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The efficacy of clove oil as an anaesthetic and in euthanasia procedure for small-sized tropical fishes

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(With 1 figure)

Abstract

Clove oil is used as a fish anesthetic because it is a natural and inexpensive product with low toxicity risks. The goal of the present study was to determine the appropriate concentration of clove oil for small-sized tropical fish to be used in mark-recapture studies or when individuals are to be sacrificed. We applied three different clove oil concentrations (D1=0.05 mL, D2=0.10 mL and D3=0.20 mL per 500 mL of water) on three small-sized fish species. We found a negative relationship between induction time and treatment for two species (*Hyphessobrycon* sp.1 and *Hemigrammus* sp.), while concentration was unrelated to recovery time. Fish body length was positively related to induction time in the D2 treatment for *Hemigrammus* sp., and negatively for *Hyphessobrycon* sp.1 in the D1 treatment, but was unrelated to recovery time for three species and treatments. Mortality rates varied across treatments, but higher rates were observed with higher clove oil concentrations. We conclude that 0.05 mL of clove oil per 500 mL of water is the most efficient dose for studies where fish will be released back to their natural habitats, while 0.20 mL of clove oil is recommended for studies that require fish euthanization for further laboratory analyses.

Keywords: Eugenol, anesthesia, euthanasia, field experiment, fish manipulation.

A eficiência do óleo de cravo como anestésico e em procedimentos de eutanaisa em peixes de pequeno porte

Resumo

O óleo de cravo é recomendado como anestésico para peixes por ser produto de origem natural, baixo custo e apresentar poucos riscos de intoxicação. O objetivo deste trabalho foi determinar concentrações adequadas de óleo de cravo para anestesiar ou eutanasiar peixes de pequeno porte em ambiente natural. Foram testadas três concentrações do anestésico (D1=0,05 mL, D2=0,10 mL e D3=0,20 mL) em três espécies de peixes de pequeno. Houve uma relação negativa entre o tempo para a sedação dos indivíduos e a concentração para duas espécies (*Hyphessobrycon* sp.1 e *Hemigrammus* sp.), porém não foi encontrada relação entre o tempo para recuperação e as concentrações. Os exemplares maiores de *Hemigrammus* sp. levaram mais tempo para serem sedados no tratamento D2, já o contrário foi observado para *Hyphessobrycon* sp.1 no tratamento D1, enquanto que não houve efeito do comprimento no tempo de recuperação das três espécies. A mortalidade dos indivíduos variou entre as três concentrações do anestésico e as maiores taxas de mortalidade ocorreram nas maiores concentrações. Desse modo, a concentração de 0,05 mL é eficiente para estudos que envolvem manuseio e a soltura dos peixes, enquanto que a concentração de 0,20 mL é recomendada em estudos onde os peixes precisam ser sacrificados.

Palavras-chave: Eugenol, anestesia, eutanásia, experimento de campo, manipulação de peixes.

1. Introduction

Capture, handling, and transport of fish can have a negative impact on their health and their growth. To resolve this problem, fish biologists have used a variety of anesthetics in attempts to reduce handling stress (Perdikaris et al., 2010). An emerging and efficacious anesthetic for use with fish is clove oil, which is extracted from flower buds, leaves and stems of clove trees (*Syzygium aromaticum* (L.) Merr. & L.M. Perry and *Myrcianthes fragrans* (Sw.) McVaugh), (both Myrtaceae). The active ingredients are eugenol (4-allyl-2-methoxyphenol) and iso-eugenol (4-propenyl-2-methoxyphenol) (Keene et al., 1998; Prince and Powell, 2000). It has been used as fish anaesthetics in the aquaculture industry and has shown to have low intoxication and mortality risks (Soto and Burhanuddin, 1995; Griffiths, 2000; Inoue et al., 2011). Moreover, the use of this anesthetic is advantageous because it is relatively inexpensive, efficacious, cost effectiveness, safe and does not present risks for users or to the environment (Soto and Burhanuddin, 1995; Munday and Wilson, 1997; Guénette et al., 2007; Hisano et al., 2008; Lucena et al., 2013; Öğretmen et al., 2014).

Clove oil have been increasingly used as anaesthetic in mark-recapture studies in order to reduce handling stress in freshwater fish, allowing them to be collected, identified, measured, weighed and subsequently released back to their natural habitats (Munday and Wilson, 1997; Javahery et al., 2012). In addition, this anesthetic is used in artificial reproduction and chirurgic processes, as well as with live fish transportation (Javahery et al., 2012; Inoue et al., 2005) because it mitigates fish stress levels. It also reduces potential negative effects on fish homeostasis, so decreasing mortality rates (Inoue et al., 2005; Becker et al., 2012, 2013).

Like other anaesthetics, clove oil decreases fish neurosensory functions by acting upon the nervous system, mainly the cerebral cortex (Schreck and Moyle, 1990). It also has an inhibitory effect on the respiratory system of fish, resulting in a slowing of respiration rate (Keene et al., 1998). However, high concentrations of clove oil affect the brainstem, the respiratory system and the spinal cord, potentially causing respiratory failure and medullary collapse, which may lead to death (Schreck and Moyle, 1990). Therefore, better understanding the relationship between the anesthetic concentration and its effects on fish is critical for the success of studies where individuals are to be returned alive to their natural environment.

The concentration of the anesthetic will depend on whether the fish is to be anaesthetized or euthanized, according to the goals of the study (Silva et al., 2009). Smaller fishes respond to lower concentrations than larger fishes, thus the effect of clove oil will vary depending on the fish species and their sizes (Woody et al., 2002; Ross and Ross, 2008). Although clove oil is broadly utilized, information on its anesthetic effects on small fishes from tropical regions is currently lacking (Lucena et al., 2013). Therefore, determining the appropriate concentration of the anaesthetic will allow a reduction in unintentional fish mortality in studies that aim to return specimens alive to their natural habitat (e.g., mark-recapture studies or population monitoring). As a consequence, impacts on studied populations and communities will be minimized.

In the present study, we set out to determine empirically the appropriate concentration of clove oil to anaesthetize or euthanize three small-sized tropical fishes species. More precisely, we asked the following questions: (i) What is the ideal concentration for anesthesia and euthanasia of the tropical small fish? (ii) Is there variation in induction and recovery times across different clove oil concentrations and species? (iii) Do induction and recovery times depend on fish size and species? And (iv), do mortality rates differ across different clove oil concentrations and species?

2. Material and Methods

2.1. Study area and sampling

The experiment was conducted in the São Nicolau farm, localized at the left margin of the Juruena River, upper Tapajós River, Mato Grosso, Brazil. Data was collected in an artificial lake that was built on the farm to provide water for cattle (9º 49› 28”S and 58º 15› 27” W). Fish were captured using a trawl (6 m × 2.8 m; 2.5 mm mesh) applied on the bottom of the lake and kept in containers filled with water from the same lake. For the experiment, we used 120 specimens from three abundant Characidae species: *Hyphessobrycon* sp.1 (40 individuals), *Hyphessobrycon* sp.2 (40 individuals) e *Hemigrammus* sp. (40 individuals) (See species details in Carvalho et al., 2013). The standard length varied between 8.19 and 34.92 mm, with an average of 24.70 mm for *Hyphessobrycon* sp.1. In *Hyphessobrycon* sp.2 the standard length ranged from 26.93 to 40.22 mm average of the 31.79 mm, and for *Hemigrammus* sp. from 6.03 to 41 mm, with an average of 27.49 mm. *Hyphessobrycon* and *Hemigrammus* are two speciose genus of small-sized fishes from the Characidae family occurring from northern cis-Andean South America, including the Amazon, Orinoco, La Plata, and São Francisco river basins, and rivers of Guyana, Suriname, French Guyana, and northeastern Brazil. They have, respectively, 136 and 54 species recognized as valid (Britski and Lima, 2008; Lima et al., 2014; Ota et al., 2014). The experiment was carried out using the environmental conditions found in the lagoon in order to minimize the stress caused on individuals and, more importantly, to replicate the conditions experienced by fishes in mark-recapture studies.

2.2. Protocol and experiment

Due to its incomplete solubility in water, pure clove oil (90-95% eugenol) was first dissolved in ethyl alcohol (92.8%) in 1:9 ratio (clove oil: ethyl alcohol) following Anderson et al. (1997). This solution was then diluted in water in order to obtain concentrations of 0.05 mL (50 mg), 0.10 mL (100 mg), and 0.20 mL (200 mg) of clove oil per 500 mL of water. These concentrations were selected after reference to other studies (Anderson et al., 1997; Keene et al.,

1998; Griffiths, 2000). In order to control for the effects of stress and hypoxia, a control experiment was set up where the behavior and mortality rate of fishes were observed without the anaesthetic. Therefore, we had a total of four treatments (D1 = 0.05 mL (50 mg), D2 = 0.10 mL (100 mg), D3 = 0.20 mL (200 mg) and control) and 30 replicates for each of them (ten individuals of the each species per treatment, a total of 120 individuals).

The experiment consisted of placing one individual fish into a container filled with 500 mL of water with a specific concentration of clove oil solution. We then timed both induction time (i.e., the time required for the fish to become completely anaesthetized) and recovery time (i.e., the amount of time for the fish to recover after sedation). For more details about stage of anaesthesia in fish see Iversen et al. (2003). This procedure was repeated for each replicate across all treatments. Fish were considered anaesthetized when they exhibited balance loss as well as reduced opercular pumping and pectoral fin beating. In order to determine recovery time, the anaesthetized individual was then transferred into a container filled only with lake water and we recorded the time taken for it to recover balance and regular movements. Individuals that did not recover balance and regular movements in 3 minutes (in the D1 treatment - 0.05 mL), 7 minutes (in the D2 treatment - 0.10 mL) and 10 minutes (in the D3 treatment - 0.2 mL) were immediately transferred for 30 minutes to another container in which water was constantly renewed. Individuals that did not exhibit any vital signs after this period were considered dead. These time period were chosen because the time required for the fish recovery is faster in low than high concentration of the clove oil (Anderson et al., 1997; Griffiths, 2000). When individuals were anaesthetized, we measured their standard length with calipers. After recovery, individuals were returned to the lake. Fish that died during the experiment were fixed in 10% formalin solution, preserved in 70% ethanol and stored in the Mato Grosso Federal University (UFMT) fish collection.

2.3. Statistical analysis

ANOVA permutation tests were used to assess whether standard length, induction and recovery time of the *Hyphessobrycon* sp.1, *Hyphessobrycon* sp.2 and *Hemigrammus* sp. varied across of the three treatments and to evaluate the effect of treatment on induction and recovery time of each species) and a multiple comparison Tukey post-hoc tests was used (Sokal and Rohlf, 1995). This test was chosen instead of ANOVA because the residuals of the ANOVA model were not normally distributed and it was not possible to normalize the data. Note that, since all individuals died on the 0.20 mL treatment, this treatment was excluded from the analysis of recovery time.

The relationships between standard length, induction time and recovery time were evaluated with linear regressions. As both induction and recovery times varied across treatments, regression models were built separately for each treatment and species. Residual normality was assessed with Lilliefors test (Kolmogorov-Smirnov) (Sokal and Rohlf, 1995). We were unable to use ANCOVA models in this analysis because regression lines exhibited non-parallelism, thus suggesting interactions between the different treatments and the standard length. Finally, differences among treatments in mortality rates were assessed using a Fisher's exact test (Sokal and Rohlf, 1995). All assumptions (i.e., homogeneity and linearity) for the parametric tests were met and analyses were performed in the R 2.15.2 statistical software (R Development Core Team, 2013).

3. Results

The standard length of individuals given treatment D1 (ANOVA: $F_{2,27}$=2.28; p=0.12) e D2 (ANOVA: $F_{2,27}$=2.21; p=0.12) showed no difference among species. However individuals of *Hyphessobrycon* sp.1 used in treatment D3 (ANOVA: $F_{2,27}$=15.92; p<0.001) and in the control ($F_{2,27}$=3.84; p<0.034) were smaller than *Hyphessobrycon* sp.2 and *Hemigrammus* sp. (p<0.001, Tukey post-hoc tests). The induction time did not vary among species in either treatment D1 (ANOVA: $F_{2,27}$=0.64; p=0.53), D2 (ANOVA: $F_{2,27}$=0.68; p=0.51) or D3 (ANOVA: $F_{2,27}$=1.68; p=0.20). Recovery time of individuals showed no difference between the three fish species for the D1(ANOVA: $F_{2,27}$=0.27; p=0.76) and D2 treatments (ANOVA: $F_{2,27}$=2.57; p=0.09).

Induction time varied across the three treatments only for *Hyphessobrycon* sp.1 and *Hemigrammus* sp. (Table 1). As expected, individuals submitted to the highest dose (D3) were anaesthetized significantly faster than individuals submitted to the two lower concentrations of clove oil (D1 and D2) (Table 1). However, there was no significant difference between individual recovery times at the two lower clove oil concentrations (D1 and D2) (Table 1). The average time for individuals to fully recover balance and movement was 6.03 and 6.65 minutes for *Hyphessobrycon* sp.1, 5.3 and 4. 9 minutes for *Hyphessobrycon* sp.2 and 6.2 and 6.3 minutes for *Hemigrammus* sp. in treatment D1 and D2, respectively (Table 1). In the treatment with the highest clove oil concentration (D3) all individuals died thus recovery time could not be evaluated.

The analysis performed on fish standard length indicated that larger individuals became anaesthetized more quickly than smaller ones during the D1 treatment on *Hyphessobrycon* sp.1 (r^2=0.35; df=8; p=0.04; Table 2), while the converse occurred with treatment D2 on *Hemigrammus* sp. (r^2=0.60; df=8; p=0.005; Table 2). No such affects were observed with the other treatments or species (Table 2). Additionally, smaller individual *Hyphessobrycon* sp.1 and *Hemigrammus* sp. exhibited a longer recovery time in treatment D1 (r^2=0.78; df=8; p<0.001; Table 2 and r^2=0.37; df=8; p=0.03; Table 2) but not on treatment D2 (Table 2).

Survival rates varied across treatments and species (Fisher's exact test; p < 0.001). In the control treatment we did not observe any mortality, while all fish died under treatment D3. In the other two treatments, the number of

Table 1. Mean, standard deviation (sd) of the standard length, induction time (minutes), recovery time (minutes) and survived rate for *Hyphessobrycon* sp.1, *Hyphessobrycon* sp.2 and *Hemigrammus* sp. with each treatment and results of the Analysis of Variance (ANOVA).

Species	Control	D1 (0.05 mL)	D2 (0.1 mL)	D3 (0.2 mL)	F	df	p
Hyphessobrycon sp.1		Mean±sd					
Standard length (mm)	28.6±3.2[a]	28.2±3.06[a]	29.4±2.5[a]	18.3±7.6[b]	**12.87**	**3.36**	**<0.001**
Induction time	na	0.19±0.08[a]	0.37±0.25[b]	0.13±0.03[a]	**6.23**	**2.27**	**0.005**
Recovery time	na	6.03±3.1	6.65±1.9	---	0.28	1.18	0.603
Survived*	10	7	6	0	---	---	**<0.001**
Hyphessobrycon sp.2		Mean±sd					
Standard length (mm)	32.0±1.1	31.7±2.0	31.5±2.9	32.0±3.8	0.09	3.36	0.965
Induction time	na	0.28±0.29	0.52±0.58	0.11±0.02	2.95	2.27	0.069
Recovery time	na	5.3±2.7	4.9±2.3	na	0.011	1.18	0.744
Survived*	10	9	7	0	na	na	**<0.001**
Hemigrammus sp.		Mean±sd					
Standard length (mm)	30.0±3.2	29.6±5.13	29.0±3.14	23.8±9.6	0.53	3.36	0.661
Induction time	na	0.23±0.05[ab]	0.32±0.02[a]	0.10±0.02[b]	**5.77**	**2.27**	**0.008**
Recovery time	na	6.2±2.9	6.3±1.0	na	0.01	1.18	0.890
Survived*	10	7	5	0	na	na	**<0.001**

Different letters above value indicate significant statistical differences (Tukey post-hoc tests). --- all fish died. na = not applicable. *Fisher's exact test was used.

Table 2. Results of the linear regression of standard length on induction and recovery times for *Hyphessobrycon* sp.1, *Hyphessobrycon* sp.2 and *Hemigrammus* sp. for each treatment.

Species	D1 (0.05 mL)		D2 (0.1 mL)		D3 (0.2 mL)	
Hyphessobrycon sp.1	r^2	p	r^2	p	r^2	p
Standard length vs. induction time	**0.35**	**0.04**	0.09	0.67	0.07	0.55
Standard length vs. recovery time	**0.78**	**<0.001**	0.13	0.16	na	na
Hyphessobrycon sp.2						
Standard length vs. induction time	0.07	0.54	0.03	0.29	0.12	0.86
Standard length vs. recovery time	0.09	0.62	0.08	0.58	na	na
Hemigrammus sp.						
Standard length vs. induction time	0.11	0.18	**0.60**	**0.005**	0.25	0.07
Standard length vs. recovery time	**0.37**	**0.03**	0.12	0.91	na	na

individuals that died after anesthesia changed between species. In *Hyphessobrycon* sp.1 30% died in treatment D1, and 60% died in treatment D2 (Figure 1a), 10% died in treatment D1 and 20% of *Hyphessobrycon* sp.2 died in treatment D2 (Figure 1b). For *Hemigrammus* sp., 30% died in treatment D1, and 50% died in treatment D2 (Figure 1c).

4. Discussion

Even though induction time and recovery time did not vary between species, induction time varied across the three treatment for two species (*Hyphessobrycon* sp.1 and *Hemigrammus* sp.), with larger individuals exhibiting a longer induction time under treatment D2 for *Hemigrammus* sp., and a shorter recovery time under treatment D1 for *Hyphessobrycon* sp.1 and *Hemigrammus* sp. On the other hand, larger individuals of *Hyphessobrycon* sp.1 became anaesthetized faster than smaller ones with treatment D1. Treatment D3 was not efficient for fish anaesthesia because it caused mortality of fish across all replicates. In contrast, 70% of individuals of *Hyphessobrycon* sp.1 and *Hemigrammus* sp., and 90% of individuals from *Hyphessobrycon* sp.2 survived after being anaesthetized under treatment D1, while mortality reached 20% for *Hyphessobrycon* sp.2, 50% for *Hemigrammus* sp. and 60% for *Hyphessobrycon* sp.1 with treatment D2.

Although we did not observe a negative relationship between concentration and induction time for all treatments, a concentration-dependant pattern is well known (Soto and Burhanuddin, 1995; Keene et al., 1998; Hisano et al., 2008; Woody et al., 2002; Walsh and Pease, 2002), including for other types of anaesthetics (Silva et al., 2013), so that increasing anaesthetic dose results in a decreased induction time (Hoskonen and Pirhonen, 2004; Roubach et al., 2005; Öğretmen et al., 2014). It was, consequently, unexpected that the longest induction time for *Hyphessobrycon* sp.1 and *Hemigrammus* sp., were observed with treatment D2 .

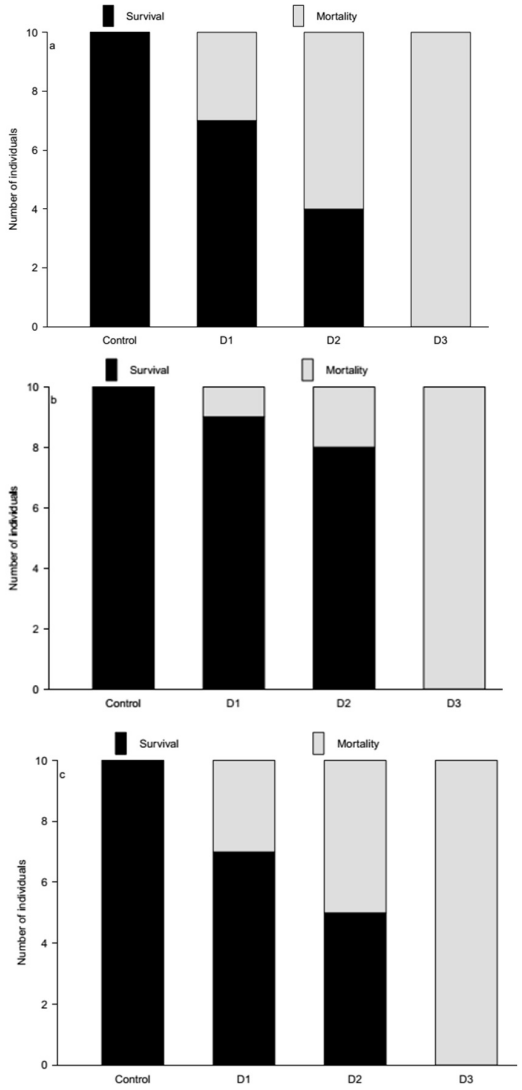

Figure 1. Number of individuals that survived and died in each treatment for *Hyphressobrycon* sp.1 (a), *Hyphessobrycon* sp.2 (b) and *Hemigrammus* sp. (c).

However, this probably occurred because we did not take into account the time of the day at which each experiment was performed. Treatment D2 was performed between 0800 and 1000 hours, which is a relatively cool period of the day. The other two treatments were performed in periods with higher temperatures: between 1000 and 1200 hours for treatment D1, and 1400 and 1600 hours for treatment D3. The higher temperatures occurring after 1000 hours increase fish metabolic rate and consequently, the effects of anaesthetic will be faster than lower temperatures (Hoskonen and Pirhonen, 2004). Opercular rates at high temperature are greater than those at low temperature. As the gills in fish are the main route of entry and the excretion of anaesthetics, increasing gill ventilation and cardiac rates at high temperature would increase the gill permeability of anaesthetic and result in increasing the efficacy of anaesthetic (Javahery et al., 2012). Thus, temperature is important environmental factor what could be considered when using clove oil as anaesthetic (Iversen et al., 2003; Javahery et al., 2012). Furthermore, individuals from *Hyphessobrycon* sp.1 specie used in treatment D3 are statically lower than treatment D1 and D2.

Studies analyzing the effect of standard length on induction and recovery times have reported conflicting results (Prince and Powell, 2000; Walsh and Pease, 2002; Woody et al., 2002; Hoskonen and Pirhonen, 2004; Perdikaris et al., 2010). As with our dissenting results for *Hyphessobrycon* sp.1 and *Hemigrammus* sp., larger bodied Whitefish (*Coregonus lavaretus*) had shorter induction times than smaller ones, while the opposite occurred in Rainbow Trout (*Oncorhynchus mykiss*) (Hoskonen and Pirhonen, 2004). The same study found that Brown Trout (*Salmo trutta*) and Atlantic Salmon (*Salmo salar*) of different body sizes did not differ in induction times (as we found for *Hyphessobrycon* sp.2). Of the few studies that have assessed the relationship between fish size and recovery time, two found no relationship (Hoskonen and Pirhonen, 2004; Prince and Powell, 2000), and one found that larger individuals had quicker recovery times than smaller ones (Woody et al., 2002). This parallels the current results for *Hyphessobrycon* sp.1 and *Hemigrammus* sp.

In general, clove oil was an efficient anaesthetic at low concentrations. However, the survival rates of fish decreased sharply at higher concentrations (Soto and Burhanuddin, 1995; Munday and Wilson, 1997; Iversen et al., 2003; Hisano et al., 2008; Inoue et al., 2011; Javahery et al., 2012), which was observed in part of the replicates of the D2 and all of the D3 treatments. Thus, the use of a 0.05 mL (or less) dose of clove oil (per 500 mL of water) as an anaesthetic should be recommended for mark-recapture and population monitoring studies (Ouedraogo et al., 2014). Clove oil concentrations above 0.05 mL per 500 mL of water increase considerably the mortality rates and are thus only recommended for studies in which fish species need to be sampled and sacrificed for later laboratory analyses (e.g., dietary or reproductive analysis). In such studies we suggest using the concentration of 0.20 mL (or 200 mg) clove oil per 500 mL of water in order to speed anaesthesia-induced death and so eliminate suffering when individuals are fixed in formalin solution.

In conclusion, the present study showed that the response of small bodied tropical fish to a clove oil anaesthetic are species-specific, but that all three species were anaesthetized in less than one minute and recovery the fully equilibrium and movement in less than 7 minutes, so showing close oil be an efficient anaesthetic for field studies of tropical fish. These results are similar to that found in others studies with large bodied and fisheries-exploited species in temperate (Anderson et al., 1997; Hoskonen and Pirhonen, 2004) and tropical regions (Simões et al., 2010; Bertozi Júnior et al., 2014; Sanchez et al., 2014a, b). Based on studies that have been assessed the clove oil efficiency, the appropriated anaesthetic dose is between 0.05 mL (50 mg L^{-1}) and 0.10 mL (100 mg L^{-1}) (Javahery et al.,

2012). However, in this study we had worked with three small bodied species which the largest individual sampled reached 41 mm, thus we believe that concentration more than 0.05 mL (50 mg/500 mL or 100 mg L^{-1} of water) should be not recommended for species the size of those in the current study.

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