99% of the samples and was the dominant (highest in relative abundance) N2-fixing genus in 74% of them. *Anabaena* was recorded in 78% of the samples but was dominant in only 5% . Relatively high levels of occurrence associated with low frequencies of dominance were also observed for *Calothrix* and *Fischerella*.

Table 7 . Occurrence and dominance of major groups of N_2-fixing BGA in the samples.

Groups	Average relative occurrence (%)			% of the samples where a group was			
	Wet soils	Dry soils	All soils	dominant	second dominant	recorded but not dominant	recorded (total)
Unicellular	27.4	6.7	18.5	18	13	22	53
Anabaena	10.0	6.6	8.5	5	29	44	78
Nostoc	47.5	80.3	61.6	74	22	3	99
Scytonema	0.5	0.1	0.4	0	1	13	14
Calothrix	8.1	4.9	6.8	3	22	35	60
Gloeotrichia	0.3	0.1	0.3	0	1	21	22
Fischerella	4.3	1.1	2.9	0	11	30	41

A general trend observed among N2-fixing BGA is that strains forming mucilaginous colonies (unicellular, *Nostoc* and *Gloeotrichia* groups) are less susceptible to grazing than strains that do not form such colonies (Grant *et al.* 1985). Mucilaginous strains were dominant in more than 90% of the soils ; strains that do not form mucilaginous colonies were present in most soils but were rarely dominant. This may indicate that grazing is a major limiting factor in the development of blooms of nonmucilaginous strains active in N2-fixation in rice fields. More information regarding the selectivity of the plating method is needed before definite conclusions can be drawn.

Besides a higher abundance of *Nostoc* in dry soil, no significant correlation was observed between the relative abundance of the various groups of heterocystous BGA and the soil physicochemical properties.

34. Correlation between the abundance of N2-fixing BGA and soil properties.

Highly significant positive correlations were observed between the abundance of heterocystous BGA and pH, as well as available P (Table 8). The correlation with pH was significant in soils having a pH lower than 6.5 but not higher (Fig 9). Correlation with available P took into account some unusually high values from highly P-fertilized plots, but still remained significant when only values lower than 50 ppm were considered. These two correlations agree with other reports (Matsuguchi *et al.*1975; Roger and Reynaud,1977) and the observation that N2-fixing BGA are usually more abundant in neutral to alkaline soils rich in P (Roger and Kulasooriya,1980).

A highly significant positive correlation was observed between C content of the soil and 1) total algae, and 2) the ratio between total algae and heterocystous BGA. However there was no significant correlation between C and heterocystous BGA. A similar trend was observed for N. These results indicate that soils rich in organic matter have higher total algal populations and lower relative populations of heterocystous BGA.

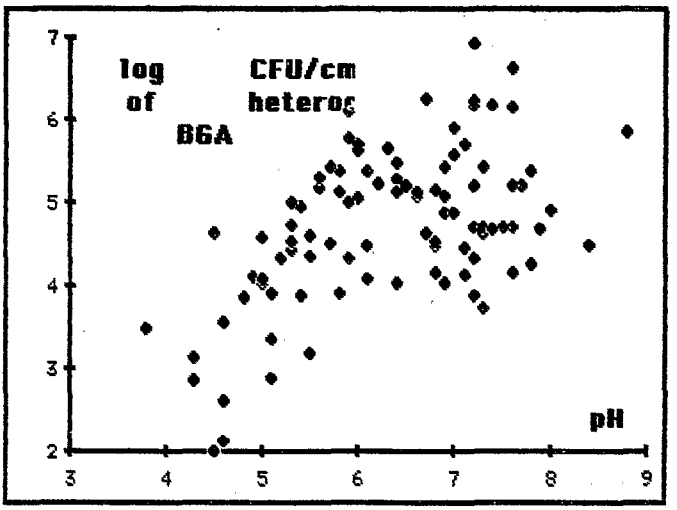
A negative correlation between C:N and the abundance of heterocystous BGA was in agreement with a positive correlation between C:N and the ratio between total algae and heterocystous BGA. These correlations may partially be explained by the negative correlation between C:N and available P which was observed among the soils studied. Similarly the positive correlation between CEC and the abundance of heterocystous BGA may partly be due to the highly significant correlation observed between CEC and pH.

Table 8 . Correlation between soil properties and algal counts (CFU/g dw) (Pearson's correlation coefficient)[a].

	C	N	C:N	P	CEC	pH	Tot.[b]	Hcy.[c]	Tot. Hcy.
Carbon		++	ns	ns	ns	ns	++	ns	++
Nitrogen			ns	ns	ns	ns	++	ns	+
C:N				--	ns	ns	ns	-	+
Available P(Olsen)					ns	ns	ns	++	ns
CEC						++	ns	++	ns
pH							ns	++	--

[a] : + and ++ : positive correlations significant at the 5 and 1% level respectively ; - and -- : negative correlations significant at the 5 and 1% level respectively.
[b] : Tot. = total algal population.
[c] : Hcy. = Heterocystous BGA.

Figure 9. Abundance of heterocystous BGA (log CFU/cm^2) in soils as a function of soil pH.

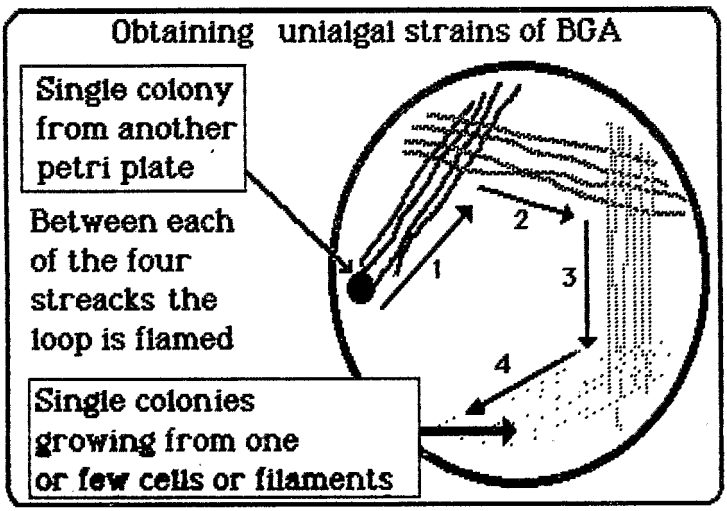


4. Isolation and conservation of the strains

41. Method of isolation

Strains are isolated by picking isolated colonies from plates used for counting, using preferentially higher dilutions. Strains are made unialgal by successive cross streaking on agar medium (Fig 10).

Figure 10



42. The IRRI collection

The collection comprises 195 strains of 20 genera originating from 21 countries. About 45% of the strains are from Africa and 40% from Asia. Dominant genera are *Nostoc, Anabaena,* and *Calothrix* which reflects the rice field origin of the collection (Table 9).

43. Conservation of the strains

In most cases strains recorded during ecological and agronomical studies are not kept by the authors because the maintenance of a culture collection is expensive and time consuming. Also the acceptance of a strain in a collection such as ATCC requires purification of the strain and prerequisites which refrain the authors from depositing their material.

Table 9: Number and origin of the collection of blue-green algae. IRRI, 1986.

Genera	Africa		Asia		Europe	Other regions	TOTAL
	Senegal	Other	Philipp.		Other		
Anabaena	20	2	5	11	9	3	**50**
Aphanothece	0	1	1	0	0	0	**2**
Aulosira	1	1	0	1	0	1	**4**
Calothrix	14	2	5	3	0	1	**25**
Cylindrospermum	4	1	1	0	0	0	**6**
Gloeotrichia	1	0	3	0	0	0	**4**
Fischerella	0	3	7	0	0	0	**10**
Nodularia	2	0	1	1	0	0	**4**
Nostoc	20	6	13	13	2	5	**59**
Scytonema	6	0	1	1	0	0	**8**
Tolypothrix	0	0	0	4	0	0	**4**
Wollea	0	0	1	0	0	0	**1**
Westiellopsis	0	1	0	0	0	0	**1**
N2-fixing	68	16	38	34	11	10	**177**
Nonfixing	16	0	0	1	1	0	**18**
TOTAL	**84**	**16**	**38**	**35**	**12**	**10**	**195**

The availability of pure cultures is essential for effective microbiological work on any microorganism. However, in agronomical studies of BGA, the major purpose of a strain collection is to have a range of organisms available for producing inocula for experiments. Axenic strains are not needed. Therefore strains isolated during the experiments, if kept, are usually not axenized and are maintained only as unialgal material.

431. Traditional methods

For short term preservation, stock cultures incubated at laboratory temperature under continuous low light, either on liquid medium or on

agar slants, generally proved satisfactory with axenic and non-axenic strains. Generally BGA grow slowly and generation times of about 24 hours are common. Therefore inoculation of a very small quantity of culture avoid too frequent transfers. Cultures are examined once a week in order to detect changes, especially bleaching. Transfers are done when necessary (usually once every 2 months) by inoculating a loopful of culture into 50 ml medium in a 125 ml Erlenmeyer flask. Newly inoculated flasks are kept without light for 24 hours and then incubated 25 cm under 20-watt fluorescent tubes in open shelves, at laboratory temperature.Old cultures are kept to safeguard against possible losses of newly transferred cultures. Cultures to be discarded are first autoclaved at 15 lbs/in^2 for 15 minutes.

This method, which requires subculturing about every two months, is time consuming and might lead to significant changes of the strain properties after long term cultivation under artificial conditions in the laboratory.

For long term preservation of axenic and non-axenic strains, deep freezing in liquid nitrogen has been successfully used.

432. Methods using dried material

As most of the heterocystous BGA form spores known to be resistant to dessication, conservation of N2-fixing BGA in dry state was tested using three techniques.

4321. Soil based inoculum

A simple method for keeping non-axenic strains is to produce "soil-based" inoculum and to dry it as algal flakes. When the strain is needed, suspension dilutions of the algal flakes are plated on Petri dishes and isolated colonies are then grown in liquid medium.

Dried soil-based inocula produced, in erlenmeyer flasks or on petri plates, on soil previously autoclaved at 120ºC for 30 minutes for three consecutive days exhibited a good viability. Out of 70 strains of 11 genera tested, 67 could be regrown after 20 months of storage in plastic bottles a laboratory temperature. However about 30% of the strain was contaminated with algae from the soil, mostly diatoms, which indicates that special attention should be paid to the sterilization of soil to be used as preserving support.

4322. Powdered mass culture

Strains are grown in 20-l carboys in BG-11o medium. The concentration of Na_2CO_3 is increased five-fold and the culture is

continuously bubbled with air enriched with CO_2 to maintain a pH of about 7.0-7.5. Light intensity is progressively increased from about 1klux to15klux when the density of the culture increases.

Drying at room temperature of BGA produced in mass culture and harvested by decantation, is a convenient way of keeping fair amounts of inoculum that can be later used for producing rapidly large quantities of BGA material for field experiments. This method has been proven successful with all of the10 strains of heterocystous we have currently tested..

Dried and powdered cultures produced with this method could regrow after 50 to 56 months of storage. However this method requires culturing of large quantities of algal material .

4323. Deposition on paper strips

Conservation in dry state of dense cultures produced in 125 ml erlenmeyer flasks, deposited on strips of sterile Whatman chromatography paper(nº3), dried in a sterile hood at room temperature, and placed in sealed polyethylene bags proved to be most convenient and efficient. Among twenty unialgal strains prepared in October 1985, 18 are still viable. The nonviability of the two other strains was recognized one month after preparation. Sets of these strains have been mailed to 12 laboratories all over the world with information on how to revive the material and a questionnaire. In half of the laboratories, all of the strains could be regrown. In other laboratories most of the strains were regrown.

From March to October1986, 136 N_2-fixing strains of the IRRI's BGA collection were transferred on paper strips. In January1987,121 of the strains could be regrown; a second trial with the15 strains that failed to grow was unsuccesfull. In September 1987, 33 strains among 136 failed to grow; a second test on these strains permitted regrowth of 7 of those.

5. Summary and conclusions

Among available methods for quantitative estimation of algal populations, only the plating of suspension-dilutions of soil permits the simultaneous enumeration, identification and isolation of most algae present in the sample. The method was adapted to wetland soils and standardized.

Because of the inadequacy of existing taxonomical keys for ecological

studies with BGA, a simplified classification into broad taxa according to morphological criteria directly observed on the material growing on Petri dishes was developed. This grouping has no taxonomical pretension but is a convenient tool for ecological studies because it permits a rapid classification of the strains and corresponds to relatively well defined growth behavior *in situ*.

The interpretation of the results of plate counts must consider the limitations of this method, which 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. Also, because of competition among too many colonies on petri dishes, quantitative estimates are biased and strains present at densities lower than 1% of the total CFU are usually not recorded. The method is, therefore, suitable only for quantitative estimation of the major strains present in a soil.

The method was applied to study BGA populations in 102 rice soils from 5 countries, with regard to the major chemical properties of the soils. Results showed the presence of N2-fixing strains in all samples studied. Heterocystous BGA comprised, on an average, 9% of the total algal population, ranging from 1×10^2 to 8×10^6 CFU/cm^2 (median 6×10^4).

Heterocystous BGA abundance was positively correlated with soil pH and available P. *Nostoc* was recorded in 99% of the samples and was the dominant N2-fixing genus in 74% of them. Relatively high levels of occurrence associated with low frequencies of dominance were observed for *Anabaena*, *Calothrix* and *Fischerella*. A general trend observed among N2-fixing BGA is that strains forming mucilaginous colonies are less susceptible to grazing than strains that do not form such colonies (Grant *et al.* 1985). Mucilaginous strains were dominant in more than 90% of the soils; strains that do not form mucilaginous colonies were present in most soils but were rarely dominant. This indicates that grazing is a major limiting factor in the development of blooms of non-mucilaginous strains active in N2-fixation in rice fields.

Strains isolated during these studies are maintained in a collection which comprises 195 strains of 20 genera originating from 21 countries.

Conservation in dry state as soil based inoculum, powdered mass culture or after deposition on paper strips was tested and proven to be an efficient method for short- to medium-term conservation.

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