APPLICATION OF THE SERIAL DILUTION TECHNIQUE TO ESTIMATE THE BIOMASS OF N_2-FIXING BLUE-GREEN ALGAE UNDER FIELD CONDITIONS

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Abstract

The serial-dilution method developed to estimate algal biomass in field samples is described. This method is illustrated by a transect experiment in a dry rice field. It shows a log-normal distribution law for algal material. The effect of taxon volume unit, enumeration and sampling on the accuracy of the method is determined.

INTRODUCTION

The main problem in the measurement of the effect of inoculation with blue-green algae in rice fields is to determine the principal factors which might be involved in the inoculation effect. The review by Roger and Kulasooriya (1980) indicates that most work on algalization has been performed to compare the grain yield in treatments inoculated or non-inoculated with algae. Experiments conducted on this "black box" basis give no additional information on the qualitative and quantitative development of the algal inoculum and of the phototrophic nitrogen--fixing activity although these parameters are important and may have explained why the algalization effect was positive, negative or residual. To have a better understanding of the evolution of blue-green algae during the rice cultivation cycle, it is necessary to estimate the total algal biomass along the development of the crop from seedling stage to harvest.

Algal abundance has been estimated by three principal methods: direct observation, measurement of pigments, and plating techniques. The direct microscopic examination is generally used for qualitative determinations, whereas the pigment analysis does not indicate the composition of the algal flora. Plating techniques, which are more frequently used, are advantageous in providing both qualitative and

quantitative information simultaneously, but the accuracy of such a method depends on the dilution procedure.

The purpose of the present paper, is to describe an improved serial-dilution method developed in our lab, to estimate algal biomass in field samples.

1. METHOD

The experiment was conducted in a dry rice field just after cropping; 57 samples consisting of three soil cores of one cm diameter and about 1 cm depth each were collected along a transect of 18.5 m. They were stored in closed and dry bottles under lab temperature.

Each sample was weighed, crushed and resuspended in 30 ml deionised water and thoroughly stirred. Total nitrogen, protein concentration, and pH of each suspension was measured. The suspension was diluted from 10^{-1} to 10^{-6} in glass tubes and one ml of each dilution was spread onto a petri dish containing 30 ml of algal medium solidified with 1% agar. We used the BG 11 medium (Allen and Stanier 1968) for the 10^{-4}, 10^{-5} and 10^{-6} dilutions to enumerate the total algal constituants and the BG 11 medium minus nitrogen with 10^{-3}, 10^{-4}, 10^{-5} dilutions to enumerate specifically nitrogen fixing blue-green algae. For each dilution three petri dishes were used. Incubations were conducted in a light chamber (500 lux, 30°C) for 21 days. Counts were performed with a stereomicroscope WILD M5. The petri dish was divided by a frame into 1 cm squares, and each square was examined under a x 12 magnification. Enumeration of algal colonies was performed on the entire surface of each petri dish.

II. RESULTS

A. Volume unit

In this method of enumeration each algal colony is presumed to have arisen from a propagule which could either be a short piece of filament or an akinete. To convert the algal numbers to biomass, it is necessary to calculate the mean volume of this propagule unit and the following method was adopted for this purpose. From these enumerations, 14 principal taxons were identified (Table 1).

Several colonies belonging to each taxon were picked from the petri dish and vigourously stirred for 10 minutes. With this suspension we measured, under microscope, the size of a hundred pieces of filament or the diameter of cell aggregates. From these measurements, a volume unit was calculated for each taxon by assuming that the form of the broken filaments corresponded roughly to either a cylinder, sphere or cone. The value of each taxon volume unit is thus specific to the algae collected in this ecosystem but can be used for other ecosystems if the taxon is well identified.

The relative error determined here for r=5% is closely related to the ability to fragment the colonies and to the dispersion of broken filaments during the stirring and diluting steps. For instance the size of filaments of Nostoc punctiforme (relative error = 9%) is more homogenous than those of filaments of Anabaena spherica (relative error = 20%) and the relative error of the estimation of this taxon biomass would be higher than for N. punctiforme.

B. Enumeration of taxons

Enumeration of taxons was normally conducted using two succesive dilutions and the result was expressed as the mean of the six petri dishes.

On the medium BG 11 minus nitrogen, eucaryotic algae and non nitrogen fixing blue-green algae grew normally during the first week, then their growth stopped and colonies became yellow confirming their inability to fix N_2 (Reynaud and Roger 1977).

For N_2-fixing blue-green algae (N_2-fix BGA) there was a good correlation between the number of colonies which developed, either on medium with or without nitrogen source.

The use of the two media and three dilutions allowed us to:

- distinguish slow growing taxons from fast growing ones
- count each taxon as its optimal dilution

- reduce possible competition between algal groups
- easily isolate each taxon.

Petersen (1932) pointed out that the dilution method was not reliable for filamentous type and that some spreading types also do not form individual colonies in the agar medium. To have an estimation of these biomasses we have settled on a scale of the density of separation of filaments for these taxons on 1 cm^2 of agar plate; we have compared it with a direct enumeration of filaments under microscope for a suspension of Lyngbya sp. Means of triplicates estimations were identical with the two methods.

C. Biomass determination

The accuracy of algal estimation and in situ ARA measurements depend upon the density of sampling and of the distribution law of the variable. Earlier studies on the correlation between means and variances of enumeration of soil microorganisms and in situ ARA measurements, indicates that these variables have approximately a log-normal distribution (Roger et al., 1977, Roger and Reynaud, 1978). The first implication of this law was that the confidence interval and parametric statistical variable (i.e.: t variable of Student Fisher) must be calculated using the logarithms of algal enumerations or ARA measurements. The confidence interval was dissymmetrical, its inferior limit was generally slightly lower than that incorrectly calculated using the t variable of Student Fisher; the upper limit was generally higher. The validity of the logarithmic transformation must be checked by a method based on the ratio between two correction coefficients established by Neyman and Scott (1960) and programmed by Roger et al. (1978); transformation is considered as valid when the ratio $\bar{c}/c$ is included between 0.66 and 1.33.

The transect experiment is a good demonstration of the log-normal distribution law for algal material. When variables such as pH, weight of samples and N concentration in soils, are distributed along the transect as a normal distribution, the log-normal transformation is justifiable for 10 taxons out of 11 (Table 2). In fact, Anabaena sphaerica has a $\bar{c}/c$ ratio of 3.465 depending of

two very high values, but this ratio diminished to 0.988 with their supression.

Most results are expressed as number of algae per gram of soil; these data don't take into account algae present in the flood water of submerged soils and do not permit extrapolation to the field level. A more satisfactory way to evaluate algal population is to determine the number of algae per cm^2, each core sample includes the first centimetre of soil and the corresponding flood water column. As the soil has been sampled in a dry area we compared the two expressions: the precision was the same; the confidence interval on the weight of 57 samples was 5%, r=5%.

On the transect the total algal biomass was estimated at 2970 kg/ha with a confidence interval of 10 (lower limit) and 19 (upper limit) for r = 5%. As we have determined that there is no autocorrelation function between algal material of two next samples we have calculated the confidence interval with less and less samples within 18.5 meters.

	lower limit	upper limit
57 samples	10	19
29 samples	34	25
19 samples	53	32
15 samples	69	35
12 samples	83	39

The accuracy was very high with 57 soil samples (that is every 30 cm intervals), and since biological evaluations could tolerate an accuracy of about 50%, it seems convenient in this transect to collect a sample every meter.

The serial-dilution method, is currently used as a standard procedure in our lab. This allows us to compare the algal biomasses studied here with those of other areas in Senegal: on 97 soils estimated: 21 are covered by less than 10 kg of algae, 57 between 10 and 100 kg, 18 between 100 kg and 10 tons and one by 12 tons. An average dry weight is equal to 3.85% of fresh weight, on the average of the dry weight 5.5% is nitrogen (Roger, Tirol and Watanabe,

unpublished); thus, the algae supplied the rice field with 6.2 kg of nitrogen.

CONCLUSIONS

The serial-dilution method is not an universial technique for the quantitative evaluation of the algal biomass: when there is a thick algal bloom it is easier and more accurate to collect the algae and measure the pigment concentration.

The method described in this paper is time consuming and could lead to errors due to spore germination. However, it is useful in its application to large scale studies of non-bloom forming algae after their introduction to fields and for the identification of dominant taxons. To compensate for the log normal distribution of these organims, algal enumerations are best carried out on composite samples prepared by mixing several surface soil cores removed from a field.

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TABLE 1

Taxons	Shape and size measured	Number of elements measured	Volume unit $\mu 3$
Pseudanabaena sp.	Length and diameter of	112	$1.5010^{2} \pm 11\ \%$
Narrow L.P.P. species	cylindrical	132	$1.4510^{3} \pm 17\ \%$
Lyngbya sp.	trichomes	245	$1.3510^{4} \pm 11\ \%$
Anabaena ambigua		103	$3.2010^{3} \pm 20\ \%$
Anabaena vaginicola		105	$8.1310^{3} \pm 13\ \%$
Scytonema millei		100	$1.3010^{4} \pm 22\ \%$
Spirogyra spp.		138	$6.5010^{4} \pm 24\ \%$
Nostoc microscopicum	Trichomes length and	134	$5.1010^{2} \pm 17\ \%$
Nostoc punctiforme	diameter of hemispherical	134	$4.9010^{2} \pm 9\ \%$
Anabaena sphaerica	cells	105	$7.9010^{2} \pm 20\ \%$
Calothrix spp.	Height and diameter of conical trichomes	72	$1.0010^{3} \pm 18\ \%$
Gloeothece samoensis	Diameter of cells aggregates	98	$1.3410^{4} \pm 23\ \%$
Unicellular green algae		20	$3.8010^{1} \pm 15\ \%$
Navicula sp.	Double cone: length and diameter of frustule	20	$3.5010^{2} \pm 10\ \%$

Calculated volume unit and relative accurency to 95% confidence interval of principal taxons determined in a Senegal rice-field.

Table 2

Taxons	Mean value kg/ha	% Confidence interval Lower Limit	Upper Limit	C'/C
Pseudanabaena sp.	193	6	21	1.032
Narrow L.P.P. strains	615	22	27	1.099
Lyngbya sp.	678	3	32	0.880
Total homocystous	1664	7	21	1.034
Nostoc microscopium	4.8	4	30	0.857
Nostoc punctiforme	23.2	3	32	0.807
Total Calothrix sp.	198.9	9	30	0.792
Gloeothece samoensis	94	21	32	0.891
Anabaena ambigua	35.7	13	33	0.823
Anabaena vaginicola	100.3	118	42	0.840
Anabaena spherica	104.4	960	39	3.465
Scytonema millei	43.2	14	44	0.970
Total N_2-fixing forms	632.5	6	24	0.987
Total algal biomass	2970	10	19	1.029

Biomass estimations, confidence interval with a log-normal distribution, valid when $0.66 \leq C'/C \leq 1.33$.

Means are obtained from 57 samplings on a paddy field transect in the Fleuve region Senegal.