MONITORING OF BIOLOGICAL VARIABLES IN *Chironomus sancticaroli* CULTURING AS TEST ORGANISM

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ABSTRACT

International protocols such as USEPA, ASTM, OECD describe procedures for ecotoxicity tests with sediment samples using benthic organisms such as insect larvae of the genus *Chironomus sp*. The criteria for acceptability of the test are based on variables observed in control. The same protocols adopt the sensibility to the reference toxicant to ensure the quality of the test organisms before their use in testing, but do not describe methods for monitoring biological variables in cultures of these organisms. This study aims to monitor the variables: survival, mentum deformity, fecundity, fertility and hatching rate of the species *Chironomus sancticaroli* cultivated in laboratory under specific conditions in order to prepare a database for the future establishment of acceptance criteria for these variables. The average percentage of survival obtained was 89.3%(σ = 12.8). The average mentum deformity found was 13% (σ = 13.9), while the fecundity (530 eggs, σ = 116) and fertility (225 larvae, σ = 135) resulted in an average hatching rate of 42% (σ = 21.1). The variation data found although consistent with data from other author suggesting the influence of external factors such as the presence of contaminants in culture water, in addition to genetic interference caused by the inbreeding. The broad range of these variables suggest the need for further studies, especially regarding the quality of the natural water used in cultivation and genetic interference, such as "inbreeding," in mentum deformities.

1. INTRODUCTION

Surface and ground water quality have been extensively changed by natural and anthropic activities (USEPA 1989; Dornfeld, 2006; CETESB, 2008), causing the imbalance of aquatic ecosystems reflected by aquatic organisms. These changes are constantly monitored on programs for assessing the quality of ecosystems in USA, Canada, Europe, Brazil, etc.

The sediment, in aquatic ecosystems, acts as a storage for organic and inorganic substances, mixtures of metals introduced by natural geochemical processes or anthropogenic activity and it is a habitat for many aquatic communities such as benthic organisms used as test organisms in bioassays and biomonitoring.

Test organisms, such as the *Chironomus* genus (Insecta, Diptera, Chironomidae), have also been used as test organisms in ecotoxicity tests with sediment, since they are good indicators of environmental quality and are present abundantly in the benthic fauna in aquatic and

marine environments. (Warwick, 1990).

There are several protocols described by USEPA (2000), ASTM (2005), OECD (2004; 2010), Environment Canada (1997), Sibley (2001) among others, for ecotoxicity testing with integral or fortified sediment samples using larvae of *Chironomus tentans, Chironomus riparius* and *Chironomus sp*. Endpoints observed in these protocols are growth, survival, fecundity, fertility, hatching rate. Mentum deformity also has been used as a tool in biomonitoring (Environmental Canada, 1990; Bird, 1995; Hudson, 1996; Janssens de Bisthoven, 1998; Servia, 2000; Meregalli, 2002; Environment Canada, 1997; Bonani, 2010).

The specie *Chironomus sancticaroli* has great ecological significance because it is a native species found in São Paulo State. Described by Strixino & Strixino in 1981, was considered by Speis & Reiss (1996) a junior synonym of *Chironomus xanthus* Rempel 1939 (Correia, 2004) and has been cultivated in laboratory and used in ecotoxicity tests with sediment, among others, by Fonseca (1997); Dornfeld (2002); Bramorski (2004), Silva (2005); Dornfeld (2006), Costa (2007), Barbosa (2008); Pusceddu (2009), Sales (2009), Bonani (2010).

The variability of results observed in bioassays is related to the skill technical to conduct the test, variations in abiotic conditions, variability of sensitivity of the test organisms (Zagatto, 2006) and even changes of biological variables in culturing conditions. However, in the known protocols it is not a mandatory to perform the monitoring of biological variables such as the same endpoints in ecotoxicity tests other than sensibility to reference toxicant.

Test protocols and other studies cited describe the procedures adopted for tests with chironomids and the conditions for culturing them suggesting as criteria for monitoring the culture´s health the observation of the first emergence; lipid content; dry weight (USEPA, 2000; ASTM, 2005); culturing abiotic characteristics; organisms behavior; feeding; ecotoxicity test acceptability criteria (Environment Canada, 1997); reproduction rate; resistant forms and sensibility to reference toxicant (Zagatto, 2006). To other test organisms there is an acceptability criteria for survival in cultured organisms (ABNT, 2007; 2009; 2010).

This study was designed with the purpose of monitoring biological variables such as survival, mentum deformity, fecundity, fertility and hatching rate in chironomids cultivated in the laboratory using a specific methodology. The results will be used in order to prepare a database for the future establishment of acceptance criteria for them, ensuring the availability of organisms with known quality for tests and when to start new cultures.

2. MATERIALS AND METHODS

Larvae of *Chironomus sancticaroli*, originally obtained from the Centro de Recursos Hídricos e Ecologia Aplicada da Escola de Engenharia de São Carlos - USP were cultivated based on the procedures developed by Fonseca (1997) and USEPA (2000).

2.1. Obtaining the Larvae

Egg masses produced were placed in trays containing 4 liters of dilution water, sifted sand as substrate (<500 mm), mild aeration and daily feeding after 24 hours with 3 ml of fish meal (20 g / L), keeping up daily to use in tests.

A week after the separation of egg masses, the larvae (from second to third instar) were counted. The identification of the instars of this specie was based only on the duration, in days, of each instar as identified by Fonseca (1997). This specie develops throughout the larval cycle in 13 days, in an average temperature of 25 °C: 4 days for the first instar, 2 for the second, 2 for the third and 5 for the fourth.

2.2. Organisms Survival and Mentum Deformity

The determination of the percentage of survival of organisms was conducted with random sampling of 50 larvae and transferring them to trays containing sand as substrate, 1.5 L of dilution water, mild aeration and 2 mL of solution fish meal daily. The luminosity and temperature were the same used in cultures. After one week, the survival organisms were recovered and counted to determine the percentage of survival and mentum deformity.

Organisms recovered in the survival test were preserved in 70% ethanol for preparation of slides and observation of the mentum deformity as described by Khulmann (2000). Cephalic capsules were removed and placed dorso-ventrally on slides with Hoyer's medium, covered with coverslip and pressed for the mentum exhibition. The prepared slides kept at room temperature for at least 24 hours to observation in optical microscope with 200x magnification.

Only numerical changes in the mentum (excess of teeth, missing teeth, gap and central tooth split) were considered. After analysing the slides, the percentage of deformities were calculated for the cultured organisms.

2.3. Fecundity, Fertility and Hatching Rate

Methodologies were described by USEPA (2000) and Strixino (1980) for the determination of these variables in *Chironomus sp*, however, these methods demonstrated a high degree of difficulty in handling and counting of eggs. Therefore, we proposed a new methodology based on photographic images, as described below:

2.3.1. Fecundity determination

The egg masses laid in cultures trays were removed and placed in excavated glass slide covered with coverslip for observation in the dissecting microscope and subsequently obtain

the photographic image. The eggs were counted manually in Microsoft Paint ® version 5.1. The numbers of eggs obtained by manual counting corresponded to fertility.

2.3.2. Fertility determination

Egg masses photographed were placed in disposable Petri dishes containing 25 mL of dilution water, covered and incubated under the same conditions of cultivation.

After 72 hours, 5 ml of 70% ethanol and rose bengal as a preservative and coloring of larvae hatched were added on each plate One day after the preservation, larvae were counted to obtain the number of fertile eggs.

2.3.3. Hatching rate determination

The number of eggs produced (fecundity) with the number of larvae hatched (fertility) was used to calculate the hatching rate by the relationship between fertility and fecundity, as a percentage.

3. RESULTS AND DISCUSSION

3.1. Organisms Survival and Mentum Deformity

Organism survival was determined by aleatory sampling of larvae produced because the culture system established for *C. sancticaroli* prevents the manipulation of the sandy substrate for quantifying and monitoring the organisms survival during the maintenance, unlike the usual technique adopted for cladocerans and amphipods cultures.

Survival data was obtained during the years 2008 to 2010. The average calculated was 89.3% (σ = 12.8), as described in Fig. 1. Among these results, approximately 84% had percentages greater than, or equal to 80% survival.

In fertility studies with *C. sancticaroli*, Strixino (1980) observed low average percentage of survival when exposed to natural sediment and different qualities and quantities of food (43%). She also described the influence of temperature on larval survival as abiotic factor to observe in culturing (95% survival at 25 ° C).

The average percentage found in this study is similar to that established by ABNT (2007; 2009; 2010) survival of 80% in cultures of other test organisms used in ecotoxicity tests.

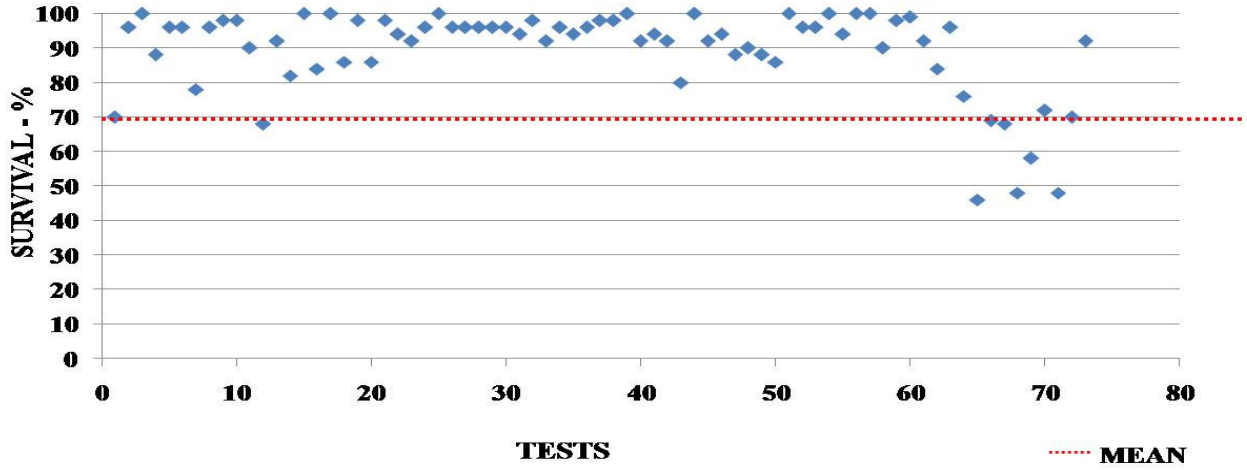

Figure 1. Weakly larvae survival percentage.

The Fig. 2 shows the percentage of mentum deformity of the organisms cultivated in the laboratory. The average deformity obtained was 13% (σ = 13.9) but values ranged between zero and 47%.

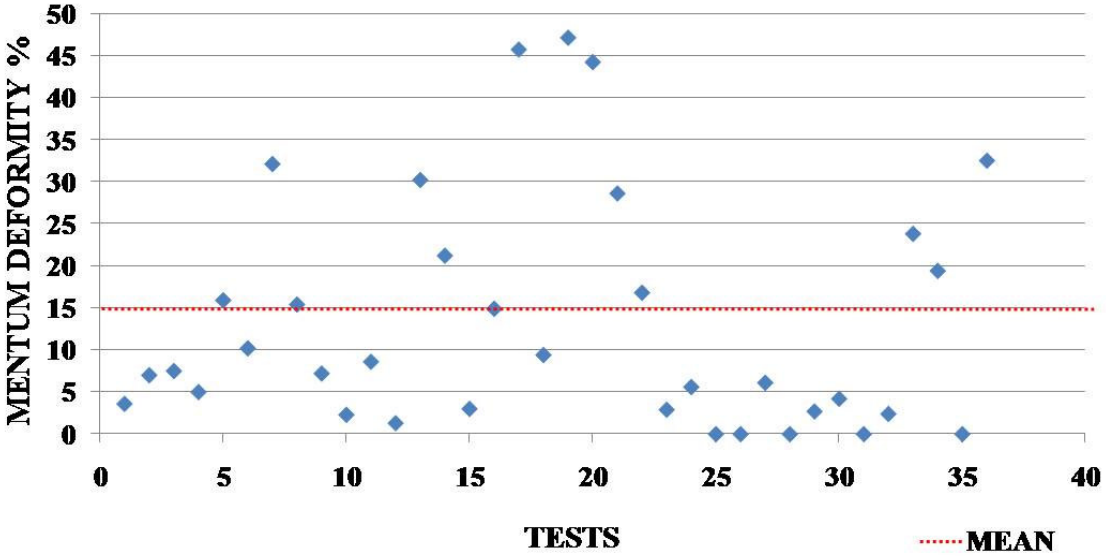

Figure 2. Mentum deformity percentage.

Janssens de Bisthoven (2001), Vermeulen (2000), among others claim that high rates of deformity in cultured organisms, as well as the presence of central tooth split in some organisms are indicative of inbreeding. The wide range of data obtained in the laboratory is not consistent with the inbreeding hypothesis because there is not a constant percentage of deformity in the population cultivated.

The last author cited studies in which the percentage of deformity in cultured organisms ranged from 0 to 52% with the use of different substrates, food and number of sample replicates.

The presence of deformities in cultures of *C. tentans* observed by Bird (1997) was independent of the characteristics of the substrate, but a result of the age of cultivated organisms, i.e., the inbreeding was observed in older cultures, without renewal of organisms.

The process of inbreeding increases the expression of recessive features including morphological abnormalities (Bird, 1995). The same author showed, in studies with organisms collected in contaminated area, a reduction of the percentage of deformity of the next generation when cultured in clean sediment, characterizing the teratogenic effect on the incidence of deformity.

Experiments conducted in the laboratory (unpublished data) indicated a decrease in the percentage of deformity in subsequent generations of the same culture, which does not match the inherited trait of inbreeding. The same reduction in frequency was observed by Jeyasingham (1997) in F2 organisms cultivated, supporting the hypothesis of interference of the site of origin and type of substrate used in cultures.

It is known that the occurrence of mentum deformity and mouthparts are influenced by the presence of potentially toxic chemicals such as metals and organic compounds. Bird (1995) describes the possible mechanisms for the induction of deformities including mutations, interference with processes of transcripts and translations; disruptions in cell division and metabolic disturbing as the disruption of proteins control during the organism development.

Procedures standardized and controlled conditions help maintain culture quality, however, the use of natural water as dilution water in the laboratory cultures cannot guarantee the stability of organisms, because changes in physical-chemical quality and water chemistry may occur.

These changes may be responsible for variations in the percentage of deformities found in organisms cultivated. Studies by Servia (2000) showed that besides the effect of inheritance, the deformities were induced by the water quality where eggs were hatched and cross-contamination of eggs as a result of the accumulation of contaminants by the female parent.

3.2. Fecundity, Fertility and Hatching Rate

From April to May 2010, 23 egg masses were analysed. Fig. 3 presents data obtained with tests to determine the fecundity, fertility and hatching rate.

The fecundity average obtained in the laboratory during this period was 530 eggs (with a minimum of 340, maximum of 812 eggs and $\sigma = 116$). Strixino (1980) obtained an average of 744.9 eggs, with a minimum of 500 and maximum of 1045, while Fonseca (1997) obtained values from 500 to 600 eggs in the first oviposition.

The variation in the number of eggs found in these studies may have been influenced by environmental factors such as temperature and food, reported by the authors. These factors

acting during the larval and pupal stage, in addition to hereditary constitution of organisms. In the larval stage, the organism had to absorb a large amount of nutrients because the adult do not feed. The temperature according Strixino (1980; 1985) affected the development time.

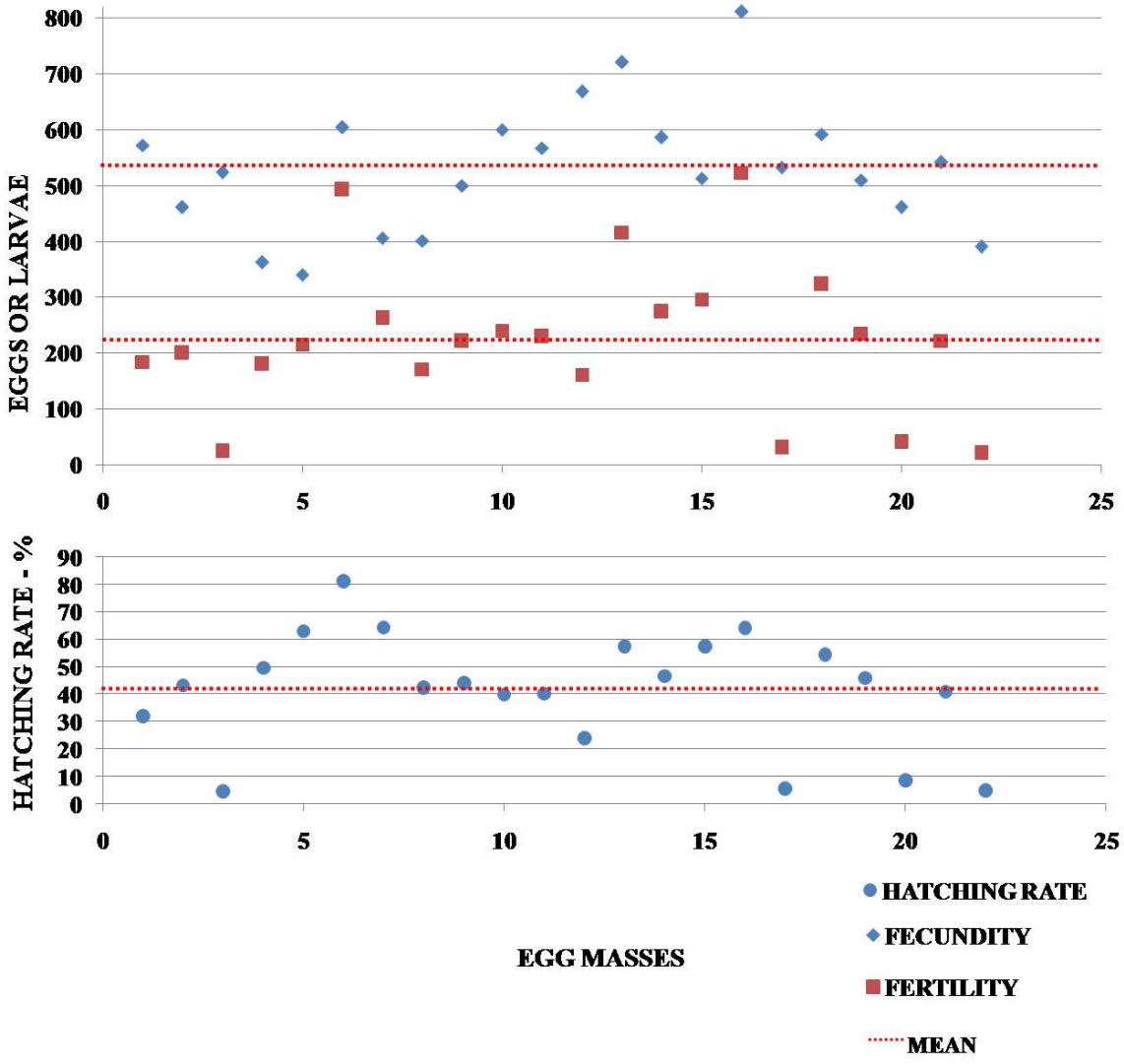

Figure 3. Results of fecundity (eggs), fertility (larvae) and hatching rate (percentage) determination.

Larvae grown at low temperatures have slowed metabolism, staying for a longer period of time in the larval stage and accumulating greater amounts of nutrients during this period. Consequently had a high mortality and fecundity because they are related to food availability. The opposite occurred with organisms cultured at optimum temperatures (25 ° C) where there was good survival and a reasonable number of eggs when fed with quality and quantity known food.

Another important factor is the fecundity per female. In the same study mentioned above, the

author demonstrated a reduction in the number of eggs per female during the reproductive cycle, because they were able to produce up to three egg masses.

Organisms were cultivated in the laboratory under controlled conditions of temperature (average 25 ° C) and known food quality (Tetramim ®), as proposed by Fonseca (1997). The variation in the number of eggs produced compared with data obtained by the authors cited can be justified by the amount of food given to the different cultures of the laboratory, no renewal of organisms, which would prevent the increase of genetic variability and the sampling of egg masses of second or third oviposition.

The average fertility found was 225, ranging from 24 to 523 larvae and $\sigma = 135$, while Strixino (1980) obtained in her experiments, 496.7 larvae. The hatching rate obtained in the study was 42% ($\sigma = 21.1$), very different from the 99.7% obtained by the same author.

She reported that the hatching rate cannot be influenced by environmental factors, however, the values obtained in the laboratory experiment were not consistent with this statement. The possibility of predation of eggs by hidracarins described by the author was not observed in this study.

An important point to be presented is the observation of effects on these variables in life cycle tests with *Chironomus tentans* and other chironomids. (USEPA, 2000; OECD, 2010). With these protocols, it is possible to identify the influence of contaminant in the production of eggs, larvae and consequently, hatching rate.

The low fertility and hatching rate observer in the laboratory tests suggests the interference of chemical compounds present in the dilution water used cultures because it´s a natural water and not reconstituted, since the culturing conditions are very similar to those exhibited by the authors cited.

4. CONCLUSIONS

The biological variables of importance for monitoring the quality of organisms cultivated in the laboratory showed the mean baseline for these cultures conditions: survival of 89.3% of exposed organisms; 530 eggs in each egg mass (fecundity); 225 larvae hatched per egg mass (fertility), hatching rate of 42% and 13% of organisms with mentum deformity.

It is worth mentioning the need for further investigation of the biotic factors (heredity) and abiotic (water quality) that hypothetically would be promoting the change from baseline of these variables obtained when compared with the existing literature.

The initial determination of the average baseline values allows to identify the situation of the organisms cultivated in accordance to the methodology of each laboratory. These data will form the basis for the calculation of an acceptability criteria for each variable, allowing the use of organisms with known quality in ecotoxicity tests and establishment of new cultures.

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