A total of about 10,340 flies belonging to different genera of family Drosophilidae were collected since July 2006 to July 2009 (Table 1). Our observations show that the members of Drosophilidae are fairly distributed in these areas. Some of the species, *viz*, *Drosophila immigrans* and *Drosophila nepalensis*, have marked preference to the colder climate. Besides known species, some species of Drosophilidae were not identified and are supposed to be new species.

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Wild stocks of Drosophilidae:

1. *Drosophila immigrans*
2. *Drosophila buscki*
3. *Drosophila nepalensis*
4. *Drosophila melanogaster*
5. *Drosophila repleta*
6. *Drosophila jambulina*
7. *Drosophila takahashii*
8. *Zaprionus indianus*

Coomassie Brilliant blue dye toxicity screen using *Drosophila melanogaster* (Diptera - Drosophilidae).

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Abstract

Synthetic dyes are often found among the wastewater. Among the chromogenic groups, the most common is the azo type, like RBBR (Remazol Brilliant Blue R or Reactive Blue 19). In the family of brilliant blue some of them are used as colorants in textile and leather, others are biochemically relevant dyes commonly used in laboratories for protein determination and gel electrophoresis. Little is known about the health risks of most anthraquinone dyes, motivating us to evaluate the toxicity of Coomassie Brilliant Blue (CBB) using *Drosophila melanogaster* as an insect model. This work investigated the long-term toxic effects of continuous and single exposure of

Drosophila melanogaster to Coomassie brilliant blue added to the culture medium. Determination of LC_{50} was done with ten different concentrations; for other experiments we used 50 μM CBB, that represents a concentration around 20 times higher than that used in the Bradford method. The estimated LC_{50} would be 38 mM for oral ingestion. The results from enzymatic assays and behavioral tests suggest that Coomassie is not degraded by carboxylesterase and is not neurotoxic. The results confirm previous observation about the low toxicity of Coomassie brilliant blue in a single or continuous exposition.

Introduction

The substantial increase in industrial generation industrial of solid wastes is of considerable concern for scientists all over the world and may affect not only specific species of flora and fauna, but also the structure and function of entire ecosystems (Fent, 2003).

Wastewaters generated by some industries are hazardous, because they may contain heavy metals and pigments. These are usually aromatic compounds difficult to degrade (Moser *et al*., 1981) and possessing a high potential to accumulate in the alimentary chain (Tonogai, 1980; Tiedge *et al*., 1986).

Synthetic dyes are often found among the wastewater. Color change is the first modification to be recognized in water; the presence of even small amounts of dyes is highly visible and undesirable (Marchant *et al*., 1996; Nigam *et al*., 2001). The structural diversity of dyes comes from the use of different chromophoric groups (*e.g*., azo, anthraquinone, triarylmethane, and phthalocyanine groups) and different application technologies (*e.g*., reactive, direct, disperse, and vat dyeing) (Heinfling *et al*., 1998).

Among these classes of chromogenic groups of dyes, the most common group is the azo type, which makes up to 70% of all textile dyestuff produced, followed by the anthraquinone type (Vestraete *et al*., 1998). The color of anthraquinone dyes is partially associated with the anthraquinone nucleus and modified by the type, number, and position of substituents (Zollinger, 1991). Anthraquinones are a diverse group of naturally occurring and synthetic chemical compounds used widely in industry as colorants in foods, drugs, cosmetics, hair dyes, and textiles and in medicine as purgative, antimicrobial, and antitumor preparations (Sendelbach, 1989).

RBBR (Remazol Brilliant Blue R or Reactive Blue 19), representative of anthraquinone dye, is frequently used as a starting material in the production of polymeric dyes (Eichlerová *et al.*, 2007). In the family of brilliant blue, the Brilliant Blue FCF (bis{4-(N-ethyl-N-3-sulfophenylmethyl)aminophenyl}-2-sulfophenyl methylium disodium salt) is a triphenylmethane dye, which is used as a colorant in textile and leather. Before its ban, particularly in developed countries, it was used to act as a common food additive in beverages, dairy products, powders, jellies, confections, icings, syrups, extracts, and condiments for several years (Gupta *et al*., 2006). Afterward were produced other types of brilliant blue, such as brilliant blue G 250 – BBG, Coomassie Brilliant Blue R-250, and Coomassie Violet R-150.

Brilliant Blue G or R is a biochemically relevant dye commonly used in laboratories for protein determination and staining after gel electrophoresis, given its affinity for binding nonspecifically to virtually all proteins; nevertheless, the most commonly used are Coomassie Blue G-250, more sensitive.

Little information is available concerning the health risks of most anthraquinones. Bioassays can provide valuable information to reflect the toxicity of mixed solution on living organisms (Huang

et al., 2009), and *Drosophila melanogaster* is a well-established insect model, recommended by the European Center for the validation of Alternative Methods (ECVAM).

This holometabolous insect has a complex life cycle with different stages. Larvae and adults have a different shape and may have a different alimentary regime. Similar environmental stresses can affect each life stage in a different manner, and the adaptive strategies may depend on a specific stage of life (Loeshcke *et al*., 1996). *D. melanogaster* has been used extensively for studies in genetics and developmental biology (Chowdhuri *et al*., 2005), and over the last decade, *Drosophila* has emerged as one of the most powerful models for human diseases (Bonini *et al*., 2002; Thompson *et al*., 2002) and toxicological research (Chowdhuri *et al*., 2005). This fruit fly has several advantages, such as a short life cycle (one generation is complete within two weeks) and easiness to perform genetic crossing studies (Wilson, 2005).

Although some information about toxicity is known about Coomassie brilliant blue, this work analyzed toxic effects in *D. melanogaster*, *i*n order to verify if this chemical could be harmful to the ecosystem as well as for humans. We also investigated the long-term toxic effects of continuous exposure to Coomassie brilliant blue added to the culture medium, an approach previously used to analyze the toxic effects of ethidium bromide (Ouchi *et al*., 2007).

Materials and Methods

Stock Culture

Specimens of *Drosophila melanogaster* were collected using appropriate traps (Medeiros and Klaczko, 1999) in São José do Rio Preto, State of São Paulo, Brazil. The individuals were maintained in vials with banana-agar medium at constant temperature at 25ºC. Females were used to set up isofemale and mass stocks.

Bioassays to determine LC_{50}

In assay tubes about 10 mL of culture medium were placed ten second instar larvae of *D. melanogaster*, and then accompanied their life cycle. The emerged adults were counted and morphologically analyzed (tergites' pattern and color, wings opening and shape, and eye color). Coomassie brilliant blue (Sigma) was added to the culture medium in ten different final concentrations (0, 0.055, 0.175, 0.250, 0.5, 0.553, 1, 5, 10, and 15 mM). Every experiment was carried out in three replicates, including a control group fed with uncontaminated culture medium.

Bioassay to analyze bioaccumulation

Except for the control group, we added Coomassie Brilliant Blue (CBB) at a final concentration of 50 μM to the culture medium used for feeding the insects. A bioaccumulation study was performed for ten generations. Twelve virgin couples of females and males emerged from the stock were used to generate the first generation; for the other generations, 12 new couples were picked up among the animals emerged on the fifth day after the first adults emerged. The females were allowed to lay eggs for ten days. The emerged adults were counted every day for 9 days consecutively, and then a new counting was done for the 11^{th}, 13^{th}, and 15^{th} days. During the experiment, all the adults were also analyzed morphologically (tergite color and pattern, wings shape and opening, and eye color).

A similar LC_{50} was done for couples from F_8; a female and a male (from the 50 μM contaminated medium) were allowed to couple for 24 hours in a vial with culture medium. The male was removed, and the female was allowed to lay eggs for three days, being transferred afterwards to a

new identical vial. The procedure was done for seven consecutive times, and as offspring were emerging, they were scored. For the new generation (F_9), three concentrations (25, 50, and 100 μM) were tested. The experiments were performed in triplicates for each concentration and nine replicates for the control group.

Offspring analysis

In order to study the effects on the insects' weight, adults from F_{10} were separated by sex for six days consecutively, transferred to 1.5 mL assay vials, and were daily weighed using an analytical scale.

Behavioral tests

In the mating test, the adults from F_1 and F_{10} were separated for six days by sex in groups of five insects and maintained at constant temperature (25ºC). After six days, the females and males were transferred, without anesthesia, to the same vial but without the medium and observed for one hour to determine the time of pre-copulation and copulation.

Female viability experiment

In the viability experiments a couple of adults from F_{10} were placed in a 250 mL bottle containing a plastic spoon filled with agar-sugar medium. The female was allowed to lay eggs for 24 hours, and then the couple was removed. The eggs were counted, and the spoon was placed within a bottle containing medium culture. For couples picked up from a control group, the spoon was placed in a control bottle, doing a similar procedure for those exposed, transferring to a bottle containing CBB. Subsequently eggs, pupae, and adults were counted.

Carboxylesterase assay

For assays of enzymatic activities, we formed three samples, each one with five adults collected from F_5. The animals were homogenized in 100 mM phosphate buffer pH6.2. Carboxylesterase activity was measured by the method from Ellman *et al*. (1961). Absorbance readings were performed in a Varian 100 spectrophotometer, and all the assays were measured in triplicate. Protein concentration was determined according to Bradford (1976).

Statistical analysis

For the bioaccumulative experiments, the 2-proportions equality test was used (Z-normal approximation) (Moore, 2005). The graphs were represented as the proportion of emerged flies in three replicates each day per the number of flies emerged in that generation. In the other experiments, we performed the Student's *t* test (Zar, 1999). Differences were considered significant when $p < 0.05$, using the program BioEstat 4.0 (Ayres *et al*., 2005). LC_{50} was analyzed considering the death rate, and the probit analysis was done with StatPlus (AnalystSoft).

Results and Discussion

Determination of LC_{50} was done with ten concentrations (Figure 1), but the statistical analysis showed that the flies' survival was not affected by the increase in Coomassie concentration. Higher concentrations were not assayed, because we were unable to dissolve CBB above 15 mM. Estimated LC50 by extrapolation would be 38 mM.

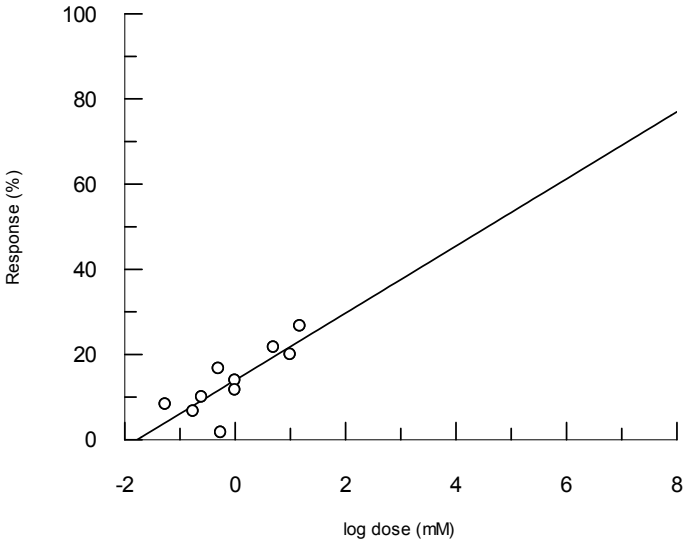

Figure 1. Coomassie induced mortality, based on LC_{50} probit analysis by StatPlus (AnalystSoft).

The massed line (ML) showed a mean emergence for the treated groups that was higher than for its control. A probable explanation could be an increase of the emergence as a strategy to increase the probability of producing adapted insects to the adverse environment, as postulated by Hirsch *et al*. (2003). For the isofemale (IL) strain in the three analyzed generations the emergence in the treated group was always lower than for the control (Figure 2).

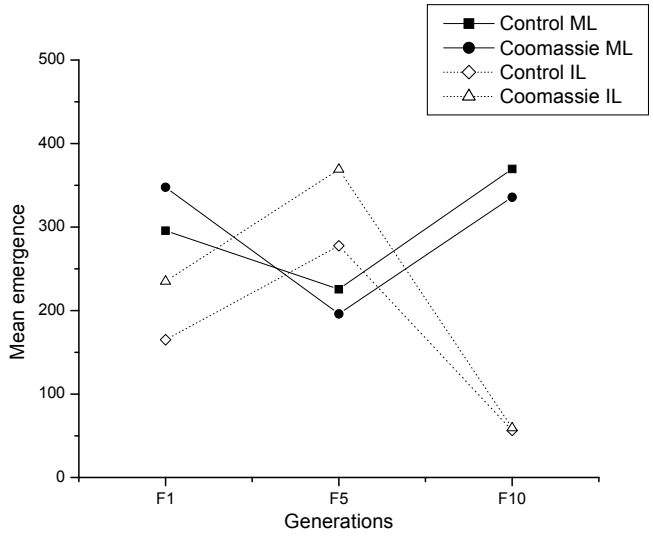

Figure 2. Comparison of the mean emergence utilizing two strains of *D. melanogaster*: isofemale (IL) and mass line (ML) for three generations, F_1, F_5, and F_{10}.

For the other experiments we chose 50 μM CBB; it represents a concentration around 20 times higher than the one used in the Bradford method. In order to verify the bioaccumulation, ten generations of the isofemale line were accompanied daily. The variance among the generations was unequally distributed. Six of the ten generations analyzed (F_1, F_2, F_3, F_5, and F_7) showed a higher proportion of emergence in the exposed groups compared to the control. Only for F_7 the differences were significant (Figure 3), however, and there was a tendency of decreasing productivity for the following generations.

For F_8 we performed a concentration test; flies emerged from the group exposed to 50 μM Coomassie blue were placed in media with 25, 50, and 100 μM of Coomassie (Figure 4). In 25 μM the emergence was the highest one. On the other hand, in 100 μM it was expected the lowest emergence. But it did not occur, in this way there was not a dose-dependent response.

In order to verify in which stage of the *Drosophila* life cycle the product had more influence, the egg viability experiments were conducted in generations F_3 and F_{10}. Results (Table 1) show that, in the presence of Coomassie, there was a decrease of the oviposition but an increase of viability.

In terms of behavioral tests, we evaluated pre-copulation and copulation times for F_1, F_5, and F_{10} (Figure 5). For the insects exposed to CBB, the mean pre-copulation time was higher (although

not significant) for the exposed insects, whereas there was no difference for copulation time between the exposed group and control, for the same generations. By observing the color of the abdomen, we observed that ingested CBB was rapidly excluded from the insect, and at least from this experiment there is no evidence that CBB can cause behavioral alterations. The literature reveals only physical indisposition in humans when the dye was ingested. It is known that after 8 hours Coomassie is entirely excreted from the organism for a single exposition (Hoffman *et al*., 1961).

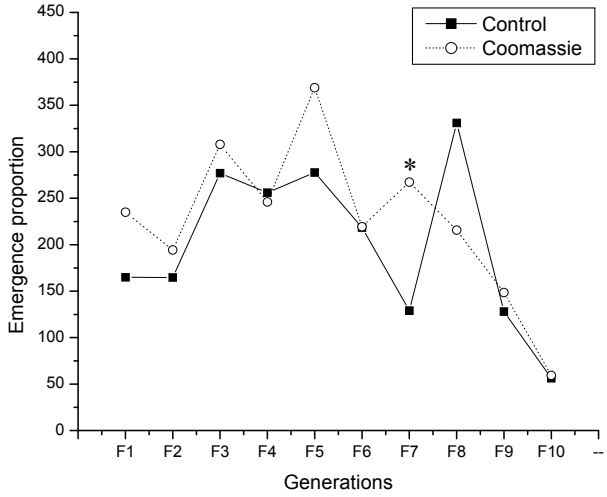

Figure 3. Graph of proportion emergence for each generation of the isofemale line. * $p < 0.05$.

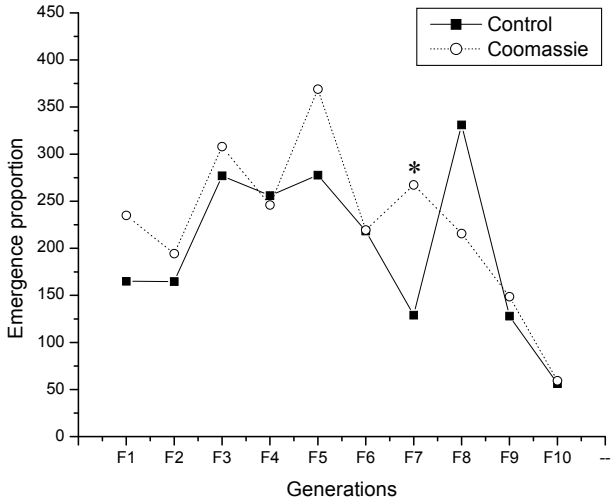

Figure 4. Mean emergence from F8 flies submitted to different acrylamide concentrations.

Table 1.

Generations	F_3				F_{10}			
	Control		Coomassie		Control		Coomassie	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Egg	47.0	12.5	53.0	13	21.5	2.5	8.5	0.5
Pupae	46.0	9.5	52.5	13.5	16.5	0.5	8.0	1.0
Adults	46.0	9.0	52.5	13.5	15.5	1.5	7.5	0.5
Viability Egg-adult (%)	97.9	5.6	98.8	1.3	72.3	1.4	88.2	0.7
Viability Egg-pupal (%)	100.0	30.3	98.8	1.3	77.3	6.7	93.8	6.3
Viability Pupal-adult (%)	100.0	94.0	100.0	0	93.8	6.3	94.4	5.6

Another possibility of toxic effect was analyzed with the influence of Coomassie on the flies' weight (Figure 6). The mean weight of treated adults is about 3 times lower than those from the control in F_{10}. The effect on the insect weight could be explained by the hypothesis that adults avoid the medium culture, because of the contaminant presence, as proposed by Trumble *et al*. (2004). Another possibility would be metabolic alterations, as proposed for flies exposed to acrylamide, for example (Yousef *et al.*, 2006).

Concerning enzymatic activity, carboxylesterase was analyzed in F_5, but there were no differences between the control (72.5 ± 23.2 U/mg) and the exposed group (70.6 ± 24.6 U/mg).

These enzymes are serine dependent esterases, which hydrolyze a wide range of xenobiotic substrates (Maxwell, 1992), providing prominent protection against neurotoxic compounds (Bonacci *et al*., 2004). The results suggest that Coomassie is not degraded by this class of enzyme and is not a neurotoxic.

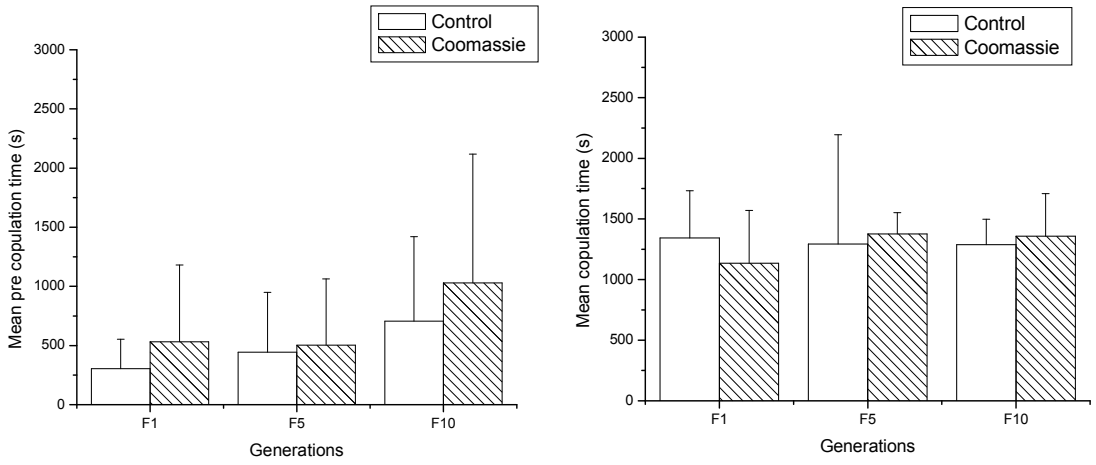

Figure 5. Behavioral tests for *D. melanogaster* exposed or not to Coomasie Blue. A: mean pre copulation time; and B: mean copulation time.

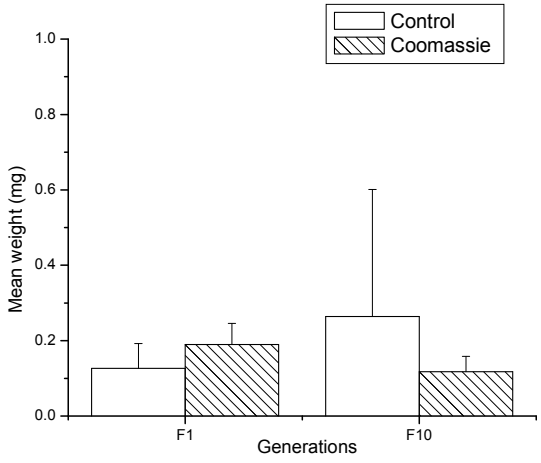

Figure 6. Graph for mean weight of *D. melanogaster* males and females in F_1 and F_{10}.

This work corroborates the small toxicity of CBB when used in low concentrations, although after a long time exposition some alterations were found for *D. melanogaster*. Following ten generations the time of pre copulation was higher than for the control group. The oviposition decreased, but the viability of these eggs was higher than the control and the eclosed adults were lean. The differences in fecundity could be interpreted in terms of changes in resource allocation by the female (Sinervo, 1999). In the case of F_{10}, females in adverse environments could postpone reproduction until the environment improves, influencing the makeup of next generation (Hirsch *et al*., 1993). In summary our results confirm the low toxicity of Coomassie brilliant blue in a single or continuous exposition.

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Feeding rates of *Drosophila mojavensis sonorensis* on native and non-native hosts.

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Drosophila mojavensis is a cactophilic fly species endemic to North American deserts (Heed, 1978). The species utilizes the necrotic tissues or "rots" of cacti during the flies' life stages and occurs as four geographically isolated subspecies (Heed, 1982; Pfeiler *et al.*, 2009). It is hypothesized that *D. mojavensis* originated in Baja California on *Stenocereus gummosus* (agria cactus) after an isolation event from *D. arizonae* (Ruiz, 1990). A northward migration is thought to have established the subspecies *D. m. wrigleyi* on Santa Catalina Island and *D. m. mojavensis* in the Mojave Desert (Pfeiler *et al.*, 2009). Because agria does not grow at these localities, the subspecies have to utilize *Opuntia littoralis* (prickly pear) and *Ferrocactus acanthodes* (barrel), respectively, in these areas. The subspecies in Baja California remained on agria and is designated as *D. m. baja*.