cosmopolitan species *D. melanogaster*[9,10]; or between geographic strains of the wide-ranging neotropical species *D. willistoni*[8,11]. On the contrary, in other species like *D. sturtevanti*[12], *D. pseudobscura*[13], *D. serrata*[14], *D. equinoxialis*[8] and the superspecies *D. paulistorum*[15] there exists a pronounced tendency toward homogamic mating. Further, in other species such as *D. prosaltans*[16] and *D. tropicalis*[8], the tendency of males and females to mate within the same strain shows a clear relation to the distance between the geographic localities from which the respective strains came.

With regard to mating isolation, *D. suboscura* seems to behave similarly to the last type of species. According to our results, different geographic strains of the species show significative homogamic tendencies in mating. This is especially clear when the 4 Chilean populations are considered. They show a North-South gradient of ethological isolation. In Europe, by a different method, Sperlich[17] demonstrated a Norh-South cline in mating preferences, which he related to the different levels of chromosomal polymorphism existing in the populations of *D. subobscura*. In general, in our experiments, the European strains show a lower tendency to homogamic mating, either when paired with each other or when combined with the Chilean populations. There is no good explanation for this. In other species, like the incipient species of the *D. paulistorum* complex[18,19] it was observed that the isolation between sympatric strains was almost without exception greater than between allopatric strains. A similar phenomenon might occur in *D. subobscura*. Although obviously all our *D. subobscura* strains are allopatric, it is important to bear in mind that the Chilean strains came from populations which are not yet geographically isolated from each other, but are, on the other hand, very far apart from their European ancestors.

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Strong mutagenic action of a bipyridylium herbicide in a N_2-fixing blue-green alga

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Summary. The herbicide paraquat (1,1′-dimethyl-4,4-bipyridylium ion) has been found to exert a growth inhibitory effect on the N_2-fixing blue-green alga *Nostoc muscorum* in nitrogen-free (N_2) and NO_3^- media, without any apparent inhibitory or stimulatory effect on the heterocyst-forming capacity of the organism. With a dose of paraquat permitting about 20 and 50% survival of this alga a reverse mutation (from *het*$^+$ *nif*$^-$ auxotrophy to *het*$^+$ *nif*$^+$ prototrophy), a forward mutation (for L-methionine-DL-sulfoximine [MSO]-resistance), and an auxotrophic mutation (for carbon-auxotrophy through methylamine [MA]-resistance) have been obtained. The toxic and mutagenic effects of this agrochemical have been compared with those of the well known mutagen MNNG (N-methyl-N′-nitro-N-nitrosoguanidine) and found to be stronger than those of the latter in each case.
Key words. Paraquat; herbicide, bipyridylium; alga, blue-green; mutagenic action; *Nostoc muscorum*.

The current extensive interest in identifying potential environmental mutagens has resulted in intensive efforts to establish reliable screening tests for examining the possible mutagenic and/or carcinogenic actions of various synthetic agricultural chemicals and food additives[2–7] and a number of pesticides (including herbicides, fungicides, insecticides, molluscicides, raticides and nematocides) have been found to be mutagenic in bacterial, mammalian and higher plant systems[8–10]. However, effects on the naturally-occurring blue-green algae, which are invariably present at the time of herbicide application in the fields, and a majority of which are highly beneficial for rice agriculture in tropical countries owing to their significant N_2-fixing properties[11–13], have received little attention from biologists and mutageticists. The mutagenicity of only 2 herbicides, i.e. butachlor (2-chloro-2,6′-diethyl-N [butoxymethyl] acetanilide) and alachlor (2-chloro-2,6′-diethyl-N [methoxymethyl] acetanilide) has been established so far in a N_2-fixing blue-green alga *Nostoc muscorum*, by the author's group[14,15].

The bipyridylium ion is known to liberate free-radicals during its interactions with living organisms, which makes it possible that it may have potent genetic effects[16]. This prompted the author to examine whether a bipyridyl compound can cause genetic alteration to any extent in a blue-green algal system. Paraquat is one of the most popularly used bipyridyl herbi-

cides in the paddy-fields of North India. The present report records the strong mutagenic effects of this chemical in different strains of *Nostoc muscorum* causing reverse, forward and auxotrophic mutations with frequencies significantly higher than the spontaneous and MNNG-induced ones. Even though MNNG belongs to the nitrosamine group, the magnitude of paraquat-stimulated mutation has been compared to that shown by MNNG because of the facts that (1) MNNG causes mutation by acting as an alkylating agent (alkyl donor) in the replicating DNA chains of microorganisms, including blue-green algae[17], and paraquat also carries alkyl groups which might be similarly potent in causing genetic alterations, besides the active free-radicals with possible mutagenic potency liberated during the interactions of paraquat with a living system[16], and (2) MNNG is the most potent mutagen known so far for blue-green algae[18], and therefore, if a chemical shows a parallel mutational yield in the same group of organisms, there are strong reasons for describing that chemical as a potent mutagen.

The filamentous, heterocystous and N_2-fixing (*het*$^+$ *nif*$^+$) parent *Nostoc muscorum*, and its heterocystous, non-N_2-fixing mutant (*het*$^+$ *nif*$^-$) strains[21] were grown routinely in modified Chu 10 medium[22] under the culture conditions described previously[20]. Both these strains form heterocysts in nitrogen-free (N_2) medium and not at all in 5 mM NO_3^- medium, since a NO_3^--metabolizing enzyme, i.e. nitrate reductase, represses the formation of heterocysts in NO_3^- medium[23]. Whereas the parent grows well in both N_2 and NO_3^- media, the *het*$^+$ *nif*$^-$ strain grows in NO_3^- medium only and not at all in N_2 medium due to its non-N_2-fixing nature. Their growth was assessed colorimetrically by optical density determinations during the optimum growth phase, and heterocyst frequency was recorded microscopically in terms of the number of heterocysts per hundred vegetative cells.

Effects of various concentrations of the herbicide paraquat (25, 50 and 75 ppm) were seen on the growth and heterocyst-forming capacity of parent *Nostoc muscorum* and its *het*$^+$ *nif*$^-$ strain in liquid N_2 and NO_3^- media. Paraquat was found to be growth-inhibitory for the 2 strains in both N_2 and NO_3^- media and no growth of either of the 2 strains could be observed with 75 ppm or higher concentrations of paraquat (table 1). However, the chemical did not exert any inhibitory/stimulatory influence on the heterocyst-forming capacity of the organism (table 1). Since the presence of heterocysts in N_2 medium is the indicator of active protein synthesis and photosynthesis[14], and the absence of heterocysts in NO_3^- medium indicates acitive NO_3^- metabolism[23] in a blue-green algal system, it can be clearly implied that the observed effect(s) of paraquat on the growth and heterocyst-forming capacity of *N. muscorum* strains may not be correlated with the dysfunction of either photosynthesis, or protein synthesis or NO_3^- metabolism. Although there is no conclusive report on the fate of the bipyridylium ion in a microbial system, studies on the effects of paraquat in higher plant systems suggest that the addition of 1 electron from photosystem I to a bipyridylium ion gives rise to a free radical which, it is believed, is reoxidized by molecular oxygen to form hydrogen peroxide. This hydrogen peroxide liberates hydroxy and peroxy free radicals which show their immediate phytotoxic action by rapid membrane disruption, leading to the lysis of the living cells[16, 24-26]. Here also, microscopic examination of the herbicide-treated cultures revealed lysis of the majority of the algal cells within 48 h, in proportion to the concentrations of the chemical applied. There is every likelihood that this abrupt killing action of paraquat could be due to its being converted into a more powerful inhibitor within the cells of the treated samples. The inhibitor could cause structural or functional damage at the genetic or non-genetic level. This consideration, along with the fact that paraquat did not cause any major physiological disorder to the organism, led us to investigate its possible genetic toxicity in terms of mutation induction in the parental and *het*$^+$ *nif*$^-$ strains of *Nostoc muscorum*.

For mutagenicity studies effects of paraquat as well as the known mutagen MNNG on the survival of the parent alga were examined on solid (1.2% difco bacto agar) N_2 medium, after Singh and Vaishampayan[14], and 20 and 50% survival doses (table 2) of the 2 chemical (60 and 20 ppm for paraquat; 150 and 120 ppm for MNNG, respectively, applied for 20 min) were used for inducing mutations for the following markers, on which studies of spontaneous mutation have been previously conducted by the author's group; i.e. (1) reverse mutation from *het*$^+$ *nif*$^-$ auxotrophy to *het*$^+$ *nif*$^+$ prototrophy, which was previously known to occur spontaneously in *N. muscorum* at a very much lower rate[14], (2) forward mutation for resistance to 400 µM MSO (50 µM MSO is toxic to *N. muscorum*) (table 1), which occurs spontaneously at a very much lower rate[19]; (3) auxotrophic mutation for carbon-requirement through 5 mM MA-resistance; 1 mM MA is toxic to *N. muscorum* (table 1), and it is known that 5 mM MA-resistant mutants, occurring spontaneously at a very much lower rate, develop carbon-auxotrophy[20].

The general methods of measurement of mutagenesis (selective plate techniques) in *N. muscorum*[14, 19, 20], established previously for scoring the 3 spontaneous mutants described above, were used here except that the paraquat- and MNNG-treated samples were run in parallel in order to have a comparative assessment of the spontaneous as well as MNNG- and

Table 1. Data* on N_2- and NO_3^--mediated growth (increase in optical density at 663 nm during the optimum growth phase, i.e. on 10th day after inoculation; initial OD in each case was 0.005) and heterocyst frequency (number of heterocysts per hundred vegetative cells)** of *Nostoc muscorum* strains: (1) Wild type parental strain, (2) *Het*$^+$ *nif*$^-$ mutant, (3) MA-resistant mutant***, (4) MSO-resistant mutant***, and (5) *Het*$^+$ *nif*$^+$ revertant of the *Het*$^+$ *nif*$^-$ mutant***

Strain	Supplement	Concentration	Growth N_2	NO_3^-	Heterocyst frequency (N_2)
(1)	Nil	Nil	0.635 ± 0.010	0.705 ± 0.012	5.6 ± 0.8
	Paraquat	25 ppm	0.145 ± 0.016	0.205 ± 0.013	5.5 ± 0.6
		50 ppm	0.105 ± 0.014	0.120 ± 0.015	5.5 ± 0.5
		75 ppm	0.0	0.0	5.4 ± 0.7
	MA	0.5 mM	0.301 ± 0.025	0.324 ± 0.019	1.2 ± 1.0
		1.0 mM	0.0	0.0	0.0
	MSO	40 µM	0.215 ± 0.015	0.230 ± 0.014	5.4 ± 0.5
		50 µM	0.0	0.0	5.2 ± 0.6
(2)	Nil	Nil	0.0	0.685 ± 0.017	5.9 ± 0.4
	Paraquat	25 ppm	0.0	0.230 ± 0.015	5.7 ± 0.6
		50 ppm	0.0	0.135 ± 0.014	5.8 ± 0.5
		75 ppm	0.0	0.0	5.3 ± 0.1
(3)	Nil	Nil	0.0	0.0	3.6 ± 0.5
	MA	5 mM	0.645 ± 0.020	0.650 ± 0.115	0.0
	Glucose	3 mM	0.590 ± 0.015	0.635 ± 0.010	5.2 ± 0.4
	Sodium acetate	1 mM	0.575 ± 0.022	0.630 ± 0.013	5.2 ± 0.5
(4)	Nil	Nil	0.505 ± 0.010	0.545 ± 0.021	12.0 ± 0.3
	MSO	400 µM	0.510 ± 0.015	0.545 ± 0.010	12.2 ± 0.4
(5)	Nil	Nil	0.475 ± 0.027	0.535 ± 0.024	4.7 ± 0.6
	Paraquat	25 ppm	0.135 ± 0.010	0.180 ± 0.023	4.5 ± 0.4
		50 ppm	0.095 ± 0.016	0.115 ± 0.021	4.5 ± 0.7
		75 ppm	0.0	0.0	4.4 ± 0.3

*The values are the means of 5 independent readings with their respective standard errors; **Under conditions of massive fragmentation heterocyst frequency was assessed as number of heterocysts per hundred vegetative cells in a microscopic field (the maximum value in each case was obtained on 3rd day of inoculation). Heterocysts were never found in NO_3^- medium; ***The phenotypic characteristics (growth, heterocyst frequency, and paraquat-sensitivity [only in the case of the 5th strain] of each strain were constant regardless of its mode of origin, i.e. isolated spontaneously or by paraquat- or MNNG-induction.

paraquat-induced mutation frequencies for the markers in question. The strains developed after paraquat- or MNNG-induction were raised in axenic clonal cultures and characterized as follows: – (1) *het*$^+$ *nif*$^+$ prototrophic characters of the revertants of the *het*$^+$ *nif*$^-$ strain of *Nostoc muscorum* were tested for their growth efficiency in N_2 medium. Their growth and heterocyst-forming characteristics were found to be parent-like (table 1) and they were thus quite similar to spontaneous revertants of *N. muscorum* studied previously[14]; (2) The MSO-resistant mutants of *N. muscorum* were found to be resistant to 400 µM MSO (table 1), and were thus quite similar in this respect to the spontaneous MSO-resistant mutants of *N. muscorum* isolated previously[19]; (3) The MA-resistant mutants of *N. muscorum* were found to utilize 5 mM MA as a carbon source and failed to grow without an exogenously supplied carbon source, e.g. 3 mM glucose, 1 mM sodium acetate, or 5 mM MA (table 1), and were thus quite similar in this respect to the spontaneous MA-resistant (carbon-requiring) mutants of *N. muscorum* isolated previously[20]. The characteristics (in all the 3 cases) were found to be stable through repeated generations.

It is obvious from table 3 that all possible categories of mutations described above, i.e. reverse, forward and auxotrophic, as suggested by Zimmermann[27], could be significantly scored with both the doses (permitting 20 and 50% survival of the organism) of paraquat and MNNG. Paraquat, like MNNG, may therefore be described as an effective mutagen for blue-green algae. Further, since paraquat-induced mutation frequencies for all the 3 markers were significantly higher than those obtained through MNNG-induction, paraquat appears to be an even stronger mutagen in this group of photosynthetic microorganisms.

Although free radicals have been shown to have mutagenic properties, their mutational yields are lower in microorganisms and yeast[28] than in the plant organisms where reduction and radical formation can take place to a greater extent[16,25]. The observed mutational yield with paraquat was more than 10 times higher in the blue-green alga *Nostoc muscorum* than was found in other microbes[28]; this may conclusively be correlated with the fact that, unlike that in other microorganisms, photosynthesis in blue-green algae is like that found in the chloroplasts of higher plants, involving O_2 evolution at a similar rate[29]. It is reasonable to suggest, therefore, that rates of reduction and radical formation in blue-green algae are identical to those found in higher plants, and this offers a convincing explanation of the considerably higher mutational yields observed in *N. muscorum*, involving a wide range of all possible mutations through paraquat-induction.

On the other hand, the paraquat-induced mutation frequencies obtained here are well comparable to the MNNG-induced mutation frequency in an *Escherichia coli* strain where auxotrophic mutations in over 40% and valine-resistant mutation in about 0.2% of the surviving cells were reported[30]. MNNG, the most potent mutagen known so far in blue-green algae[18], exerts its mutagenic effects by acting as an alkylating agent[17]. Since paraquat also possesses alkyl groups in its chemical structure, it is possible that to some extent it might act like MNNG. However, the theory that the exceptionally high mutational yield through paraquat-treatment in *N. muscorum* was most probably due to the action of bipyridylium ions, or a combination of bipyridyl and alkyl groups, may be strongly favored at the moment in view of the fact that the mutational yield obtained through exposure to MNNG, the most powerful alkylating agent known so far, was comparatively lower. Further detailed biochemical studies on the mode(s) of action of paraquat, as well as in vitro herbicide-microbe interactions are in progress in attempts to establish the strong mutagenicity of this bipyridyl herbicide. Although this agro-chemical has conferred great benefits in increasing paddy-yields in North India per hectare and per man hour[31], in view of the present results its increased discharge into the environment leads to serious concern regarding its possible toxic and mutagenic hazards in living systems. There is wide scope for thorough investigation in tropical rice-fields of the indigenous flora which provides the self-renewable (photosynthetic) natural nitrogen resources, i.e. the N_2-fixing blue-green algae, which have been consistently receiving paraquat treatment for the last few years. In addition, such chemicals may also prove deleterious to the main crop where oxygenic photosynthesis is even more rapid[32], and finally possible carcinogenic hazards to the human system must be considered.

Table 2. Data* on percentage survival of parent *Nostoc muscorum* treated** with various concentrations of paraquat and MNNG in N_2 medium

Concentration of the chemicals (ppm)	Percent survival (%) Paraquat-treated	MNNG-treated
10	61.39 ± 3.81	89.36 ± 0.28
20	48.63 ± 2.04	83.82 ± 1.64
30	43.84 ± 1.68	83.78 ± 1.45
40	35.59 ± 2.44	82.36 ± 1.25
50	27.72 ± 3.15	82.03 ± 6.17
60	21.52 ± 1.73	79.55 ± 2.32
70	8.16 ± 3.68	74.42 ± 0.34
80	0.0	67.85 ± 1.45
90	0.0	67.32 ± 2.41
100	0.0	61.40 ± 1.36
110	0.0	57.38 ± 2.44
120	0.0	50.02 ± 1.13
130	0.0	42.35 ± 3.22
140	0.0	34.02 ± 3.66
150	0.0	21.37 ± 2.69

*The values are the means of 5 independent readings with their respective standard errors; **Treatment time was 20 min. Approachingly identical pattern of survival was found in the *het*$^+$ *nif*$^-$ strain of *N. muscorum*.

Table 3. Data* on spontaneous as well as paraquat- and MNNG-induced** mutation frequencies for (1) reversion of *het*$^+$ *nif*$^-$ mutant to *het*$^+$ *nif*$^+$ prototrophy, (2) 400 µM MSO-resistance, and (3) 5 mM MA-resistance leading to carbon-auxotrophy

Induced by	Mutation frequency (1)	(2)	(3)
Nil (spontaneous)	$1.9 \pm 1.1 \times 10^{-9}$	$1.7 \pm 3.4 \times 10^{-7}$	$2.5 \pm 2.4 \times 10^{-6}$
60 ppm paraquat	$3.8 \pm 1.1 \times 10^{-2}$	$2.6 \pm 0.5 \times 10^{-3}$	$5.2 \pm 0.3 \times 10^{-3}$
20 ppm paraquat	$1.2 \pm 0.9 \times 10^{-2}$	$6.7 \pm 1.0 \times 10^{-4}$	$3.7 \pm 1.4 \times 10^{-3}$
150 ppm MNNG	$5.3 \pm 1.4 \times 10^{-3}$	$5.5 \pm 2.1 \times 10^{-5}$	$4.4 \pm 1.8 \times 10^{-4}$
120 ppm MNNG	$3.6 \pm 1.8 \times 10^{-3}$	$3.7 \pm 0.6 \times 10^{-5}$	$3.5 \pm 2.3 \times 10^{-4}$

*The values are the means of 5 independent readings with their respective standard errors; **Mutagens were used at 20 and 50% survival doses, i.e. 60 and 20 ppm for paraquat; 150 and 120 ppm for MNNG, respectively (applied for 20 min).

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A simplified method for silanization of doublebarrelled ion-sensitive microelectrodes

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Summary. A simplified procedure is described for silanization of one shank of a doublebarrelled microelectrode. this procedure makes it possible to make more functioning electrodes in a shorter time.
Key words. Microelectrode, ion-sensitive; silanization.

For recording the intracellular ion activity with ion-selective microelectrodes it is necessary to make one shank of the doublebarrelled microelectrode hydrophobic. For this reason the hydrophile groups of the inner glass surface must be masked by silane compounds. This procedure ensures that the ion exchanger or related compound remains in the tip during the experiment. But the treatment of the electrode with silanes dissolved in organic solvents is a very delicate process, and after baking the tip of the electrode is often blocked by solid plugs of polymerized silanes[1-8].

An alternative treatment is to handle the ion-selective shank with vapor of silane instead of dissolved compounds[9]. But this method is tedious, for one shank of the doublebarrelled electrode must be perfused with N_2 while the other is perfused with silane and then heated within the same apparatus[9,10]. Therefore a quicker and simpler method is suggested which allows the production of more functioning electrodes in a shorter time, according to the following steps:

1. Use doublebarrelled tubes with inner filament only. Twist them in the middle (180–200°) and pull electrodes with a resistance of about 20 MΩ when they are filled with 3 M KCl.
2. Store the electrodes in a dry atmosphere. The inner surface is then free of water vapor.
3. One stem is blocked with dental wax (Deiberit 502, Ludwig Böhme KG, D-3423 Bad Sachsa).
4. Insert the doublebarrelled electrode into a perforated cap of a closeable vessel such as a scintillation vial, and seal it with plasticine (fig.).
5. The bottom of the scintillation vessel is covered with a silane, which has a high vapor pressure (i.e., di-methyl-di-chlorsilane; Sigma Chemie GmbH, D-8028 Taufkirchen)[11].
6. Screw the cap on to the vessel and expose the tube's free stem for 50–60 sec to the vapor of the silane (total length of the capillary about 5 cm)[12]. This procedure masks the hydrophilic groups of the inner surface[12].
7. Break off the waxed end of the capillary and be sure that both wax and plasticine are completely removed! Fire polish the broken ends to prevent damages to the Ag/AgCl electrode.
8. Bake the tubes (tip down) for about 1 h at 120–140 °C.
9. Fill them from the stem with the different solutions. First the reference stem then the ion-selective stem. If the solution does not run into the tip use a cat's whisker to push it slightly down. The whiskers are also suitable for eliminating air bubbles.

The filled electrodes can be stored for some days in a test solution. The transfer of the electrodes between the different steps should be done on microscope slides which have a piece of cat gut wrapped round them at one end and rubber bands at the other (fig.). The cat gut protects the tips from damage, and the rubber bands fix the capillaries. The microscope slides with the