

10. Nitrogen Fixation in Wetland Rice Field

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INTRODUCTION

Rice grown in flooded conditions is called wetland rice. Rice grown in moist conditions similar to those used for growing cereals like wheat and maize, is called dryland rice. Because wetland rice can support the highest population per unit area of land [1], rice is mostly grown in densely populated zones and, frequently, in subsistence agricultural systems where crops are grown without synthetic fertilizers. In such cropping systems, wetland rice yields are higher than those of dryland rice. Yield differences can be attributed partly to better water supply and partly to higher fertility of wetland soils. The higher nitrogen fertility of wetland soils is exemplified by the higher dependence of wetland rice on soil nitrogen [2]. Without an external nitrogen supply, wetland rice should deplete soil nitrogen more than dryland rice. Nevertheless, wetland rice has been grown without fertilizer application for a longer time than dryland rice, with little or no decline in yield [3]. It is likely that the higher and more consistent nitrogen fertility of wetland soils can be attributed to biological nitrogen fixation. Nitrogen balance study, described later, indicates this higher nitrogen fertility status of wetland rice cultivation. Because reviews on biological nitrogen fixation in flooded rice soils [4-5] and flooded soils [6] are already available, this chapter focuses on the recent advancement of knowledge.

THE FLOODED RICE FIELD ECOSYSTEM AS A NITROGEN FIXATION SITE

Principal characteristics of wetland rice fields are determined by flooding of the soil and presence of rice plants. The most important change caused by flooding is in the aeration of soil. Because oxygen moves ten thousand times more slowly through a water phase than through a gaseous phase, the capacity of a soil to exchange gases with the atmosphere decreases as it becomes water-saturated. Waterlogging of a soil quickly leads to anaerobic conditions that develop a few millimetres beneath the soil surface in the

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Table 1. Major nitrogen-fixing microorganisms in flooded soil-rice ecosystems

Sites	Major nitrogen-fixing microorganisms	Representative genera
Floodwater and surface soil	Free-living blue-green algae	<i>Nostoc</i> , <i>Anabaena</i> , and others
	Epiphytic blue-green algae	<i>Nostoc</i> , <i>Calothrix</i> , and others
	<i>Anabaena</i> in symbiosis with <i>Azolla</i>	<i>Anabaena azollae</i>
	Photosynthetic bacteria	<i>Rhodopseudomonas</i> , <i>Rhodospirillum</i> and others
	Methane-oxidizing bacteria	<i>Methylomonas</i> and others
	Sulphur-oxidizing bacteria	<i>Thiobacillus</i>
	Aerobic heterotrophic bacteria	<i>Azotobacter</i> , <i>Derxia</i> , <i>Beijerinckia</i>
Anaerobic soil	Microaerophilic bacteria	<i>Azospirillum</i>
	Facultatively anaerobic bacteria	<i>Bacillus</i>
	Strictly anaerobic bacteria	<i>Clostridium</i> , <i>Propionibacterium</i>
	Sulphate-reducing bacteria	<i>Desulfovibrio</i>
Plant (mostly root)	Microaerophilic bacteria	<i>Azospirillum</i> , <i>Pseudomonas</i> , <i>Alcaligenes</i>
	Facultatively anaerobic bacteria	<i>Enterobacter</i> , <i>Klebsiella</i>

reduced layer. After submergence the number of fungi, actinomycetes, and aerobic bacteria is reduced and anaerobic bacteria increase. Surface soil (a few mm) and floodwater remain in an oxidized state and are colonized by aerobic microflora and photosynthetic organisms. Rice plant provides another niche for microbial activity, in the floodwater, including shoots, roots, and the rhizosphere. The subsoil develops beneath the plough pan layer.

The major environments in paddy fields are: floodwater, surface-oxidized soil, reduced soil, rice plants, and subsoil. The reader is referred to reviews on chemistry of the submerged soil [7] and microbiology of flooded rice soil [8-9].

Nitrogen-fixing organisms are distributed in these different sites, where environmental conditions for the growth and nitrogen-fixing activities differ. Major nitrogen-fixing microorganisms in each site are summarized in Table 1.

Floodwater

The floodwater is a photic zone where aquatic communities, including bacteria, prokaryotic and eukaryotic algae, and aquatic weeds, provide organic matter to the soil surface. Little is known about the amount of organic matter contributed by aquatic phototrophs. In a paddy field in the Philippines, the primary production of floodwater communities was equivalent to the productivity values in eutrophic lakes [10]. The productivity of the aquatic photosynthetic biomass probably rarely exceeds 1000 kg dry weight per hectare [11].

In floodwater, aquatic weeds and basal portions of rice shoots are colonized by epiphytic bacteria and algae. Epiphytic nitrogen fixation becomes agronomically significant in deepwater rice where the submerged plant biomass is very high [81].

Surface soil (oxidized layer)

The surface soil is at a redox potential (Eh) higher than 300 mV. The depth of oxidized layer is from 2 to 20 mm and is dependent on the reducing capacity or oxygen-consuming capacity of the soil, owing to microbial respiration and Fe⁺² oxidation [12]. In the oxidized layer, NO₃⁻, Fe⁺³, SO₄⁻², and CO₂ are stable [13] and aerobic bacteria predominate [14-15]. Methane and hydrogen evolved from the anaerobic soil are partly oxidized in the surface soil [16].

Anaerobic soil (reduced layer)

In anaerobic soil, the reduction process predominates. Eh ranges from 300 mV to -300 mV. Takai and Kamura [17] divided the reducing process of paddy soil into two stages: before and after iron reduction is completed. In the first stage, oxygen absorption, nitrate reduction, manganese reduction, and iron reduction proceed in this order, and ammonia and carbon

dioxide are liberated. In the second stage, sulphide and methane are produced and the population of anaerobic bacteria increases. The main organic acids detected in reduced paddy soils are acetic, propionic, and butyric acids [18]. Methane formation is accompanied by the decrease of organic acids and carbon dioxide.

Organic matter particles in soil are important microsites for microbial activity. Organic matter is provided to anaerobic soil as crop residues, decomposed material from the aquatic biomass, and organic fertilizers. Wada and Kanazawa [19] developed a technique to fractionate soil particles according to size and density. They found that about 30 per cent of the organic matter in a paddy soil exists in particles larger than $37\ \mu\text{m}$ and that particle size of organic debris decreases during decomposition. Microbial activities are concentrated on the soil aggregates that contain decayed organic debris. The presence of organic debris makes anaerobic soil heterogeneous. The activities of soil fauna [20] make microaerophilic sites in anaerobic layers. As Dommergues [21] pointed out, submerged paddy soils are far from being uniformly reduced and should be regarded as complex systems formed by the juxtaposition of microenvironments that are either sites of oxidation reaction or sites of reduction reaction.

Rice root and the rhizosphere

The early concept of rhizosphere suggested that the plant exudes organic substances on which soil microorganisms grow and, consequently, the root in the soil is surrounded by these microorganisms. It now appears that besides the soil adjacent to the root (rhizosphere, in the strict sense), mucilaginous layers on the surface of the epidermis and intercellular spaces among epidermis layers as well as inner tissues of the epidermis and cortex are also inhabited by microbial colonies. These provide more or less continuous media for their activities [22]. Secretion of carbon compounds by roots provides energy sources for microbial growth and is greatly accelerated by the presence of microorganisms [23-24]. Invasion of bacteria, fungi, and protozoa in wetland rice roots was observed at later stages of rice growth [25]. As in other marsh plants, rice roots receive oxygen from aerial parts of the plant and oxidize the rhizosphere. The brownish colour of rice roots indicates oxidation of ferrous iron to ferric iron and its precipitation along the root surface [26].

Rice roots grown in submerged soil have fewer and shorter root hairs and are straighter than those found in dry soil [27]. Redox conditions in the rhizosphere are determined by the balance of oxidizing and reducing capacities of rice roots. Nutrient deficiencies, particularly in nitrogen and potassium, accelerate the reduction of rhizosphere [28-29].

Subsoil

The soil beneath the plough pan is aerobic in well-drained soils and anaerobic in poorly drained soils. Its role in providing nitrogen to rice is sometimes apparent.

Table 2. Nitrogen balance in long-term fertility trials in wetland rice soils (from Watanabe, Craswell, and App [30]) and unpublished data

Site	Cropping per year	Duration years	Treatment	Kg N ha ⁻¹ yr ⁻¹			
				Input	Soil change	Plant uptake*	Balance
Aomori, Japan (41°N)	Wetland rice	21	PK	0	-20	45	+25
			NPK	57	-35	66	-25
Kagawa, Japan (34°N)	Wetland rice and barley	21	PK	0	-42	80 (55)	+38
			NPK	157	-18	154 (96)	-21
Sorachi, Japan (45°N)	Wetland rice	12	PK	0	-44	142	+98
			NPK	39	-51	136	+46
Ishikawa, Japan (36°N)	Wetland rice	22	Unfertilized	0	-34	53	+19
			PK	0	-30	64	+34
			CaPK	0	-34	72	+38
			NPKCa	100	-15	119	+4
Shiga, Japan (35°N)	Wetland rice and wheat	40	Unfertilized	0	-1.7	41 (30)	+39
			PK	0	-13.1	67 (51)	+55
			NPK	152	+2.2	112 (74)	-37
Los Baños, Philippines (14°N)	2 wetland rices (1st-24th crops)	12	Unfertilized	0	+30	116	+146
	2 wetland rices (24th-33rd crops)	5	Unfertilized	2 (rain)	±20**	71	49-89

*Values in parentheses are N uptakes by rice.

**Soil was analyzed after the 24th and the 33rd crops. No change of soil nitrogen was observed. This value is a standard error of analysis.

QUANTIFICATION OF BIOLOGICAL NITROGEN FIXATION

Balance studies

Higher nitrogen fixation in wetland conditions has long been considered the reason for the higher nitrogen maintenance levels in wetland soils than in dryland soils. The nitrogen balance of long-term fertility experiments gives a quantitative answer to the question of nitrogen gains in wetland conditions. Table 2 indicates the results of experiments in Japan and the Philippines where soil nitrogen changes have been determined and compared with crop nitrogen removal. In non-fertilized plots, net gains of soil nitrogen ranged from 20 to 70 kg N/ha per year (in Japan) or per crop (in the Philippines) except a peat soil at Sorachi. The addition of phosphorus and potassium increased nitrogen gains, but in the most heavily nitrogen-fertilized treatments, net losses of nitrogen occurred.

Nitrogen balance data from long-term fertility plots must be examined with care because of possible errors in soil analysis and unquantified role of subsoils. Most Japanese data on soil nitrogen appear significant, because the experiments were conducted for sufficiently long periods. However, in many nitrogen balance calculations, soil nitrogen changes in the subsoil are not considered. A high positive nitrogen balance in Sorachi, where a peat layer was located below the ploughed layer, suggests that subsoil nitrogen contribution may not always be negligible [31]. It is difficult to quantify the contribution of subsoil, unlike other inputs such as rain and irrigation water which can be quantified.

Yanagisawa and Takahashi [32] reported nitrogen uptake data from fertility trials without nitrogen fertilizer in Japan. Average nitrogen removal from 15 experimental stations was 64 kg N/ha, indicating that a net addition of about 60 kg N/ha per crop is necessary to replace nitrogen used by the crop. However, unless subsoil contribution, soil nitrogen changes, volatilization, and other losses are determined, nitrogen gains estimated from nitrogen uptake in no-nitrogen plots can only give an approximate evaluation of nitrogen fixation.

In pot experiments, sources of nitrogen inputs into flooded rice systems are easily controlled. Data on nitrogen balance in pot experiments indicate that: more net nitrogen gain was obtained when the soil surface was exposed to light than when it was protected from light [34-35], more net nitrogen gain was obtained in planted pots than in unplanted pots [33-35], and wetland conditions yielded more nitrogen than dryland ones [35]. These data substantiate an early study by De [36], who showed the importance of blue-green algae in maintaining soil fertility in flooded soil. The role of rice plant in stimulating nitrogen gains or reducing losses is not well analyzed. It is highly probable that nitrogen gains are stimulated by rhizospheric (associative) nitrogen fixation and that nitrogen losses are mitigated by the continuous absorption of soil nitrogen by the plant, otherwise its nitrogen would be lost.

Nitrogen balance sheets give only the sum of nitrogen gains and losses. To estimate gross nitrogen gain, quantification of nitrogen losses is essential. Experiments by Ventura and Watanabe [35] with ¹⁵N₂ labelled soil showed that differences in nitrogen gains (dark versus light treatments, wetland versus dryland) were caused by differences in nitrogen fixation but not differences in nitrogen losses.

Acetylene reduction techniques

It is well recognized that acetylene reduction activity (ARA) assay cannot be used as a quantitative tool unless ¹⁵N₂ incorporation experiments are made in identical conditions and experimental ratio of N fixed to reduced acetylene is determined. Because ARA assay is more sensitive than ¹⁵N₂ incorporation technique, it is difficult to utilize both methods for the same period, particularly when the nitrogen-fixing activity is low. However, acetylene reduction technique is useful, especially when comparative studies are made. When this technique is used in the paddy field, the following limitations should be considered.

Acetylene diffusion into flooded soil and back diffusion of formed ethylene are slow. To introduce acetylene into the system, evacuation of the gas phase [37] and mechanical disturbance [38] were proposed. Mechanical disturbance is also necessary to recover ethylene from the soil [38].

Acetylene inhibits nitrogen-fixing activity of methane-oxidizing bacteria, therefore, the contribution of methane-oxidizing bacteria to nitrogen-fixation cannot be determined [39]. Methane oxidation occurs in paddy soils [40].

Acetylene is decomposed in anaerobic conditions. Thus, prolonged incubation under anaerobic condition leads to a high ARA value due to the stimulation of nitrogen-fixation by microorganisms that probably use the decomposition products of acetylene [41].

Spatial variation of activity is large and *in situ* ARA shows a log-normal distribution [42]. High numbers of replicates (more than 6) and composite soil samples are needed for accuracy. In addition, logarithmic transformation of data is necessary for statistical analysis [42]. Methods for algae, plant associative activity, and anaerobic soils are described by Roger et al. [43], Lee and Watanabe [38], and Matsuguchi et al. [37]. Problems in acetylene reduction assay have been extensively reviewed by Knowles [44]. Acetylene methodology has been most frequently used to assess the activity of specific components of the nitrogen-fixing biomass. Some examples of measurements are given in Table 3. ARA values of photodependent nitrogen fixation have been recently summarized by Roger and Kulasooriya [45]. *In situ* photodependent ARA values ranged from 0 to 600 $\mu\text{mol C}_2\text{H}_4\cdot\text{m}^{-2}\cdot\text{h}^{-1}$ in Senegal [43]; 0.2 to 3 $\text{m mol C}_2\text{H}_4\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ in the Philippines [48]; 0.1 to 4 $\text{m mol C}_2\text{H}_4\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ in Thailand [50]; 0.03 to 0.9 $\text{m mol C}_2\text{H}_4\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ in Indonesia [51]; and 0.8 to 3.2 $\text{m mol C}_2\text{H}_4\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ in Malaysia [52]. Data from Indonesia and Malaysia may include some surface soil activities.

Maximum value may be near $4 \text{ m mol C}_2\text{H}_4 \cdot \text{m}^{-2} \cdot \text{day}^{-1}$. ARA associated with rice plant ranged from $10 \text{ to } 50 \mu \text{ mol C}_2\text{H}_4 \cdot \text{m}^{-2} \cdot \text{plant}^{-1}$. ARA of anaerobic soil ranged from $0 \text{ to } 0.5 \text{ n mol C}_2\text{H}_4 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ [46].

¹⁵N technique

The ¹⁵N₂ incorporation technique gives a direct demonstration of nitrogen-fixation. However, the technique cannot be used in the field to quantify nitrogen-fixation during the growth cycle of rice because of its high cost, point-time measurement, and the need for sophisticated apparatus to control environmental conditions in the closed chamber. In addition, results of laboratory experiments on photodependent nitrogen-fixation are often expressed per unit weight of soil. Because photodependent nitrogen-fixing activities are determined by the surface exposed to light, it is absolutely necessary to express activity per unit of surface area. Data on ¹⁵N₂ incorporation are given in Table 4. If we assume a square metre contains 10^5 g soil and 25 plants, maximum values of heterotrophic, photodependent, and associative (rhizospheric) nitrogen fixation are 30, 43, and $7.2 \text{ mg N} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$. But no data on the ¹⁵N₂-fixing rates of various agents in the same soil are available.

Recently more attention has been paid to the ¹⁵N dilution technique (substrate labelling technique) for quantifying the contribution of nitrogen-fixation in the nitrogen nutrition of organisms or plants [61]. The method is based on the fact that an organism growing at the expense of a substrate labelled with ¹⁵N (combined nitrogen) in a system where no nitrogen-fixation occurs accumulates more ¹⁵N than a similar organism growing on the same substrate in a similar system where nitrogen-fixation occurs.

The validity of the estimation by this technique depends on the choice of the non-nitrogen-fixing control. Ventura and Watanabe [35] applied the ¹⁵N dilution technique to assess the contribution of photodependent nitrogen-fixation in wetland rice using rice plants grown in pots covered by black cloth as control. From this experiment, it was found that two rice crops absorbed 20–30 per cent of nitrogen gain in flooded soil.

No data are available for the application of this technique to assess nitrogen fixation by blue-green algae and *Azolla-Anabaena* symbiosis.

Relative importance of various nitrogen-fixing agents

Nitrogen balance data shown in Table 2 indicate that photodependent nitrogen fixation is more active than heterotrophic nitrogen-fixation in soils tested in the tropics. Watanabe et al. [48] estimated the contribution of nitrogen fixers in floodwater and on the soil surface by measuring ARA before and after the removal of floodwater and surface soil and their subsequent replacement with alga-free water. They concluded that nitrogen-fixing activity by blue-green algae, and perhaps bacteria associated with the algal biomass, was greater than that of other microorga-

Table 3. N₂-fixation measured by acetylene reduction assay and percentage contribution of floodwater, soil, and rhizosphere

Site	Treatment	Fixing rate kg N ha ⁻¹ crop ⁻¹ *	Contributions (%)			Method	References
			F	S	R**		
Japan	NPK	11	0	60	40	<i>In vitro</i>	Matsuguchi [46]
	NPK + compost	17	0	70	30		
	NPK + straw	19	5	80	15		
Japan	No fertilizer	1.1	<5	>85	<10	<i>In vitro</i>	Panicksakpatana et al. [47]
	Green manure	3.8	<5	>85	<10		
Philippines	Wet season	24	43	22	35	<i>In situ</i>	Watanabe et al. [48]
	No fertilizer (163 days)						
	Dry season	34	69	17	14		Watanabe et al. [49]
	No fertilizer (168 days)						

*Assume that 1 mol C₂H₄ reduction is equivalent to 9.3 g N-fixed.

**F=floodwater; S=reduced soil; R=rhizosphere.

nisms associated with wetland plants. Wada et al. [62] and Panichsakpatana et al. [47], on the other hand, reported very little photodependent nitrogen-fixing activity in floodwater and surface soil, and concluded that these sites were not important for nitrogen fixation in rice fields (Table 3).

Field acetylene reduction assays by Lee et al. [63] could not detect nitrogen-fixing activity of the reduced layer, because acetylene does not diffuse into the bulk of the reduced layer. Watanabe et al. [49], who adopted a soil core assay method to measure reduced soil ARA, reported an average daily ARA of $0.30 \text{ m mol C}_2\text{H}_4 \cdot \text{m}^{-2}$ in Maahas soil (pH 7.5) during a rice growing season (Table 3). The relative importance of the various nitrogen-fixing agents depends on environmental conditions and cultural practices. For example, different authors have reported a higher activity of floodwater in unfertilized plots than in fertilized plots [47, 48, 62]. The effects of environmental and agricultural factors are discussed in the following sections.

ECOLOGY OF PHOTODEPENDENT NITROGEN-FIXING ORGANISMS

Photodependent nitrogen-fixing organisms that develop in the photic zone (floodwater and soil-water interface) are free-living and epiphytic blue-green algae (BGA), *Anabaena azollae* in symbiosis with *Azolla*, and photosynthetic bacteria. The ecology of these organisms is summarized in this section. Further information is contained in recent reviews on BGA in rice fields by Roger and Kulasoorya [45] and on *Azolla* by Lumpkin and Plucknett [64-65], Peters and Calvert [66], and Watanabe [67].

Free-living blue-green algae

In paddy fields BGA growth and algal successions are governed by climatic, physicochemical, and biotic factors.

Light intensity is the most important climatic factor. BGA are sensitive to high light and develop protective mechanisms like vertical migrations in the water of submerged soils; preferential growth in shaded zones like embankments, under or inside decaying plant material, or a few millimetres below the surface; photophobotaxis; photokinesis, and stratification of the strains in algal mats where nitrogen-fixing strains grow under a layer of eukaryotic algae [68]. In areas with high incident light intensities, BGA develop later in the crop cycle when the plant cover is dense enough to protect them from excessive light [69]. On the other hand, light deficiency may also be a limiting factor. In Japan, available light under the canopy was below the compensation point of the phytoplankton during the later part of the growth cycle [70]. In the Philippines, during the wet season, under moderate light, ARA was higher in bare soil than in planted soil [48].

Temperature is rarely a limiting factor for BGA in paddy fields because

the range of temperature permitting their growth is larger than that required by rice. However, temperature influences the composition of the algal biomass and the productivity. The optimal temperature for BGA is 30–35°C. Low temperatures decrease productivity and favour eukaryotic algae. High temperatures favour BGA and increase algal productivity [45].

pH is the most important soil factor, among the soil properties, determining the composition of algal flora. Under natural conditions BGA grow best in neutral-to-alkaline environments. In rice fields positive correlations occur between water pH and BGA number, soil pH and BGA spores, soil pH and the nitrogen-fixing algal biomass in samples homogeneous for stage of rice development, and in fertilization and plant cover density [68]. The beneficial influence of high pH on BGA growth is demonstrated by the addition of lime, which increases BGA growth and N₂ fixation [45]. However, the presence of certain strains of BGA in soils with pH values between 5 and 6 has been reported [71–72].

Phosphorus availability is another important factor determining BGA growth. Okuda and Yamaguchi [73] incubated 117 submerged soils and noted that BGA growth was closely related to the available phosphorus content of the soil.

Biotic factors (organisms) that limit BGA growth are pathogens, antagonistic organisms, and grazers. Of these, only grazers have been documented. The development of zooplankton populations, especially Cladocerans, Copepods, Ostracods, and mosquito larvae prevented the establishment of algal blooms within one or two weeks [74]. Grazing rates and algal diet preferences of Ostracods were studied by Grant and Alexander [75] who estimated the potential consumption of BGA at an average field density of 10,000 Ostracods m⁻² to be about 120 kg (fresh weight) ha⁻¹ day⁻¹. An economic alternative for controlling Ostracod populations is the application of crushed seeds of neem tree (*Azadirachta indica*) [76]. Snails form another group of algal grazers in submerged paddy fields. The biomass of snails can be as much as 1.6 t/ha in some rice fields in the Philippines [45]. Commercial pesticides that can control grazers are expensive and uneconomical [76].

Agricultural practices to encourage BGA growth: The growth of nitrogen-fixing BGA in rice fields is most commonly limited by low pH, phosphorus deficiency, and grazer populations. Application of phosphorus and lime has frequently produced positive results. An increase in algal biomass has also been reported as a secondary effect of insecticide application [45].

Recently, surface application of straw was reported beneficial for BGA growth and photodependent ARA [77–78]. This may be due to an increase of CO₂ in the photic zone, a decrease of mineral nitrogen and O₂ concentration in the floodwater, and the provision of microaerobic microsites by the straw. Increased CO₂ availability and low nitrogen concentration are

known to favour the growth of nitrogen-fixing BGA. Low O_2 concentration in the photic zone may have increased their specific nitrogen-fixing activity.

Epiphytic blue-green algae

Nature and distribution of nitrogen-fixing blue-green algae: Epiphytic BGA have been observed on wetland rice [79], deepwater rice [173-174], and on weeds growing in rice fields [80]. A comparison of these different hosts [175] revealed that epiphytism and the associated ARA on wetland rice at seedling, tillering, and heading stages and on the submerged weed *Chara* were predominantly due to colonies of *Gloeotrichia* sp. visible to the naked eye. Epiphytic algae on wetland rice at maturity, on the submerged weed *Najas*, and on deepwater rice could be observed only under a microscope. The dominant algae were *Nostoc*, *Calothrix*, and *Anabaena*. A unique finding was that BGA also exist inside the cavities of senescent rice leaf sheaths. This "endophytism", however, in addition to being not confined to rice, was not present in living healthy tissues. A frequent observation was that older parts of the hosts and plants with rough surfaces supported more epiphytic BGA. It was concluded that epiphytism is possibly related to abiotic effect, of which a mechanical effect in relation to the roughness of the host surface appears to be important.

Nitrogen-fixing activity of epiphytic BGA: Rates of light-dependent ARA on wetland rice gradually diminished from seedling to maturity mainly due to the concomitant decrease of *Gloeotrichia* epiphytism and the reduction of available light. In deepwater rice there was also a decrease in specific ARA (activity per gram of host) from heading to maturity but this was compensated by an increase in the host biomass so that a constant activity per plant was observed at both stages. The results of ARA measurements indicated that nitrogen contribution by nitrogen-fixing microorganisms epiphytic on wetland rice is low but epiphytic BGA play an important role in inoculum conservation because floating algae and soil algae are frequently washed from the field during heavy rains [79-80]. On the other hand, epiphytic nitrogen fixation on deepwater rice makes a substantial nitrogen contribution to this ecosystem (10-20 kg N/ha) mainly due to the greater biomass available for colonization by epiphytic BGA [81].

Fate of epiphytically fixed nitrogen: The importance of epiphytic nitrogen-fixation and the availability of epiphytically fixed nitrogen was evaluated by Watanabe et al. [81] and Watanabe and Ventura [60], using ^{15}N techniques. Direct evidence of nitrogen-fixation associated with deepwater rice was obtained by exposing submerged parts of a plant to $^{15}N_2$ for nine days. There was higher enrichment of $^{15}N_2$ in submerged nodal roots and leaf sheaths where BGA grew epiphytically. During the nine-day-period 8 mg nitrogen was fixed by the plant and at maturity (Table 4) and 40 per cent

Table 4. Evaluation of N₂-fixation in flooded soil and/or wetland rice by ¹⁵N₂ experiments

Systems	Exposure period (days)	Daily N ₂ -fixing rate	References	Note
Heterotrophic in soil	30	0.133 (μg/g soil)	Rao [53]	
Heterotrophic in soil	30	0.13 ~ 0.3 (μg/g soil)	Kalininskaya et al. [54]	(a)
Photodependent in floodwater +soil	28	3.0 ~ 5.6 (mg/m ²)	MacRae and Castro [55]	(b)
Photodependent + heterotrophic floodwater +soil	730	0.15 (μg/g soil)	Reddy and Patrick [56]	
Photodependent in floodwater +soil	30	43 (mg/m ²)	"	
Photodependent in floodwater +soil +weed	30	5.8 ~ 7.5 (mg/m ²)	"	
Associative in plant	7	195 - 350 (μg/plant)	Ito et al. [57]	
Associative + heterotrophic in plant +soil	7 ~ 13	54 ~ 105 (μg/plant)	Yoshida and Yoneyama [58]	(c)
Associative + heterotrophic in plant		13 - 20 (μg/plant)		
Associative + heterotrophic in plant +soil	13	576 (μg/plant)	Eskew et al. [59]	(d)
Associative + heterotrophic in plant		99 (μg/plant)		
Associative + heterotrophic in plant +soil	3	238 (μg/plant)	Eskew et al. [59]	
Associative + heterotrophic in plant		40 (μg/plant)		
Photodependent epiphytic with deepwater rice	9	900 (μg/plant)	Watanabe and Ventura [60]	(e)

(a) Three kinds of planted paddy soils were used. Soils were taken at tillering stage of rice and after harvest.

(b) Original data which were expressed per g soil were converted to surface area. Ten g soil was placed in 1.1 cm diameter tube. Three kinds of soil were used.

(c) Range of data was obtained by four experiments.

(d) After exposure of plant and soil system to ¹⁵N₂, the plant was grown further to maturity in normal air. ¹⁵N₂ remaining in soil and plant might have been further fixed. Therefore, actual exposure period was longer than 13 days.

(e) Only aquatic parts of plants were exposed to ¹⁵N₂. Plant was grown further in deep water to maturity.

of the fixed nitrogen was found in parts of the plants not directly exposed to $^{15}\text{N}_2$ [60].

In the shallow-water rice, epiphytic BGA make only a small contribution to the nitrogen input but in deepwater rice they produce a substantial nitrogen input especially important in a cropping system where nitrogen fertilizer is seldom applied.

Symbiotic blue-green algae and *Azolla*

Anabaena azollae fixes molecular nitrogen in symbiosis with the water fern *Azolla*. Genus *Azolla* comprises two subgenera, *Euazolla* and *Rhizosperma*. *A. filiculoides*, *A. microphylla*, *A. caroliniana*, and *A. mexicana* belong to *Euazolla*; *A. pinnata* and *A. nilotica* belong to *Rhizosperma*. The various species are widely distributed in tropical and temperate fresh water ecosystems throughout the world. *Azolla* is indigenous in some rice fields and can grow in others when inoculated.

Azolla has been used as a green manure for wetland rice culture for centuries in northern Vietnam [83] and southeastern China [82]. Studies of *Azolla* use in paddy fields were initiated at the International Rice Research Institute [102], the Central Rice Research Institute in India [84], and the University of California [85] in the mid-1970s. Farmers recently adopted *Azolla* as green manure on more than 5,000 ha in South Cotabato, Philippines [86]. Previously, only *A. pinnata* was used as green manure for wetland rice in Asia. Various *Euazolla* species have been recently introduced to Asia and their growth and nitrogen-fixing capabilities are being examined [87-89].

The morphology, life cycle, and physiology of *Azolla-Anabaena* symbiosis are not discussed in this chapter. Reader is referred to other reviews [64, 68, 90, 91]. This section describes environmental factors that affect *Azolla* growth in flooded rice soils: temperature, light intensity, water and wind, pH, mineral nutrition, and pest pressure.

Temperature: Temperature requirement differs among *Azolla* species. The optimum for *A. pinnata*, *A. mexicana*, and *A. caroliniana*, grown at constant temperature under artificial light (15 klx), was about 30°C, whereas *A. filiculoides* required 25°C [92]. The response of nitrogenase activity to temperature from 10 to 42°C also showed that *A. filiculoides* grows best under lower temperatures. Although *A. pinnata* is widely distributed in the tropics, it grows best in cooler season [89]. Watanabe and Berja [93] examined the growth of *A. pinnata*, *A. filiculoides*, *A. mexicana*, and *A. caroliniana* at average temperatures of 22, 29, 33°C with 8°C difference between day (12 hours) and night (12 hours). Growth rate during the exponential phase was either larger at higher temperature or almost equal at the three temperature levels except in *A. filiculoides* which exhibited a lower growth rate at higher temperature. Maximum biomass at stationary phase decreased as temperature increased. At 22 and 29°C, *A. caroliniana* achieved the highest biomass. At 33°C, strains of *A. pinnata* recorded the highest biomass. This

experiment also showed that *Azolla* becomes more sensitive to higher temperature at higher plant density than at lower plant density. Temperature response is related to other factors. The lower the temperature, the lower is the optimum light intensity for growth and nitrogenase activity [94-95]. Because *Azolla* use depends on tolerance for high temperature in the tropics and tolerance for cold in the temperate region, various species were tested for their adaptability to different climatic conditions [87-89]. From trials in Hanchow, China, *Azolla* species were classified into four groups according to temperature response:

1) cold-tolerant, heat-sensitive type: *A. filiculoides* and *A. rubra* (the latter is close to *A. filiculoides*), optimum temperature about 20°C, minimum limit -5 to -8°C, maximum limit 38 to 40°C.

2) heat-tolerant, cold-sensitive type: *A. microphylla* and *A. mexicana*, optimum temperature 25-30°C, minimum limit 5 to 8°, maximum limit 45°C.

3) relatively cold-tolerant and heat-tolerant type: *A. caroliniana* and *A. pinnata* var. *imbricata* (*A. pinnata* species which is common in Asia), optimum temperature 25 to 30°C, minimum limit -3 to -5°C, maximum limit 45°.

4) cold-sensitive and heat-sensitive type: *A. pinnata* var. *africana* (*A. pinnata* which is common in Africa) and *A. nilotica*, optimum temperature about 25°, minimum limit, 8 to 3°C. Maximum limit about 38°C.

Light: Experiments with short periods of exposure to various light intensity showed that light saturation for nitrogen-fixing activity is about 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ (16 klx) [96], 5 klx [97], and 5 to 10 klx [98]. When growing *in situ*, *Azolla* requires higher light intensities because the plants overlap each other. Growth increases with light intensity up to values of 400 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ or 32 klx [92], 40 klx [98], 49 klx [94]. Further increase in light intensity was reported to retard growth [94, 99].

Although shading not only reduces light intensity but also the temperature of water and air during sunny midday, according to the experience in our laboratory, some shading is beneficial for the growth of the ferns. Further, *A. pinnata*, *A. mexicana*, and *A. caroliniana* have been observed to turn red in strong sunlight and remain green in shade.

Water and wind: *Azolla* is sensitive to drought. Ungerminated sporophyte is more tolerant of desiccation than vegetatively growing plants. The fern can grow on the surface of water-saturated soil but it grows more slowly than on water surface because abscission of leaves, which triggers further vegetative propagation, is easier in floating condition. Wind and wave action, as well as other strong turbulence, causes fragmentation and diminishes growth. Therefore, *Azolla* is not found on large lakes or swiftly moving waters [94]. It was often experienced in the Philippines that *Azolla* did not survive after a typhoon.

pH: In buffered liquid medium, *Azolla* produced equal biomass

between pH 5 and 8, but its growth was retarded at pH 9 [92]. Singh [100] reported that acid soils (pH 3 to 3.6) did not support growth and the fern died.

Nutrient supply: Except for nitrogen which can be supplied entirely by nitrogen fixation, the macronutrients essential to the symbiosis are the same as those of other photoautotrophs. Deficient levels of macronutrients in *A. pinnata* are 0.08 per cent P, 0.4 per cent K, 0.18 per cent Ca, and 0.016 per cent Fe on dry matter basis [89]. In continuous flow culture, Subudhi, and Watanabe [101] determined that threshold phosphorus concentration is between 0.06 and 0.03 ppm in water and 0.1 per cent in plants. *Euazolla* species required higher threshold phosphorus in the plant than *A. pinnata*. The growth of *Azolla* floating on floodwater is limited by the release of phosphorus from soil to floodwater which is too slow to meet its requirement. Floating *Azolla* utilizes only water-soluble phosphate (superphosphate) which is easily adsorbed by the soil. Therefore, split application of phosphate fertilizer directly on the leaves of the plant is needed to maximize the efficiency of utilization of phosphate applied to *Azolla* [102].

In some phosphate-rich and light-textured soils in the Philippines, *Azolla* can double its biomass in about three days without external source of phosphate [86]. In some alkaline soils, supplemental iron is required to support the growth of *Azolla* [96].

Pests and predators: *Azolla* is attacked by many kinds of pests—insects, snails, algae, and fungi. Insects cause the most severe damage. *Azolla* pests have been reviewed by Lumpkin and Plucknett [65]. Among Lepidoptera, *Pyralis*, *Nymphula* and *Cryptoblabes* are reported in China and South and Southeast Asia. The larvae feed on *Azolla* leaves and construct a tunnel of *Azolla* leaves and sometimes roots, and live inside the tunnels [103]. Damage by Lepidoptera is more severe at higher temperatures. Among Diptera, *Polypedilum*, *Chironomus*, and other Chironomidae are destructive pests. *Bagous* (Coleoptera) and a grasshopper, *Criotettix* (Orthoptera), are also *Azolla* pests. The latter are the most destructive among all insects [104]. Snails (*Lymnaea*) eat young roots and leaves but are less harmful than insects. Some species and strains of *Azolla* are sensitive to fungus attack. This attack is triggered or accompanied by diminished *Azolla* growth under unfavourable conditions (the authors' observation). *Rhizoctonia* and *Sclerotium* were isolated from *A. pinnata* [105]. Chemical pest control is possible, but use of pesticides limits the economical feasibility of *Azolla* as a green manure [86]. Cheap ways of controlling pest damage must be sought.

Photosynthetic bacteria

Photosynthetic bacteria are generally thought to make no significant nitrogen contribution to paddy fields, because they have a low nitrogen-fixing activity that is inhibited by O₂ whereas the floodwater and the soil-water interface are likely to be aerobic. However, these organisms are

relatively abundant and as many as 10^5 to 10^7 have been found per ml of floodwater or per gram of soil [106]. Recent results of Habte and Alexander [107] suggested that fixation by photosynthetic bacteria increases when BGA are killed by chemical treatments. Experiment at IRRI (unpublished) showed a 10^5 times increase of photosynthetic bacterial population after surface incorporation of 4 t of straw per hectare; maximum value recorded was 10^8 *Athiorodaceae* per gram of 5 mm top soil. Filters inhibiting specifically the photosynthetic activity of BGA were used in a pot experiment to assess the N_2 -fixing activity of photosynthetic bacteria. No nitrogen accumulation was detected in pots covered with filters whereas nitrogen accumulated in the surface soil of the controls (IRRI Annual Report for 1981). This indicates at least a low activity of photosynthetic bacteria compared with BGA.

ECOLOGY OF HETEROTROPHIC NITROGEN-FIXING ORGANISMS

Two types of heterotrophic nitrogen fixation are recognized. One is associated with the rice plant and the other is dependent on organic debris from crop residues and aquatic biomass.

N_2 fixing microorganisms associated with rice

As mentioned in the section on nitrogen balance, positive nitrogen gains are higher in the presence of rice. Nitrogen fixation takes place in close association with the rice plant, particularly with the roots. There is evidence that nitrogen fixation is greater in planted soil than in unplanted soil [54, 108]. This section deals with nitrogen fixation by bacteria living on or in rice tissue (associative nitrogen fixation). Recent development on associative nitrogen fixation was reviewed by Patriquin [176].

Evidences of process: Sen [109] discussed the presence of heterotrophic nitrogen-fixing bacteria in rice roots, but the significance of his suggestion was overlooked. Döbereiner and Campelo [110] studied the presence of nitrogen-fixing bacteria in or on the roots of tropical graminaceous plants. Rinaudo and Dommergues [111] and Yoshida and Ancajas [108] found that some nitrogen-fixing (acetylene reduction) activity was associated with wetland rice roots.

Initial suggestions were based on ARA of the excised root [108]. Field assays of less disturbed soil cores with plants developed [63, 113] showed ARA values which were higher in the presence of plants [63].

Field daily ARA values in the Philippines [48, 114-115], Thailand [50], Senegal [116] and the West Indies [117] were in the range of 10-50 $\mu\text{mol C}_2\text{H}_4$ per plant except in acid soils [50, 118].

Incorporation of $^{15}\text{N}_2$ into wetland rice plants enclosed in a ^{15}N -enriched atmosphere was demonstrated by Ito et al. [57], Eskew et al. [59], and Yoshida and Yoneyama [58]. Ito et al. [57] transferred field-grown rice

plants to water culture and measured ^{15}N uptake for seven days. Water culture avoids the contribution of nitrogen fixation in soil and dilution of $^{15}\text{N}_2$ by nitrogen gas in soil solution. The basal portions of shoots and roots were most enriched with ^{15}N . Transfer of the fixed ^{15}N to growing tissue was low during the seven-day exposure to $^{15}\text{N}_2$. Eskew et al. [59] exposed the rice plant at heading stage in soil for 13 days under $^{15}\text{N}_2$ gas. Immediately after the exposure, one tiller was analyzed for ^{15}N . Only a small amount was detected. The plant was grown to maturity under normal air. During this period, ^{15}N content in panicles and shoots increased. At maturity, 68 per cent of ^{15}N in the plant was recovered in the panicles, indicating that fixed nitrogen is available to rice. Compared with the results of the other authors, the results of Yoshida and Yoneyama [58] showed a much faster transfer of ^{15}N from soil and roots to shoots and panicles. The reason for this apparent discrepancy is unknown. In experiments where rice plants were grown in soil, 75–80 per cent of the fixed atmospheric nitrogen was recovered in the soil. This means that root zone soil is a more active site for nitrogen fixation in flooded-soil rice systems than the rice plant itself.

N₂ fixing bacteria associated with rice root: Various nitrogen-fixing bacteria have been isolated from the rhizosphere, roots, culms and leaf sheaths of rice. The colony forming units (CFU) of aerobic bacteria in the rhizosphere soil or roots were higher than those of anaerobic bacteria [119–120]. Rhizospheric bacteria include *Azotobacter* [121], *Azospirillum* [122–125], *Pseudomonas* [125–127], *Enterobacter* [125–128], *Klebsiella* [125] and *Alcaligenes* [128].

To determine predominant nitrogen-fixing bacteria of the rice root and rhizosphere, the rhizosphere soil (attached to the root), the washing from the root and the macerated root, and the macerated basal portion of shoots were diluted and inoculated to 0.1 per cent tryptic soy agar plates. The colonies that developed were isolated and assayed for nitrogen fixation activity on semisolid medium amended with yeast extract. About 80 per cent of the isolates from rice root gave positive N_2 -fixation results [126]. The majority of N_2 ase positive bacteria, identified as *Pseudomonas* [127], had uptake type hydrogenase activity [129], and were H_2 -dependent chemolithotrophs (unpublished). CFU of *Azospirillum* was about ten or hundred times lower than CFU of *Pseudomonas* [130]. Among *Azospirillum*, *A. lipoferum* was found at higher frequency than *A. brasilense* [124]. Predominance of *Pseudomonas* over *Azospirillum* was also determined by fluorescent antibody staining of root (unpublished).

Thomas Bauzon et al. [125] used a "spermosphere model" to study the composition of the nitrogen-fixing microflora of a rice rhizosphere (rhizosphere soil plus root). Serial dilutions of a rhizosphere sample were inoculated to semisolid inorganic medium supplemented with yeast extract, on which the germinated rice seedling (spermosphere model) was grown in the dark. N_2 -fixing bacteria were isolated from the highest dilution which

gave nitrogenase activity associated with the spermosphere. This system produced N_2 -fixing isolates with a 65 per cent frequency. Densities were 10^5 per gram of dry sample in the initial rice rhizosphere. The bacteria isolated in this system were *Klebsiella oxytoca*, *Enterobacter cloacae*, *Azospirillum* and *Pseudomonas paucimobilis*.

Factors affecting nitrogen-fixing activity and microflora

Rice growth stage: Many investigators have shown that N_2 -fixing activity per plant reached its maximum at or near the heading stage [48, 108, 115, 131-132]. A similar trend was observed with ARA of the planted soil [47, 48] and with ARA per gram of dry weight of root [133].

Moisture condition: N_2 -fixing activity (ARA) was much higher with wetland rice than with dryland rice [108, 130]. Densities of N_2 -fixing bacteria (*Pseudomonas* and *Azospirillum*) per root weight were also higher in wetland fields than in dryland fields [130]. *Pseudomonas*, which was dominant in wetland rice roots, was also found in the roots of aquatic plant *Monochoria vaginalis*, but not in the roots of dryland crops and grasses [134]. The reasons for preference for wetland conditions are not known.

Light intensities: Some data show a higher apparent ARA during daytime than at night [119, 135-136]. A higher N_2 -fixing rate during daytime possibly results from an increased exudation of photosynthetate in the rhizosphere. On the other hand, it is also possible that more active transport of gases (C_2H_2 , C_2H_4) during the daytime results in an apparent higher ARA. ARA measurement in a system where gases were forced to circulate [137] did not show a clear-cut trend about diurnal variations. To demonstrate the former possibility, it is necessary to make measurements under conditions where the transport of C_2H_2 to the root and the recovery of C_2H_4 are not limiting.

Oxygen: Because the densities of aerobic bacteria in the rhizosphere were higher than those of anaerobic bacteria and because the aerobically or anaerobically isolated N_2 -fixing bacteria were either microaerophilic or facultative anaerobic N_2 -fixing bacteria [138], it is believed that the nitrogen-fixing process is microaerophilic. By measuring ARA of rice plant in water culture, Watanabe and Cabrera [137] found that ARA in pots aerated with gas with 20 per cent O_2 almost equalled that of untreated pots where oxygen dissolved in water was less than 0.1 ppm. Whereas denitrification was completely stopped by aeration, ARA was not inhibited by aeration (unpublished). These facts suggest that nitrogen fixation associated with rice roots is protected from oxygen damage. On the other hand, van Berkum and Sloger [136], measuring ARA of intact plants with roots that were directly exposed to the atmosphere, found that 0.25 per cent O_2 completely inhibited ARA. When oxygen transport from leaves was stopped by sealing the cut ends of shoots, 0.25 per cent O_2 in the atmosphere surrounding the roots was optimum for ARA of roots. This seems to indicate a microaerophilic nature of nitrogen-fixation of rice roots.

Considering that N_2 -fixation associated with dryland rice is much lower than that associated with wetland rice, it is likely that too much aerobic condition harms N_2 -fixation. Oxygen requirement and tolerance mechanisms for oxygen associated with nitrogen fixation in rice are still unsolved.

Combined nitrogen: Experiments with isolated roots [139] and with water-cultured rice [137] showed that nitrogen-fixation associated with rice root was sensitive to NH_4^-N . ARA was inhibited by 70 per cent at 0.33 mM NH_4^+ [137]. N_2 -fixation associated with plants grown in the soil is less sensitive to the application of mineral nitrogen fertilizer. In fields where fertilizer nitrogen application did not exceed 100 kg N/ha, mineral nitrogen did not depress ARA associated with rice [49]. At the heading stage, when nitrogen fixation associated with rice root is most active, NH_4^+ content of the soil is as low as that of unfertilized soil [140]. This fact may explain the low sensitivity of associative nitrogen-fixation to mineral nitrogen fertilizer when the whole crop cycle is taken into account.

Nitrogen-fixation sites: Because repeated washing does not remove N_2 -fixing microflora from roots [126], it is postulated that nitrogen-fixing flora is located firmly on the surface of or in rice roots. However, information on intracellular and intercellular distribution of diazotrophs in rice roots is insufficient. Huang et al. [141] inoculated Gram negative rods with peritricous flagella into gnotobiotic rice seedlings and observed the presence of bacteria inside the cortex cells using an electron microscope. The basal portion of roots has been reported to be more active than the distal portion [142]

The basal portions of shoots located in the floodwater are also nitrogen fixation sites [143]. The relative importance of the basal portion of shoots is higher at earlier stages of wetland rice growth.

Differences due to species and varieties: It is expected that more nitrogen would be obtained in flooded soil by breeding rice varieties that stimulate greater nitrogen-fixation. Aiming at these goals, differences in associative N_2 -fixation between *Oryza* species and *O. sativa* varieties were sought. Many researchers reported this difference by ARA [115, 131, 132, 144-145]. However, because varietal differences change according to growth stage [115, 131], it is not clear if the observed differences reflected the nitrogen fixation rate during the entire growth period of rice. Techniques to detect differences in nitrogen fixation during the entire growth period are needed. It is not known if observed differences among varieties are genetical or physiological.

N_2 -fixing microorganisms in anaerobic soil

Heterotrophic N_2 -fixation occurs in flooded soil free from living rice roots as shown by $^{15}N_2$ incorporation or by ARA (Tables 3 and 4). Distribution of aerobic N_2 -fixing bacteria in rice soil has been extensively studied [5]. CFU of these aerobic nitrogen-fixing bacteria may decrease in flooded

conditions. However, some data did not show the decrease of CFU of aerobic N₂-fixing bacteria [146-147]. Dilution or plate counts sometimes give counts of aerobic nitrogen-fixing organisms as high as 10⁵ or more [114, 147]. Little is known, however, about predominant aerobic (probably microaerophilic) nitrogen-fixing bacteria in paddy soils.

Recently, the presence of *Azospirillum* in flooded soil has received attention. Charyulu and Rao [148] found that CFU of *Azospirillum* in the flooded soil increased during rice straw decomposition. CFU levels per gram soil were about 10⁶ except in acid saline soil (< 10⁴). This result indicates that *Azospirillum* is one of the active nitrogen-fixing organisms in flooded rice soils.

In laboratory experiments on flooded soils incubated with straw, the increase of nitrogen-fixation in anaerobic conditions was accompanied by the multiplication of *Clostridium* [149].

Sulphate-reducing bacteria are abundant in flooded soils (more than 10⁴ CFU/g soil). Nitrogen-fixation associated with straw increased with the addition of sulphate, 1-2 mg N being fixed per gram of sulphate reduced. Obviously sulphate-reducing bacteria play a role in nitrogen fixation in flooded rice soils, but contribute little to nitrogen gain [150].

Many reports show that heterotrophic nitrogen fixation is more active in flooded or anaerobic soils than in aerobic soils [151-154]. Yoneyama et al. [155] reported that lower Eh values (less than -200 mV) favour nitrogen fixation (acetylene reduction) in flooded soils amended with straw. Matsuguchi [46] and Panichsakpatana et al. [47] also reported that decrease of Eh up to -300 mV stimulated acetylene reduction in the reduced plough layer soil. However, some data indicated that more ¹⁵N₂ was fixed in nonflooded than in flooded soils [156-157]. Difference in N₂-fixation between both conditions were dependent on soils and organic matter. No explanation was given by this group of researchers.

In anaerobic plough layer, organic matter supply limits nitrogen-fixation. Reports by Rao [158] showed from 1 to 7 mg nitrogen fixed per gram straw added. From Charyulu and Rao [156] data, nitrogen fixed per gram of added rice straw (5 g/kg soil) in flooded soils ranged from 0 (acid saline soil) to 1.6 mg (acid sulphate soil). From nitrogen balance data, nitrogen gain stimulated by straw application was about 4 mg N/g straw in planted soil (IRRI 1979 Annual Report). Previous unpublished IRRI data indicated that straw incorporation into flood fallow pots did not have a statistically significant effect on nitrogen balance. Recent results (IRRI 1981 Annual Report) also indicated that nitrogen balance in the presence of rice straw was stimulated by rice plants. Charyulu, Nayak, and Rao [159] found that ¹⁵N₂ fixation stimulated by the addition of cellulose was higher in soil from the planted fields than from fallow fields. This is consistent with the hypothesis that nitrogen fixation, which depends on the externally added organic matter, is higher in the root zone than in fallow soil.

INCREASE IN RICE YIELDS BY THE USE OF NITROGEN-FIXING ORGANISMS

From agricultural point of view, the final goal is to increase nitrogen fixation in flooded soil and increase rice yields by replacing or supplementing expensive chemical fertilizer nitrogen. Possible means of increasing nitrogen fixation in flooded soils include addition of materials that stimulate nitrogen-fixation such as organic amendments and phosphorus application, inoculation of nitrogen-fixing organisms, and selection of rice cultivars that stimulate greater nitrogen fixation. Inoculation of nitrogen-fixing organisms has been tried in combination with other treatments like phosphorus application. Here, the effects of inoculation with BGA, *Azolla*, and N_2 -fixing bacteria are described and the reasons for the often reported yield increase in rice are discussed.

Effect of algal inoculation

Reviewing literature on BGA and rice, Roger and Kulasoorya [45] concluded the following effects of algal inoculation. Algalization may effect plant size, nitrogen content, and the number of tillers, ears, spikelets, and filled grains per panicle. Grain yield has been the most frequently used criterion for assessing the effects of algalization. Results of field experiments report an average yield increase of about 14 per cent over the control corresponding to about 450 kg grain per hectare per crop where algal inoculation was effective. Results of pot experiments report an average yield increase of about 42 per cent. Because of better BGA growth in pots than *in situ*, pot experiments may be suitable only for quantitative studies.

From field experiments where algalization was done with and without mineral fertilizers (NPK) it appears that yield increase by mineral fertilizers is always higher than that strictly due to algalization. Average yield increase in the presence of nitrogen fertilizers (14.6 per cent) does not significantly differ from that in the absence of nitrogen fertilizers (14.3 per cent). Because biological N_2 fixation is known to be inhibited by inorganic nitrogen, the beneficial effect of algalization in the presence of nitrogen fertilizers was most frequently interpreted as resulting from growth-promoting substances produced by BGA. Such a hypothesis needs to be proven because algalization experiments have been conducted on a "black box" basis, where only the last indirect effect (yield) of an agronomic practice (algalization) was observed. No data on nitrogen fixation and algal biomass measurements in an inoculated paddy field are available. Therefore, the relative importance of nitrogen fixation by inoculated BGA in increasing rice yield, compared with other possible effects like auxinic effects, effects on soil properties, increase of phosphorus availability, etc. is still unknown.

If yield increase is due to nitrogen fixation, part of fixed nitrogen must be utilized by the rice plant. A recent experiment, described below, has quantified the utilization of algal nitrogen by rice.

Availability of algal nitrogen to rice

Uptake by rice of nitrogen fixed by BGA was demonstrated on a qualitative basis by Renaut et al. [160] and Venkataraman [161], using ¹⁵N tracer technique. In a quantitative experiment Wilson et al. [162] recovered 37 per cent of the nitrogen from a rice crop from ¹⁵N labelled *Aulosira* sp. spread on the soil and 51 per cent of the nitrogen from the same material incorporated into the soil. The study was conducted on a laboratory scale and did not include analysis of ¹⁵N remaining in the soil.

Pot and field experiments at IRRI, using ¹⁵N labelled *Nostoc* sp., showed that availability of nitrogen from dried BGA incorporated in the soil was between 23 and 28 per cent for the first crop and between 27 and 35 per cent for two crops. Surface application of the algal material reduced the availability to 14-23 per cent for the first crop and 21-27 per cent for two crops [163]. Availability of nitrogen from fresh algal material was similar to that of dried material when surface applied (14 per cent) but much higher (38 per cent) when incorporated [164]. The pot experiment demonstrated that algal nitrogen was less available than ammonium sulphate for the first crop, but for two crops algal nitrogen availability was very similar to that of ammonium sulphate [165]. That indicates a slow-release nature of BGA nitrogen, which reflects the cumulative effects of algal inoculation [45]. The ¹⁵N balance in plants and soil after two crops (pot experiment, dried algae) showed that losses from ¹⁵N ammonium sulphate were more than twice that from BGA regardless of the mode of application. From these results it was concluded that the BGA material, because of its organic nature, is less susceptible to nitrogen losses than inorganic fertilizer and that its low C/N ratio (5-7) gives it a better nitrogen availability than an organic fertilizer like green manure. The relative availability of algal nitrogen to rice depends on susceptibility to decomposition of the algal material which varies not only with the strains [165], but also with the physiological state as demonstrated by the discrepancy between the values reported by Wilson et al. [162] and those reported by Tirol et al. [163]. Wilson et al. used an algal material collected directly from the flask culture and blended after resuspension in distilled water, whereas Tirol et al. used an algal material dried at room temperature, comprising mainly vegetative cells in dormancy and akinetes, and therefore less susceptible to decomposition.

Effect of *Azolla* inoculation

The effect of *Azolla* on rice yield is principally determined by the biomass and its nitrogen content. Table 5 shows the reported values of maximum biomass and its nitrogen content in the field. However, nitrogen gain in *Azolla* biomass may not be exclusively from atmospheric nitrogen. Soil nitrogen may contribute to a nonnegligible extent. No experimental data on the contribution of soil nitrogen to the nitrogen nutrition of *Azolla* in the field are available. Under favourable experimental conditions,

Table 5. Azolla biomass, its nitrogen and daily N-accumulating rate

Species	Conditions and site	Maximum biomass			Average daily N accumulation	Source
		Dry matter (t/ha)	N content (kg/ha)	Days		
<i>A. filiculoides</i>	Fallow paddy, USA	1.7	52	35	1.5	Talley and Rains [166]
	Shallow pond, USA	1.8	105	—	—	Talley et al. [96]
	Fallow paddy, China	2.2	75	20	3.8	Li et al. [88]
	Rice canopy, China	1.7	66	16	4.1	"
<i>A. mexicana</i>	Pond, USA	0.8	39	39	1.0	Talley et al. [96]
	Fallow paddy, USA	1.1	38	—	—	Talley and Rains [166]
<i>A. caroliniana</i>	Fallow paddy, Philippines	—	40	26	1.5	Watanabe et al. [89]
	Fallow paddy, China	1.8	73	20	3.6	Li et al. [88]
<i>A. pinnata</i>	Fallow, paddy, India	2.3	104	30	3.5	Singh [84]
	Fallow paddy, Philippines	1.1	48	30	1.6	Watanabe et al. [89]
	Rice canopy, Philippines	—	73	91	0.8	Watanabe et al. [89]
	(4 crops)					
	Rice canopy, Vietnam	—	25	60	0.4	Than and Thuyet [83]
	Rice paddy, China	1.8	67	20	3.4	Li et al. [88]
	Rice canopy, China	0.81	36	16	2.2	"

Table 6. Effect of *Azolla* on rice yields in Asia

	Yield (t/ha)			Increase due to <i>Azolla</i>	Source
	Control ^a	N fertilized ^b	<i>Azolla</i> plot ^c		
China, Zhejiang, 1964	3.7	ND ^d	4.4 w/o I, BT	0.7	Liu [82]
			4.4 I, 1 time, BT	0.7	
			4.9 I, 2 times, BT	1.2	
			5.0 I, 3 times, BT	1.3	
China, Zhejiang	4.7	5.6 (60)	5.2 I, AT	0.5	Li et al. ^e [88]
			5.7 I, BT	1.0	
			5.0 I, BT, AT	1.3	
Vietnam, 1958-67	2.4	ND	2.8 I, BT	0.4	Dao and Do [167]
India, 1976 (Kharif)	4.2	5.5 (40)	4.9 I, BT	0.7	Singh [84]
1977 (Rabi)	1.7	3.2 (40)	2.6 I, BT	0.9	"
Thailand, 1977	2.6	2.9 (37.5)	3.5 I, BT	0.9	Sawatdee et al. [168]
IRRI, 1979-80	4.2	5.2 (77)	5.4 I, BT, AT	1.2	Watanabe et al. [89]
4 Asian countries	2.9	3.5 (30)	3.6 w/o I, AT	0.7	Kikuchi et al. ^f [86]
			3.6 I, BT	0.7	
			3.5 I, BT	0.6	
			4.0 I, BT, AT	1.1	

^a No *Azolla* and no chemical N.

^b Figures in parentheses are levels of chemical N applied (kg N/ha).

^c I=incorporated; w/o I=without incorporation; BT=before transplanting; AT=after transplanting.

^d ND=Data not available.

^e Means of 4 species of *Azolla*.

^f Thailand, India, China and Nepal of all sites for 2 years.

Azolla can accumulate 40-120 kg/ha nitrogen within 30 days (Table 5). Gains can be enhanced by growing a second or a third *Azolla* crop after the first crop is incorporated or collected. Annual nitrogen production by *Azolla* thus cultivated throughout the year reaches 450 kg N/ha in the Philippines [102] and 800 kg in India [84] by *A. pinnata*, and 1.2 ton in China by *A. filiculoides* [88, 169].

Azolla is grown either before or after rice transplanting (dual culture) or both. Practices in *Azolla* use have been reviewed by Watanabe [67]. Table 6 summarizes yield responses to *Azolla*. Results at many sites of the International Network Soil Fertility and Fertilizer Evaluation for Rice (INSFFER), which was participated in by many rice growing countries, showed that one *Azolla* crop and its incorporation increased rice yield to the same extent as application of 30 kg N/ha of urea. Growing four or more *Azolla* crops within the wide row spaced rice canopy and incorporating them in soil gave the same yield as 70-100 kg N/ha of chemical nitrogen fertilizer [89]. The increased yield effect of *Azolla* is primarily due to its nitrogen.

Availability of *Azolla* nitrogen to rice

Azolla's nitrogen becomes available to rice after decomposition. Dried *Azolla* releases 47 per cent of its nitrogen as ammonium for four weeks, while fresh *Azolla* releases 60 per cent for the same period. Availability of nitrogen from dried *Azolla* to one rice crop is about 60 per cent of that of ammonium sulphate [177]. The decomposition rate of *Azolla* depends on its nitrogen content and the presence of other components. The lower the nitrogen content, the less available is its nitrogen [89]. Shi et al. [170] compared nitrogen availability of milk vetch, water hyacinth, and *Azolla* labelled with ^{15}N to wetland rice and the following crop of buckwheat. Although *Azolla* had the highest nitrogen content among the green manures, the availability of nitrogen from *Azolla* to wetland rice was the lowest. This was attributed to a high content in lignin, which is about 30 per cent or more on a dry matter basis [87]. Watanabe et al. [89] studied ^{15}N uptake by rice from *Azolla* in pot and field experiments. In pots, 50 per cent of nitrogen from incorporated *Azolla* was absorbed by the plant, whereas 10 per cent was absorbed when *Azolla* floated on the water. In the field, 28-26 per cent of *Azolla* nitrogen was absorbed by rice when *Azolla* was incorporated 30 to 53 days after transplanting. But when *Azolla* was kept on the surface only 15 per cent was absorbed by the plant. Thus, incorporation of *Azolla* showed higher efficiency, as found for inorganic nitrogen fertilizer.

Inoculation of *Azospirillum*

The positive effects of this bacterium to the yields of dryland crops and grasses were summarized by Boddey and Döbereiner [171]. In India, Subba Rao [172] reported the cases of *Azospirillum* inoculation to rice grown in the fields at five sites. This bacterium was grown in farmyard-soil mixture.

Rice seedlings were dipped in the slurry of the inoculant before planting. In some cases, yield increase by inoculation was observed at no nitrogen-fertilizer or at 40 kg N/ha. It is not known if the yield increase was due to nitrogen fixation or other factors.

CONCLUSION

The flood-soil-rice ecosystem is favourable for biological nitrogen fixation. Nitrogen fixation by photodependent microorganisms in the photic zone and by heterotrophic microorganisms in the reduced layer and in association with wetland rice has been studied extensively. Despite the enormous volume of these studies, the nitrogen-fixing rate in flooded rice soils has not been satisfactorily quantified. Inoculation with blue-green algae, *Azolla*, and some nitrogen-fixing bacteria has been made and positive responses of rice yield have been reported. However, the mechanism of increasing yield, particularly with blue-green algae, is still poorly understood. Despite great agricultural use of *Azolla*, the botany, physiological, and improvement of agronomical properties of *Azolla-Anabaena* symbiosis are much less developed than those of legume-rhizobia symbiosis. Considering the fact that almost half of the world population uses rice as a major source of calories, research on nitrogen fixation in flooded-soil rice systems should be further strengthened, particularly through cooperation among scientists in developed and developing countries.

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