Protective role of *Spirulina* feed in a freshwater fish (*Poecilia reticulata* Peters) exposed to an azo dye-methyl red

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Acute toxicity of an azo dye-methyl red (5-40 ppm) was examined under starving conditions, on two groups of *Poecilia reticulata*—a freshwater fish, fed on different diets prior to their exposure to dye. Besides natural feed, fish of group-1 also received *Spirulina* feed for one month (feed population), whereas those of group-2 received only natural feed (non-feed population). The mortality data revealed non-feed population to be more tolerant to feed stress during acute toxicity study, whereas feed population exhibited better tolerance to the combined stress of both feed and methyl red; especially at higher concentrations of the latter. RBCs in methyl red treatments acquired different shapes (poikilocytosis) and an increase in their size (anisocytosis) was also noticed. Percentage of such abnormal RBCs was almost equal in both feed and non-feed populations, except at a lower concentration (5 ppm), at which percentage of poikilocytic RBCs was lesser in the feed population. RBC counts in the control non-feed fish (34.5 x 10^4/mm^3) were significantly lower than control feed population (50.0 x 10^4/mm^3). Their number decreased with an increase in methyl red concentrations in non-feed population (9-26%), but percent reduction in RBC counts was almost similar (20-26%) at various concentrations of methyl red (5-30 ppm) in the feed population. Despite reduction in RBC counts, feed population did not suffer from anemia in methyl red treatments, as evident by their RBC counts which were almost equal to control fish of non-feed population. The results suggest that *Spirulina* feed improves tolerance of test organism towards methyl red manifested by noticeable reduction in the cytotoxic effects on RBCs and a lower mortality rate at higher concentrations of dye.

Keywords: Acute toxicity, Anisocytosis, Methyl red, *Poecilia reticulata*, Poikilocytosis, *Spirulina*

Azo dyes are one of the major pollutants in the wastewaters discharged from industries such as textile, paper and pulp and tannery. During chronic exposure of organisms to sublethal concentrations (5 and 10 ppm) of an azo dye methyl red in the microcosms, Sharma et al. reported its adsorption in the organisms. The consumption of such organism by fish alongwith contaminated water, lead to their mortality, which was attributed to reduction in their RBC counts, including of histopathological abnormalities in the gills, liver and intestine. Further, a pronounced deposition of methyl red in the gills, lateral line system and intestine of dead fish, and blackish brown matter in their brain was also observed. These abnormalities were dose dependent, being higher in 10 ppm treatment in comparison to 5 ppm treatment.

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In recent years, scientists have reported nutritional and medicinal properties of *Spirulina* relevant to several vertebrates, including human beings. Few publications on *Spirulina* also deal with reduction in genotoxicity and oxidative stress of cisplatin, cyclophosphamide, mitomycin-c and urethane in mice, its protective role in Pb exposed rats and improvement in immunoresponce in mice. Such an improvement in immunoresponce has been attributed to the availability of phycocyanin pigment in *Spirulina*. Improvement in general health, alongwith an increase in RBC counts and protein content of a freshwater fish *Poecilia reticulata* on supplementing their natural diet with *Spirulina* has been reported. This feed may also protect fish from pollutants such as azo dyes. With this backdrop, the effects of *Spirulina* supplementation in natural diet of *Poecilia reticulata*; especially on their mortality and cytotoxic effects on RBC during acute exposure to methyl red have been examined in this study.
Materials and Methods

The Guppy fish (*Poecilia reticulata* Peters; length = 18.5-22.0 mm; width = 2.4-3.0 mm) caught from the tank (6.0x3.7x2.0 m) in the Department of Botany were acclimatized for 15 days in plastic troughs (40 l) containing a good amount of natural population of plankton (both phyto and zooplankton) to serve as food and *Ceratophyllum demersum* (submerged hydrophyte) to oxygenate water. The details of six microcosms used for rearing uniform sized fish from the plastic troughs, for a period of one month, is described elsewhere. During this period, water in the microcosms was replaced daily with tank water to provide natural feed to fish, which was also available to them from periphyton growing over *Ceratophyllum* plants, and also on the under water surface of microcosms. Besides, fish in microcosms were fed daily with *Spirulina* suspension (5 ml/microcosm) prepared by adding 110 mg of its dried powder from Sunova capsule (Dabur Research Foundation) in 30 ml tap water. Interestingly, fish moved fast towards *Spirulina* suspension pipetted in the microcosms, suggesting their preference for it. Hereafter, these microcosm-reared fish are referred to in the text as feed population.

A stock solution of methyl red (1000 ppm) was prepared by dissolving 1 g methyl red in 10-15 ml of ethanol and made up to 1000 ml using tap water. After one-month of stay in the microcosms, the healthy mature fish (length = 21.4-22.6 mm; width = 2.7-3.5 mm) from the microcosms were shifted to plastic troughs filled with de-chlorinated tap water from a bore well in the University Campus and starved for 24 hr. These were then transferred to 5 different concentrations of methyl red prepared by diluting the stock solution (1000 ppm) with tap water and kept for a period of 96 hr for assessing acute toxicity, as detailed earlier. Fish kept similarly in the tap water served as control. Both control and methyl red exposed fish were starved during the period of acute exposure, as per standard convention. Dead fish were removed immediately. After 96 hr of exposure, autopsy of surviving fish was performed for RBC counts and blood smear preparation. The percentage of morphologically abnormal RBCs was calculated by observing approximately 200 RBCs in 20 microscopic fields (10x x 100x) using an oil immersion. The measurements of RBCs were made by an ocular meter standardized with a micrometer scale as parallel magnification.

In another set of experiment, fish caught from the tank were also acclimatized for 15 days, as described earlier. This population, reared only on naturally available feed (plankton) has been termed as non-feed population (without *Spirulina* feed) hereafter in the text. Healthy mature fish (length = 19.8-22.0 mm; width = 2.4-3.0 mm) were used directly for examining acute toxicity of methyl red, as described earlier. All data presented are mean values of three replicates in each treatment. Student’s *t* test was calculated using Systat Version 5.

Results and Discussion

Mortality—Fish mortality was nil in the control fish of non-feed population and was pronounced in the feed population (Fig. 1a,b). Anne documented that natural selection is severe in the early generations, following a switch over from wild to captivity or captivity to the wild. It is important to note here that feed population reared in microcosms had abundance of food (natural + *Spirulina*), prior to their acute exposure under starving conditions in comparison to non-feed population, who fed only on natural feed in the tank. Thus, transfer of control fish of feed population to a new environment (devoid of...
feed) impaired their starvation tolerance in comparison to that of non-feed population, often encountering such situations in nature.

In methyl red treatments however, mortality of non-feed population increased both with concentration and exposure period (Fig. 1b), while the feed population exhibited almost an opposite trend, being maximum at lower concentration (5 ppm) and comparatively lower than non-feed population at higher concentrations (Fig. 1a). Thus, non-feed population was found to be more tolerant to feed stress, as evident by mortality data of control sets of non-feed and feed populations, whereas the tolerance of feed population was better towards the combined stress of feed and methyl red, especially at higher concentrations of the latter. Such an improvement in the immune system has been ascribed to the availability of phycocyanin pigment in *Spirulina* [17].

High protein content of *Spirulina* [18] may have improved their tolerance towards methyl red, as noted by Kushwaha *et al.* [23] in albino rats exposed to monocrotophos.

**Effects on morphology of RBC**—Mature RBCs in control fish were oval, having a distinct centrally placed nucleus. A sizable percentage of them however, acquired different shapes (beaked, reniform, triangular, quadrilateral and pentagonal: poikilocytosis) irrespective of methyl red concentrations in both feed and non-feed populations (Fig. 2). Further, their percentage was almost equal in them, except at a lower concentration (5 ppm) at which their percentage was relatively less in the feed population (Fig. 3a,b). Nucleus in the abnormal RBCs was usually eccentric. Various reasons assigned to RBC poikilocytosis are—an increase in membrane fluidity [24], reduction in ATP levels [25] and inhibition of activity of membrane bound enzymes [26]. It is likely that these changes may decrease the oxygen carrying capacity of the RBC, leading to oxygen stress in the methyl red exposed fish. The lower percentage of abnormal RBCs at 5 ppm in the feed population indicates protective role of *Spirulina*.

**Effect on RBC size**—The RBC size of both feed and non-feed populations increased (macrocytic condition) in methyl red treatments (Table 1), with the exception of 5 ppm concentration, wherein it decreased (microcytic condition) in non-feed population (anisocytosis). The anisocytosis may be the consequence of inhibition of activities of membrane bound enzymes, largely responsible for maintaining the integrity of erythrocyte membrane and its structure [6,27].

**Effect on RBC counts**—The RBC counts in the non-feed control fish (34.5x10^4/mm^3) were significantly lower than feed population (50.0 x 10^4/mm^3). Their number decreased in methyl red treatments, being dose dependent (9-26%) in non-feed population, but almost similar (20-26%) at various concentrations of methyl red (5-30ppm) in feed population (Fig. 4a, b). Thus, cytotoxicity of methyl red to RBC was higher in feed population. It is interesting to note that despite higher percentage reduction, the RBC counts in the feed population were still greater than the control population of non-feed population. Thus, feed population did not suffer from
anemia similar to non-feed population even after exposure to methyl red. This explains lower fish mortality at higher concentrations of methyl red in the feed population, in comparison to non-feed population.

It is thus evident that *Spirulina* feed improves tolerance of fish towards methyl red, which may be ascribed to their better health and increase in RBCs and total protein content. Thus, besides nutritional and medicinal properties, *Spirulina* also enhances fish immunity and hence their tolerance towards methyl red.

### Table 1—RBC measurements (µ) in the feed and non-feed population of *Poecilia*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>5 ppm</th>
<th>10 ppm</th>
<th>20 ppm</th>
<th>30 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feed population</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>7.2±0.53</td>
<td>6.7±0.64* (-7)</td>
<td>7.6±0.83 (+5.5)</td>
<td>8.4±0.96 (+17)</td>
<td>7.3±0.61 (+1.3)</td>
</tr>
<tr>
<td>Width</td>
<td>5.8±0.25</td>
<td>5.2±0.72* (-10)</td>
<td>5.9±1.0 (+1.7)</td>
<td>6.7±0.67* (+15.5)</td>
<td>6.3±0.67* (+8.6)</td>
</tr>
<tr>
<td><strong>Non-feed population</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>7.1±0.24</td>
<td>7.8±0.7* (+9.9)</td>
<td>7.9±0.84* (+11.3)</td>
<td>8.1±0.8* (+14)</td>
<td>8.0±0.93* (+12.7)</td>
</tr>
<tr>
<td>Width</td>
<td>5.3±0.68</td>
<td>6.0±0.78 (+13)</td>
<td>5.4±0.62 (+1.9)</td>
<td>6.1±0.68** (+15)</td>
<td>6.2±0.57 (+17)</td>
</tr>
</tbody>
</table>

*p* values: Significantly different from control at *5%*; **1%**.

### Acknowledgement

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### References

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