



Allergenicity of airborne cyanobacteria *Phormidium fragile* and *Nostoc muscorum*

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Abstract

In recent times, airborne microorganisms and their constituents have become prominent safety and health concern. Ongoing climatic changes coupled with unwarranted human activities have significantly deteriorated the ambient air quality. In certain environments, airborne algae contribute significantly to the total biological load of the atmosphere, hitherto dominated by bacteria and fungi. Present study was aimed to investigate the allergenic potency of two frequent viable algal forms i.e., *Phormidium fragile* and *Nostoc muscorum* found in the atmosphere of Varanasi City, India. To test the allergenic potency, crude extracts of these strains were subjected to intra-dermal allergy test and subsequent leukocyte counts, which revealed their allergenic nature. Both the species varied in their allergenic potency. *N. muscorum* appeared to be more allergenic than *P. fragile*. However, when the allergens were mixed in equal amounts, the severity of allergenicity increased significantly. A limited pattern of cross-reactivity between the species was also evident.

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1. Introduction

The quality of air has serious implications on human health. Environments of agriculture, biotechnology, industrial settings as well as the non-industrial indoor environments present unique exposure concerns due to the nature and concentration of the microorganisms encountered, and the susceptibility of the exposed population (Burge and Rogers, 2000). Aerobiological studies conducted in different parts of the world have demonstrated the presence of different microalgal groups in the air (Schlichting, 1969; Brown, 1971; Schlichting et al., 1973; Carson and Brown, 1976, 1978). Microalgae of the class Chlorophyceae, Xanthophyceae, Bacillariophyceae, and Cyanophyceae are common components of the outdoor airborne algal flora (Schlichting, 1961; Brown et al., 1964).

Cyanobacteria may become abundant in the air of tropic zones and warm atmospheres (Schlichting, 1974). Toxins produced by cyanobacteria could be a serious health

hazard to the exposed population and animals coming in direct contact and/or when airborne cyanobacterial toxins are inhaled. Isolated incidents of cyanobacterial toxicity have been documented over the centuries but reports have become more frequent in the recent years (Chorus, 2001).

Though the role of airborne algae in inducing certain type of respiratory problems has long been known (Woodcock, 1948), the question whether airborne microalgae can act as inhalant allergen was first raised by McElhenney et al. (1962). Under normal conditions, an adult human inhales about 240 algal cells^{-h} in tidal breathing zone (Schlichting, 1969). Heise (1949, 1951) could correlate airborne algae with symptoms of hay fever. Bernstein and Safferman (1970) have documented the role of algae in causing allergic reaction to humans. Influenza-like reaction in humans after inhalation of cyanobacterial aerosols has been reported from three Swedish towns (Annadotter et al., 2005).

In general, airborne algae reported from the atmosphere range between 0.3 and 15.0 μm. The size is quite appropriate to be retained by the mucosa membrane of the respiratory system and consequently may cause respiratory

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disease and allergy (Schlichting, 1974). In addition, large airborne residence time of small particles due to the low settling velocity further increases the risk of exposure to the associated allergens markedly (Burge and Rogers, 2000). They have emphasized that in due course of time the ongoing climatic changes could potentially alter the timing and abundance of airborne allergens, including algae. In nature, airborne algae never exist in pure form but mixed with biotic and abiotic particles, and might be producing much severe affects than anticipated (Burge and Rogers, 2000; Villalobosa-Pietrini et al., 1995).

Over the 24-month period of sampling (March 2003–February 2005), we have recorded 34 algal genera from the atmosphere of the Varanasi City (Sharma, 2005). Cyanobacteria appeared to be the dominant aero-algal flora accounting for 72% of the total airborne algal catches represented by 23 genera, followed by green algae and diatoms (26% and 1% with 8 and 3 genera, respectively). *Phormidium* was present in the air through out the year. *Nostoc* was the next dominant one. Among the species *Phormidium fragile* and *Nostoc muscorum* were recorded in all of the sampling months (Sharma, 2005). Present investigation describes the allergenic potency of the two abundant cyanobacterial species *P. fragile* and *N. muscorum*. Allergic response of mice was evaluated against the crude algal extract using intra-dermal test and subsequent leukocyte analysis.

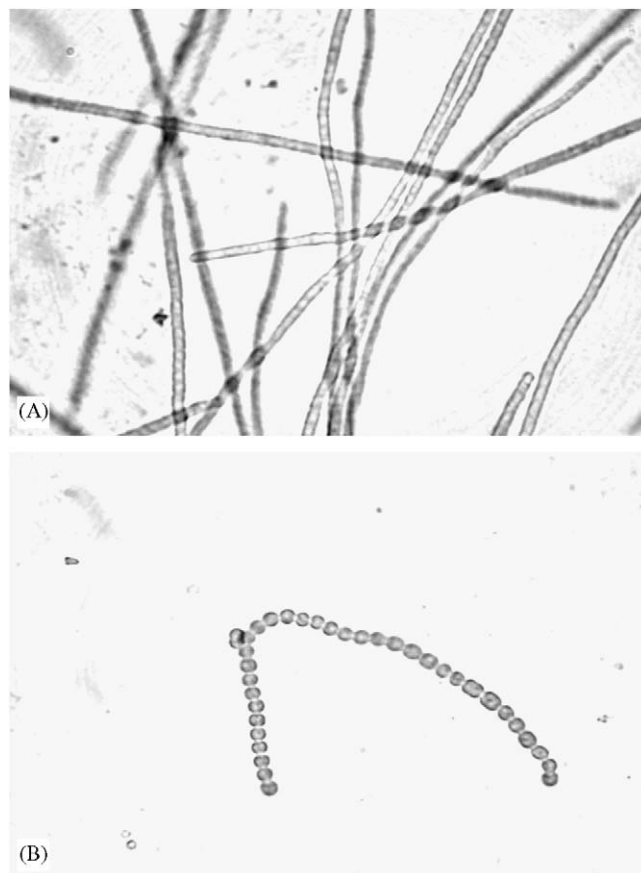


Fig. 1. Microphotograph of *P. fragile* (A) and *N. muscorum* (B). The original magnification was 100 × .

2. Materials and methods

2.1. Test organisms

The two cyanobacterial species *P. fragile* (Meneghini) Gomont (Nostocales, Oscillatoriaceae) and *N. muscorum* Ag. ex Bor. et Flah (Nostocales, Nostocaceae) (Desikachary, 1959) isolated from the airborne algal samples were used as the experimental organisms (Fig. 1). Axenic cultures of *P. fragile* and *N. muscorum* were raised using standard microbiological methods. Cultures were grown in BG 11 medium (Rippka et al., 1979) at $28 \pm 1^\circ\text{C}$ and illuminated with 40 W day light fluorescent lamps giving an average light intensity of $70 \mu\text{E m}^{-2} \text{s}^{-1}$ on the surface of the vessels with a 14:10 light:dark rhythm. Liquid cultures were shaken manually twice a day. Cyanobacterial cultures of late exponential phase were harvested by centrifugation at $10,000g$ for 10 min, washed twice with sterile double-distilled water, lyophilized and stored at -20°C until used.

2.2. Preparation of cyanobacterial extracts and total protein estimation

A known amount (0.5 g) of freeze-dried cyanobacterial cells were suspended in extraction buffer, NH_4CO_3 , 0.1 M, pH 7.8 (1:10, w/v) and sonicated for 5×3 min on ice with cooling intervals of 1 min. The suspensions were centrifuged at $18,000g$ for 30 min at 4°C . The clear supernatants were filtered with sterile Millipore filters ($0.45 \mu\text{m}$) and made to a final concentration of $600 \mu\text{g protein mL}^{-1}$ with extraction buffer. The crude extracts thus obtained were referred to as crude allergen and stored at -20°C until use (Einarsson, 1985). Protein content of the cyanobacterial crude extracts was measured as Lowry et al. (1951).

2.3. Animals

Male AKR mice, 6–8 weeks old obtained from Central Drug Research Institute, Lucknow, were used for the test. Mice were kept at $25 \pm 2^\circ\text{C}$ and 12:12 light:dark schedule in the Department of Zoology, Banaras Hindu University. All the mice were provided with standard mice feed, water ad libitum. The mice were identified by tail tattoo. The study protocol was approved by Animal Ethics Committee, Banaras Hindu University.

2.4. Sensitization of mice

For sensitization, AKR mice were divided into three categories, each group was sensitized by intra-dermal injection of $50 \mu\text{L}$ crude extract ($30 \mu\text{g}$ protein) of *P. fragile* (group A), *N. muscorum* (group B) and both mixed i.e., $25 \mu\text{L}$ each of *P. fragile* and *N. muscorum* (group C), with added equal amount of Freund's complete adjuvant. Process was repeated once a week for 5 weeks (Majd et al., 2004). Sensitized mice were used as experimental models.

2.5. Intra-dermal allergy test

To test the allergenic response of mice against the cyanobacterial strains, crude cyanobacterial extracts (without adjuvant) were injected intra-dermally into the sensitized mice. Experiments were performed in triplicate. Mice Nos. 1–3 (group A) were given $50 \mu\text{L}$ ($30 \mu\text{g}$ protein) of *P. fragile* crude extract, while mice Nos. 4–6 and 7–9 (groups B and C) received similar amount of *N. muscorum* and mixed crude extracts ($25 \mu\text{L}$ each of *P. fragile* and *N. muscorum*), respectively, on the right side of their

bodies. Similarly, six mice (Nos. 10–15), three each from the groups A and B received 50 µL of mixed crude extract on the right side of their bodies.

2.6. Cross-reactivity test

Three mice, Nos. 16–18 from group A were tested with *N. muscorum* crude extract (hereinafter referred to as CRT₁) while, mice Nos. 19–21, belonging to group B were given *P. fragile* extract (CRT₂). The skin reactions like swelling, redness, and/or wheal and flare at the injected site were recorded at 0, 10, 20, 30, 60, and 90 min of injection. The wheal diameter was measured by outlining the margin of affected area with a ballpoint pen at the time of administration and any change in the wheal diameter was marked by repeating the pen drawing at different time intervals. The response was preserved by transferring onto the sellotapes by lifting the ballpoint marks. Extraction buffer (NH₄CO₃, 0.1 M, pH 7.8) was used as negative control, while histamine acid phosphate served as positive control (Majd et al., 2004). A wheal with 4 mm diameter was regarded as the lower limit for positivity, as no reactions of that size were observed with negative control solution. Similarly, 50% reddening of the skin was treated as positive reaction.

2.7. Eosinophils and neutrophils counting

A week after intra-dermal injection of the crude cyanobacterial extract, blood samples were drawn from the heart of the treated mice (Aberg, 1989) and stored at –76 °C until further use. Blood from non-sensitized mice was taken as control. Eosinophils and neutrophils were counted with the help of Neubaur haemocytometer and compared with control mice.

3. Results and discussion

In nature, airborne algal population consists largely of dried algal cells of late growth phase (Schlichting, 1974). Keeping this in view, extraction of allergens was performed using late exponential growth phase cultures. Early and late exponential phase cultures of *Chlorella* are reported to produce almost similar amount of allergen but appeared to be partially degraded during the stationary growth phase (Tiberg et al., 1990). Since, we used crude extracts; allergenicity was tested using intra-dermal method, which was 10,000–30,000 times more sensitive than that of the conventional skin prick test (Turkeltaub et al., 1982).

Both the cyanobacterial species, *P. fragile* and *N. muscorum* were capable to produce allergy in mice (Tables 1 and 2). The parameters used for allergenic response, changes in the wheal diameter and the severity of redness of the tested skin gave almost identical results. Allergic response of the mice injected with *P. fragile* extract was noticed at 30 min of application, whereas the response was fast in the case of mice injected with *N. muscorum* extract, i.e., 20 min of the intra-dermal injection of crude extract. Wheal diameter was maximal at 90 min of crude cyanobacterial extract administration, whether it was from *P. fragile* or *N. muscorum*. However, *N. muscorum* appeared to be more allergenic (wheal diameter 9.6 mm) than that of *P. fragile* (wheal diameter 9 mm). Both the extracts mixed together in equal amount (final concentration 30 µg protein in 50 µL) produced an increase in the wheal diameter within 10 min of the application. The maximal wheal diameter attained at 90 min of administration was 1.6 and

Table 1

Allergic response of mice in relation to time after administering the crude extracts of *P. fragile*, *N. muscorum*, and *P. fragile* and *N. muscorum* mixed, measured as change in wheal diameter

Wheal diameter (mm)					
Time (min)	<i>P. fragile</i>	<i>N. muscorum</i>	<i>P. fragile</i> + <i>N. muscorum</i>	CRT ₁	CRT ₂
0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0
10	4 ± 0	4 ± 0	7 ± 0	4 ± 0	4 ± 0
20	4 ± 0	5 ± 0.58	8 ± 0	4 ± 0	4 ± 0
30	5 ± 0	6 ± 0.58	8 ± 0.58	4 ± 0	4 ± 0
40	5 ± 0	7 ± 0.58	10 ± 1.15	4 ± 0	4 ± 0
50	6 ± 0	8 ± 0.58	11 ± 0.58	5 ± 0.58	5 ± 0.58
60	8 ± 0.58	8 ± 0.58	12.5 ± 0.58	6.3 ± 0.58	5 ± 1.15
90	9 ± 1	9.6 ± 0.58	14.9 ± 0.58	7 ± 0	6 ± 0.58
120	9 ± 1	9.6 ± 0.58	14.9 ± 0.58	7 ± 0.58	6 ± 0

CRT₁ and CRT₂ denotes for cross reactivity test, first (*P. fragile* sensitized mice was injected *N. muscorum* crude extract) and second (*N. muscorum* sensitized mice was injected *P. fragile* crude extract), respectively.

Values are the means ± SD of three replicates, except in the case of mixed extract, where six replicates are used.

Table 2

Allergic response of mice in relation to time after administering the crude extracts of *P. fragile*, *N. muscorum*, and *P. fragile* and *N. muscorum* mixed, measured as intensity of the skin redness

Redness of the skin					
Time (min)	<i>P. fragile</i>	<i>N. muscorum</i>	<i>P. fragile</i> + <i>N. muscorum</i>	CRT ₁	CRT ₂
0	–	–	–	–	–
10	–	–	+	–	–
20	–	+	++	–	–
30	+	+	++	–	–
40	+	++	+++	–	–
50	++	++	+++	+	+
60	+++	+++	+++	+	+
90	+++	+++	+++	++	++
120	+++	+++	+++	++	++

CRT₁ and CRT₂ denotes for cross reactivity test, first (*P. fragile* sensitized mice was injected *N. muscorum* crude extract) and second (*N. muscorum* sensitized mice was injected *P. fragile* crude extract), respectively.

Three replicates are used except in the case of mixed extract, where six replicates are used.

–, No response; +, mild; ++, average; +++, severe.

1.5 times, respectively, than that of the individually injected *P. fragile* and *N. muscorum*, indicating for quicker and more effectiveness of the mixed extract than that of *N. muscorum* and *P. fragile* allergens administered individually. Leukocyte count revealed that the number of eosinophils and neutrophils increased considerably in all the cases (Fig. 2). In mice administered with extracts of *P. fragile*, *N. muscorum* and both mixed, the increase in eosinophils count to that of control were 1.6, 2.6, and 4.2 folds, while neutrophils were 1.7, 2.4, and 2.6 folds, respectively.

Thus, all the three treatments of *P. fragile*, *N. muscorum*, individually and in combination produced varied responses. Despite equal amounts of proteins, quicker

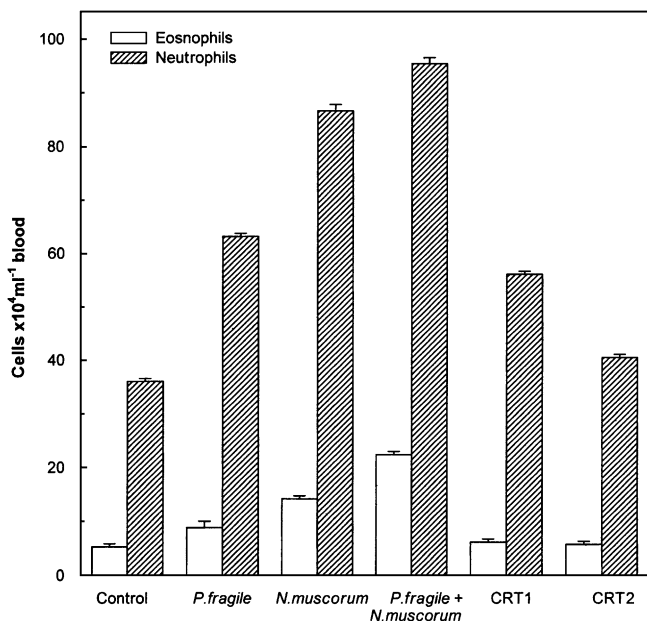


Fig. 2. Eosinophils and neutrophils counts in the peripheral blood of mice administered with crude allergens of *P. fragile*, *N. muscorum*, and *P. fragile* + *N. muscorum* mixed. CRT₁ and CRT₂ denotes for cross-reactivity test, first (*P. fragile* sensitized mice was injected *N. muscorum* crude extract) and second (*N. muscorum* sensitized mice was injected *P. fragile* crude extract), respectively. Values are the means $\pm$ SD of three replicates, except in the case of mixed extract, where six replicates are used.

response to *N. muscorum* allergen than that to *P. fragile* indicates that the potency of allergens is not linked with protein abundance, albeit shows species-specific variation. Howlett and Clark (1981) have found that allergenicity has no relation with protein content in rye grass pollen. On the other hand, no correlation has been found between the toxin content and the allergenic character of cyanobacterial strains. The most toxic *Microcystis aeruginosa* was not the most allergenic sample, but the non-toxic *Aphanizomenon* was the most allergenic one (Torokne et al., 2001). Furthermore, it also suggests that structural and compositional differences of the extracts might be responsible for the difference in allergenicity.

Administration of mixed allergens into mice, sensitized either by *P. fragile* (Nos. 10–12) or *N. muscorum* separately (Nos. 13–15) produced the fastest (maximum wheal diameter attained at 10–30 min of injection) and enhanced (1.6–1.5 times over the individual extracts) response than the mice receiving allergens individually. This synergistic effect might be due to the production of different antibodies against the different antigenic determinants of *P. fragile* and *N. muscorum*. Brown and Lester (1965) have reported immunogenic cross-reactivity between two green algal genera *Tetracystis* and *Chlorococcum*. However, Bernstein et al. (1969) have observed a limited cross-reactivity among different strains of green algae and several non-algal antigens.

To verify the occurrence of cross-reactivity between the selected genera, *P. fragile* sensitized mice were given crude

allergenic extract of *N. muscorum* (mice Nos. 16–18), and *N. muscorum* sensitized mice were treated with *P. fragile* extract (mice Nos. 19–21). The allergenic symptoms were apparent, but slow (50 min of administration) and weak (wheal diameter 7 and 6 mm, respectively). Identical results were obtained for leukocyte counts of the treated mice revealing the weak nature of cross-reactivity. However, the increase in number of eosinophils and neutrophils was comparatively more (1.2 and 1.5 times) when *P. fragile* sensitized mice received crude extract of *N. muscorum* than *N. muscorum* sensitized mice receiving *P. fragile* extract (1.1 and 1.2 folds). The weak nature of cross-reactivity indicates the structural difference in IgE binding fraction of the allergens of both genera.

Sensitization against algae has clinical significance as many a time the amount of airborne algae in the air can exceed the amount of other common allergens (Brown et al., 1964). It has been found that *Chlorella* sensitized children were often sensitized for other common allergens especially molds, possibly due to immunological cross-reactivity (Tiberg and Einarsson, 1989). This simultaneous sensitization is plausible as the growth of mold (one of the most common allergens) and algae is favored by humidity.

4. Conclusions

P. fragile and *N. muscorum* are the frequent cyanobacteria in the air samples of Varanasi city, India. When crude allergenic extracts of these strains were subjected for intra-dermal allergy test and leukocyte counts revealed their allergenic nature. Both the species varied in their allergenic potency. *N. muscorum* appeared to be more allergenic than *P. fragile*. However, when the allergens were mixed in equal amounts the severity of allergenicity increased significantly. A limited pattern of cross-reactivity between the species was also evident.

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