

ISSN 1678-3921

Journal homepage: www.embrapa.br/pab

For manuscript submission and journal contents,
access: www.scielo.br/pab

Peppermint essential oil as an anesthetic for and toxicity to juvenile silver catfish







Abstract – The objective of this work was to evaluate peppermint (*Mentha piperita*) essential oil toxicity to and potential as an anesthetic for juvenile silver catfish (*Rhamdia quelen*). To determine the lethal concentration at 4 hours (LC_{50-4h}), 210 fish (3.08±0.8 g and 7.59±0.67 cm) were exposed to 0, 20, 50, 80, 110, and 140 mg L⁻¹ essential oil. To evaluate the anesthetic potential, nine fish were individually exposed to each oil concentration (50, 80, 110, and 140 mg L⁻¹) used. Water quality parameters were controlled. The mortality rate and the severity and extent of the gill injuries of silver catfish juveniles at 4 hours of exposure increased with increasing peppermint essential oil concentrations, with LC_{50-4h} estimated to be 75.06 mg L⁻¹. The main gill injuries were: congestion of the venous sinus of the primary lamella and at the base of the secondary lamella; interlamellar hyperplasia with fusion of lamellae; epithelial detachment; dilation of the venous sinus; edema adjacent to the lamella; and aneurysm. However, this oil is an effective anesthetic for silver catfish juveniles at the concentration of 80 mg L⁻¹, with a short time of anesthesia (<4 min) and recovery (<10 min), with no mortality.

Index terms: *Mentha piperita*, *Rhamdia quelen*, anesthesia, gill, histology, lethal concentration.

Óleo essencial de hortelã-pimenta como anestésico e sua toxicidade para juvenis de jundiá

Resumo – O objetivo deste trabalho foi avaliar a toxicidade e o potencial como anestésico do óleo essencial de hortelã-pimenta (*Mentha piperita*) para juvenis de jundiá (*Rhamdia quelen*). Para determinar a concentração letal em 4 horas (CL_{50-4h}), 210 peixes (3,08±0,8 g e 7,59±0,67 cm) foram expostos a 0, 20, 50, 80, 110 e 140 mg L⁻¹ do óleo essencial. Para avaliar o potencial anestésico, nove peixes foram individualmente expostos à cada concentração (50, 80, 110 e 140 mg L⁻¹) de óleo utilizada. Os parâmetros de qualidade da água foram controlados. A taxa de mortalidade e a severidade e a abrangência das lesões branquiais de juvenis de jundiá em 4 horas de exposição cresceu com o aumento da concentração do óleo essencial de hortelã-pimenta, com CL_{50-4h} estimada em 75,06 mg L⁻¹. As principais lesões branquiais foram: congestão do seio venoso da lamela primária e na base da lamela secundária; hiperplasia interlamelar e com fusão das lamelas; descolamento do epitélio; dilatação do seio venoso; edema justalamelar; e aneurisma. Entretanto, esse óleo é eficiente anestésico para juvenis de jundiá na concentração de 80 mg L⁻¹, com reduzido tempo de anestesia (<4 min) e de recuperação (<10 min), sem mortalidade.

Termos para indexação: *Mentha piperita*, *Rhamdia quelen*, anestesia, brânquias, histologia, concentração letal.

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Received

December 8, 2017

Accepted

January 23, 2019

How to cite

SPANGHERO, D.B.N.; SPANGHERO,
E.C.A. de M.; PEDRON, J. dos S.;
CHAGAS, E.C.; CHAVES, F.C.M.;
ZANIBONI-FILHO, E. Peppermint essential
oil as an anesthetic for and toxicity to juvenile
silver catfish. *Pesquisa Agropecuária
Brasileira*, v.54, e00367, 2019. DOI: <https://doi.org/10.1590/S1678-3921.pab2019.v54.00367>.

Introduction

Currently, there are few safe, effective, and practical sedative options available for fish (Trushenski et al., 2013). Benzocaine and tricaine methanesulfonate (MS-222), for example, which are the most commonly used synthetic anesthetics in fish, can both cause adverse effects. Benzocaine can induce methemoglobinemia, which can interfere with oxygen transport through the blood, and MS-222, although effective, is limited by regulatory agencies such as the US Food and Drug Administration (FDA), which prohibits the consumption of treated fish for a period of 21 days (Trushenski et al., 2013). The effects of prolonged MS-222 exposure include hypoxia (inadequate oxygen supply at the tissue or whole-body level), increased plasma lactate concentrations, hyperglycemia (elevated blood glucose levels), increased urinary output, and electrolyte loss. In addition, it can be continuously absorbed throughout immersion, leading to a lethal overdose (Treves-Brown, 2000).

Among natural anesthetics, eugenol – the active ingredient in clove [*Syzygium aromaticum* (L.) Merr. & L.M.Perry] oil – is the most widely used for fish and is approved in several countries, including Australia, New Zealand, and Chile (Trushenski et al., 2013). However, the FDA's Center for Veterinary Medicine (CVM) does not recommend the use of clove oil or of any of its components in fish (United States, 2007) because of its carcinogenic potential in rats (NTP, 1983).

Therefore, there is a need to find alternative anesthetics for fish. Previous studies have investigated essential oils from: clove basil (*Ocimum gratissimum* L.), *Hesperozygis ringens* (Benth.) Epling, pepper-rosmarin (*Lippia sidoides* Cham.), and *Ocotea acutifolia* (Ness) Mez for silver catfish (*Rhamdia quelen*) (Silva et al., 2012, 2013); and peppermint (*Mentha piperita* L.) for Nile tilapia (*Oreochromis niloticus*) (Hashimoto et al., 2016) and “pirarucu” (*Arapaima gigas*) (Malheiros et al., 2016).

The latter oil is obtained from a hybrid plant, resulting from a crossing between spearmint (*Mentha spicata* L.) and water mint (*Mentha aquatica* L.) (Isacan et al., 2002). Its anesthetic potential has been attributed to its main component, menthol, an anesthetic that has great interest for aquaculture (Pádua et al., 2010). Other authors also reported anthelmintic activity of

peppermint essential oil against *Dawestrema* spp. (Malheiros et al., 2016), as well as a cytotoxic effect against *Ichthyophthirius multifiliis* (Valladão et al., 2016) in in vitro studies. Likewise, for an in vivo test, a reduction of up to 70% in monogenean parasites was observed when infected Nile tilapia were bathed three times at 40 mg L⁻¹ for 10 min at a 24-hour interval (Hashimoto et al., 2016). However, any substance dissolved or suspended in water, i.e., therapeutic baths, may be irritating to fish, even when applied in short-time intervals, as noted in gills, for example, which are directly affected due to their external location, making them particularly sensitive to stressors and susceptible to immediate lesions (Roberts, 2012).

Among the fish species that present great potential for continental aquaculture in Southern Brazil, the silver catfish stands out as the main native species produced in the region (Baldisserotto, 2009). Although peppermint oil is already used as an anesthetic, its effects are species-specific (Ross & Ross, 2008); therefore, it is important to test its toxicity for its safe use in short-time therapeutic baths of silver catfish, specifically regarding gill structure.

The objective of this work was to evaluate peppermint essential oil toxicity to and potential as an anesthetic for juvenile silver catfish.

Materials and Methods

The experiment was conducted in August 2017 at the freshwater fish biology and culture laboratory of Universidade Federal de Santa Catarina, located in the state of Santa Catarina, Brazil. Silver catfish juveniles were obtained from the induced reproduction of wild broodstock from the Upper Uruguay River and kept in captivity. Shortly after mouth-opening (72 hours after hatching), larvae were fed *Artemia* sp. nauplii for 15 days. After this period, the fish were placed in 1,000-L glass fiber boxes, where they were fed a balanced diet (45% crude protein) and kept for 60 days, under laboratory conditions, with continuous water renewal in a recirculation system, constant aeration, and a temperature of 26±1°C (mean±standard deviation), until they reached 3.08±0.80 g in weight and 7.59±0.67 cm in total length, when the toxicity and anesthesia experiments were started.

The peppermint essential oil was obtained from the laboratory of medicinal plants and phytochemistry of

Embrapa Amazônia Ocidental, located in Manaus, in the state of Amazonas, Brazil. Peppermint specimens were grown at the medicinal plant collection also of Embrapa Amazônia Ocidental. Plant shoot (leaves and/or inflorescences) was removed for essential oil extraction by hydrodistillation in a Clevenger apparatus for 2 hours. In each distillation, 500 g leaves and inflorescences were used. The essential oil was stored at 4°C until analyses (Potzernheim et al., 2012).

The chemical analysis of the essential oil was conducted using gas chromatography with mass spectrometry, according to Morais et al. (2012). The main components found were: 27.5% menthol, 22.5% menthofuran, 12.8% pulegone, 12.5% menthyl acetate, 11% menthone, 3.5% limonene, and 2.1% 1,8-cineole. The hydrophobic nature of the essential oil required that a stock solution diluted with 99.8% ethyl alcohol be used at a ratio of 1:9 essential oil:alcohol (v:v) to facilitate dilution in water.

For the acute toxicity test, 210 silver catfish juveniles were placed in 21 experimental units containing 1 L culture water in a static system with constant aeration. The fish were fasted for 24 hours prior to the start of the experiment and kept without feed during the test. Each treatment consisted of three replicates, and the experimental design was completely randomized. Five treatments were applied, consisting of increasing essential oil concentrations (20, 50, 80, 110, and 140 mg L⁻¹) in the culture water. Two controls were used: one containing only culture water (water) and the other, culture water plus 1.26 mL L⁻¹ ethyl alcohol. This amount of alcohol was equal to the maximum amount used in the treatment containing the highest essential oil concentration. The period of exposure to the essential oil was 4 hours, after which mortality was recorded and lethal concentration (LC_{50-4h}) determined. Fish were considered dead if they did not exhibit opercular or caudal movements, or if they did not respond to a touch stimulus after 1 hour of being transferred to culture water without the addition of essential oil.

The anesthesia and anesthesia recovery tests were also performed in experimental units containing 1 L water in a static system with constant aeration. For this, four concentrations of peppermint essential oil were tested: 50, 80, 110, and 140 mg L⁻¹. Nine fish were individually exposed to each concentration after fasting for 24 hours. The stages of anesthesia and

recovery were monitored, and the time when the fish reached each stage was recorded with a digital timer. These stages were defined using characteristic fish behavior, based on Park et al. (2009). The stages of anesthesia were: slow swimming (A1); loss of balance (A2); rest (A3); and paralysis of opercular beat, i.e., deep anesthesia (A4). At the last stage of anesthesia, the fish were immediately withdrawn from the container with essential oil and transferred for recovery to the ones with only water. The recovery stages were: return of opercular movement (R1), beginning of movement (R2), beginning of swimming (R3), and recovery (R4).

At the beginning of each experiment, water quality parameters were measured using the multiparameter probe YSI 556 MPS (YSI Incorporated, Yellow Springs, OH, USA). In the acute toxicity test, the following values were found: 26.2±0.5°C temperature, 2.0±0.0 g L⁻¹ salinity, 7.6±0.7 mg L⁻¹ dissolved oxygen, pH 8.0±0.1, and 417.0±3.6 µS cm⁻¹ conductivity. In the anesthetic test, 26.7±0.3°C temperature, 2.0±0.0 g L⁻¹ salinity, 7.0±0.3 mg L⁻¹ dissolved oxygen, pH 7.8±0.3, and 393.0±2.8 µS cm⁻¹ conductivity.

Immediately after toxicity testing, samples from the second, right-hand-side gill arch of three fish per replicate were collected for the histological analysis. The collected material was identified, fixed in 10% buffered formalin solution for 24 hours, washed in 70% alcohol for 3×15 min, and stored in 70% alcohol until the analysis. Gill arches were decalcified and dehydrated in an ascending series of alcohol solutions (70, 80, 90, and 100%), and then embedded in paraffin following a routine technique. Subsequently, 3-µm-thick cross sections were taken, using the Leica RM2245 semi-motorized rotary microtome (Leica Biosystems, Wetzlar, Germany), and stained with Harris hematoxylin and eosin. Images were captured using the Zeiss AxioVert.A1 light microscope with the AxioCam ERc 5s camera and analyzed with the Zen Lite 2012 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

The gill analysis consisted of identifying tissue alterations such as: hyperplasia of secondary lamella and interlamella, with fusion of secondary lamellae; epithelial detachment in the secondary lamella; edema adjacent to the lamella; dilation of the venous sinus; aneurysm; and congestion at the base of the secondary lamella and of the venous sinus of the primary lamella. These changes were classified according to

the method of Poleksić & Mitrović-Tutundžić (1994), which considers the degree of severity in relation to the intensity (mild, moderate, or severe) and location (focal, multifocal, or coalescent) of the lesion.

In order to compare the intensity and extent of the lesions among the different treatments, numerical scales were used. For lesion intensity: 0, absent; 1, mild; 2, moderate; and 3, severe. For extent: 0, absent; 1, focal; 2, multifocal; and 3, coalescent. The degree of alteration in each gill was calculated by multiplying lesion intensity and extent values. In this case, the degree of injury varied on a scale of 0 to 9, with 0 indicating no injury and 9, maximum lesion intensity and extent.

In the acute toxicity test, the LC_{50-4h} value was determined by the trimmed Spearman-Kärber method with 95% confidence intervals (CIs) (Hamilton et al., 1977) and complemented by the linear regression analysis. To compare the treatment means, the data were tested in conformity with the assumptions of the analysis of variance (Anova) by performing Shapiro-Wilk's and Levene's tests for normality and homoscedasticity, respectively. If the subsequent Anova showed significant differences, the treatment means were compared by Tukey's test, at 5% probability.

Results and Discussion

In the acute toxicity test, no mortality was observed in the control groups or in the treatment with the lowest essential oil concentration (20 mg L⁻¹) after 4 hours of exposure. The mortality rates of fish exposed to 50, 80, 110, and 140 mg L⁻¹ peppermint essential oil were 6.7±0.5, 56.7±1.7, 83.3±1.2, and 100.0±0.0%, respectively. Mortality was directly associated with increasing essential oil concentrations ($p < 0.05$), with an estimated LC_{50-4h} of 75.06 mg L⁻¹ (95% CIs = 67.49–83.17 mg L⁻¹) (Figure 1). This value was greater than the LC_{50-4h} of 38 mg L⁻¹ obtained for juvenile “pirarucu”, weighing approximately 35 g (Malheiros et al., 2016), indicating that these fish are less tolerant than silver catfish to exposure to this same essential oil. According to these results, concentrations of up to 20 mg L⁻¹ peppermint essential oil can be safely used in 4-hour therapeutic baths of silver catfish.

The intensity and extent of gill damage increased with increasing concentrations of peppermint essential

oil. After 4 hours of exposure, the control groups showed no epithelial detachment in the secondary lamella, hyperplasia with fusion of secondary lamellae, aneurysm, venous sinus dilation, or congestion in the secondary lamella (Table 1). Venous sinus dilation remained constant at 20 and 140 mg L⁻¹, and aneurysms were similar at ≥ 50 mg L⁻¹. When compared with the fish from the control groups, a greater congestion of the venous sinus of the primary lamella was only observed at the essential oil concentration of 110 mg L⁻¹.

Morphological changes (Figure 2) may indicate the development of adaptive strategies of fish for the protection of important physiological functions, including gas exchange, acid-base balance, osmoregulation, and the excretion of nitrogen compounds (Barbieri & Bondioli, 2015). It should be pointed out that aneurysms were only observed in fish exposed to peppermint essential oil and probably resulted from the rupture of xenobiotic cells (Winkaler et al., 2007), which damages vascular integrity by releasing large quantities of blood (Heath, 1987). This type of lesion can also be caused by primary alterations such as hyperplasia of the secondary lamella or edema adjacent to the lamella (Bernet et al., 1999).

Gill lesions may cause a reduction in respiratory capacity, leading to fish death. However, it is important to note that healthy fish are not necessarily characterized by the absence of tissue diseases, since they may also exhibit moderate structural disturbances or mild inflammatory reactions (Bernet et al., 2004),

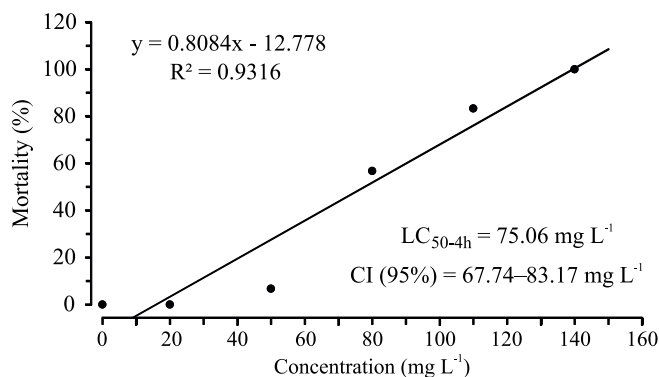


Figure 1. Linear equation showing the relationship between the concentration of peppermint (*Mentha piperita*) essential oil and the mortality of silver catfish (*Rhamdia quelen*). LC_{50-4h} , lethal concentration of the essential oil after 4 hours of exposure; and CI, confidence interval.

such as those found in the control groups in the present study, in which maximum lesion severity was 2. Here, the continuous exposure for 4 hours to the essential oil caused tissue alterations and even gill lesions, indicating stress.

During the anesthetic evaluation, all of the peppermint essential oil concentrations induced fish to the last behavioral stage of anesthesia (A4) and recovery (R4) (Table 2). Furthermore, no mortality was recorded during the test or even 24 hours after exposure. Concentrations ≥ 80 mg L⁻¹ resulted in deep anesthesia (A4) at about 4 min of exposure, whereas for 50 mg L⁻¹ ~6 min were required. Recovery at 140 mg L⁻¹ took the longest time, ~11 min.

The depressant effect of peppermint essential oil may be, in part, due to the effects of menthol and pulegone on gamma-aminobutyric acid type A (GABA_A), which is an inhibitory neurotransmitter of the central nervous system (Zhang et al., 2008; Tong & Coats, 2010). GABA_A stimulation by agonist drugs promotes cell membrane hyperpolarization, causing central nervous system depression and consequent anesthesia (Guénette et al., 2007). The anesthetic effect of menthol has also been observed in juvenile “dourado” (*Salminus brasiliensis*), weighing approximately 194 g, subjected to concentrations ranging from 60 to 150 mg L⁻¹ (Pádua et al., 2010), and in juvenile “tambaqui” (*Colossoma macropomum*), weighing approximately 89 g, treated with concentrations between 50 and 250 mg L⁻¹ (Façanha & Gomes, 2005). Pulegone has

been used in juvenile silver catfish (93.9±3.9 g) via concentrations of 30 to 50 µL L⁻¹ of an essential oil from *H. ringens*, in which pulegone was the main component (81.4%) (Toni et al., 2015).

The stabilization of the time to induce anesthesia was observed at ≥ 80 mg L⁻¹ peppermint essential oil. A similar behavior was reported after exposure of juvenile “tambaqui” to 150 mg L⁻¹ menthol (Façanha & Gomes, 2005). In the present study, 80 mg L⁻¹ peppermint essential oil induced deep anesthesia in juvenile silver catfish, requiring an exposure time similar to that needed for the higher concentrations tested. It should be highlighted that excessive anesthesia is very stressful to fish and causes abnormal metabolic rates, oxygen consumption, blood pressure, and blood physiological responses (Park et al., 2009); these side effects can last for several hours after fish recover from anesthesia (Summerfelt & Smith, 1990). The optimal anesthetic concentrations can minimize any negative impacts and, therefore, reduce stress (Park et al., 2009).

According to the criteria for selecting an effective anesthetic agent, it is recommended that anesthesia should be induced rapidly (<3 min) and that should take a quick recovery (<10 min) (Park et al., 2003). A condition close to that was observed in silver catfish juveniles subjected to concentrations between 80 and 110 mg L⁻¹ peppermint essential oil, and 80 mg L⁻¹ was the lowest concentration that met the criteria recommended for the selection of an effective anesthetic.

Table 1. Gill lesion observed in juvenile silver catfish (*Rhamdia quelen*) exposed to different concentrations of peppermint (*Mentha piperita*) essential oil for 4 hours⁽¹⁾.

Gill lesion ⁽²⁾	Concentration (mg L ⁻¹)						
	0 + alcohol	0	20	50	80	110	140
EDSL	0.0±0.0d	0.0±0.0d	1.8±0.4c	1.9±0.3c	2.0±0.0c	4.0±0.0b	6.0±0.0a
HSLF	0.0±0.0d	0.0±0.0d	1.0±0.0c	2.8±0.4b	3.0±0.0b	6.0±0.0a	6.0±0.0a
Aneurysm	0.0±0.0c	0.0±0.0c	1.1±0.3b	2.0±0.0a	2.2±0.6a	2.2±0.6a	2.2±0.6a
VSD	0.0±0.0b	0.0±0.0b	1.9±0.3a	2.0±0.0a	2.0±0.0a	2.0±0.0a	2.0±0.0a
CBSL	0.0±0.0c	0.0±0.0c	1.0±0.0b	1.0±0.0b	2.0±0.0a	2.0±0.0a	2.0±0.0a
HSL	1.1±0.5d	1.0±0.3d	2.0±0.0c	2.8±0.4b	3.0±0.0b	5.8±0.6a	5.8±0.6a
IH	2.0±0.0c	1.9±0.3c	1.9±0.3c	4.0±0.0b	4.0±0.0b	5.8±0.6a	5.8±0.6a
EL	1.2±0.4d	1.1±0.3d	2.0±0.0c	4.0±0.0b	4.0±0.0b	4.0±0.0b	6.0±0.0a
CVSPL	1.7±0.5b	2.0±0.0b	1.9±0.3b	2.0±0.0b	2.1±0.3b	4.2±0.6a	4.2±0.6a

⁽¹⁾Values followed by equal letters, in the same rows, do not differ significantly by Tukey's test, at 5% probability. ⁽²⁾The degree of injury was calculated by multiplying the intensity and extent of the lesion, classified according to numerical scales for lesion intensity (0, absent; 1, mild; 2, moderate; and 3, severe) and extent (0, absent; 1, focal; 2, multifocal; and 3, coalescent). Sampling of nine fish, three per replicate. EDSL, epithelial detachment in the secondary lamella; HSLF, hyperplasia with fusion of secondary lamellae; VSD, venous sinus dilation; CBSL, congestion at the base of the secondary lamella; HSL, hyperplasia of the secondary lamella; IH, interlamellar hyperplasia; EL, edema adjacent to the lamella; and CVSPL, congestion of the venous sinus of the primary lamella.

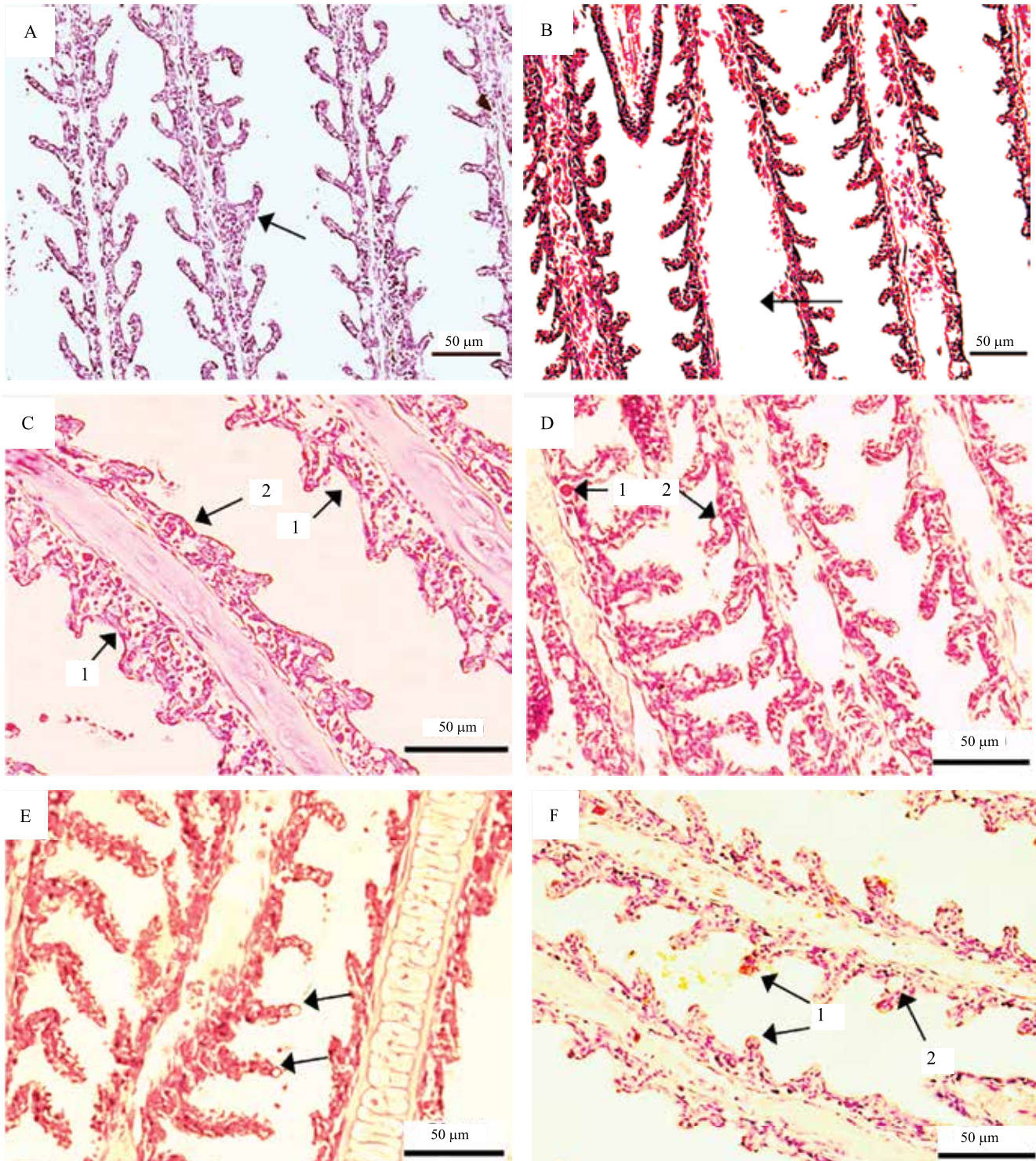


Figure 2. Photomicrograph of lesions (arrowed) in the gills of silver catfish (*Rhamdia quelen*) exposed for 4 hours to culture water with different concentrations of peppermint (*Mentha piperita*) essential oil. A, exposure only to culture water (control), hyperplasia of the secondary lamella; B, exposure to 20 mg L⁻¹ essential oil, congestion of the venous sinus of the primary lamella; C, exposure to 50 mg L⁻¹ essential oil, interlamellar hyperplasia (1) and fusion of the secondary lamella (2); D, exposure to 80 mg L⁻¹ essential oil, congestion at the base of the secondary lamella (1) and epithelial detachment in the secondary lamella (2); E, exposure to 110 mg L⁻¹ essential oil, venous sinus dilation; and F, exposure to 140 mg L⁻¹ essential oil, aneurysm (1) and edema adjacent to the lamella.

Table 2. Induction to different stages of anesthesia and recovery in juvenile silver catfish (*Rhamdia quelen*) exposed to different concentrations of peppermint (*Mentha piperita*) essential oil⁽¹⁾.

Concentration (mg L ⁻¹)	Anesthesia stage ⁽²⁾				Recovery stage ⁽³⁾			
	A1	A2	A3	A4	R1	R2	R3	R4
50	60.3±6.0a	90.3±25.3a	221.3±59.7a	355.0±15.7a	19.0±13.9a	127.7±113.5a	185.3±113.7b	222.7±110.6b
80	81.0±19.8a	97.7±22.2a	141.3±65.9a	247.0±13.9b	62.0±25.1a	153.7±104.7a	335.3±51.0b	413.0±56.8b
110	64.7±27.2a	91.0±24.6a	141.7±49.1a	270.7±18.6b	87.0±69.7a	184.7±81.7a	383.7±25.0ab	409.0±15.2b
140	49.7±11.5a	75.7±9.5a	82.3±11.7a	242.3±12.5b	158.0±63.9a	281.7±20.5a	609.0±113.3a	663.0±59.7a

⁽¹⁾Values followed by different letters, in the same columns, differ statistically according to Tukey's test, at 5% probability. Sampling of nine fish for each essential oil concentration. ⁽²⁾A1, slow swimming; A2, loss of balance; A3, rest; and A4, paralysis of opercular beat (deep anesthesia). ⁽³⁾R1, return of opercular movement; R2, beginning of movement; R3, beginning of swimming; and R4, recovery.

Conclusions

1. Peppermint (*Mentha piperita*) essential oil is toxic to juvenile silver catfish (*Rhamdia quelen*) exposed for 4 hours to concentrations greater than 50 mg L⁻¹, with a lethal concentration of 75.06 mg L⁻¹.

2. The gills of silver catfish juveniles may be considered good indicators of the toxicity caused by prolonged exposure to peppermint essential oil, and concentrations ≥ 20 mg L⁻¹ cause lesions in gill tissues after 4 hours of exposure.

3. An 80-mg L⁻¹ concentration of peppermint essential oil is recommended to induce anesthesia in juvenile silver catfish, causing deep anesthesia after 4 min of exposure and complete recovery after 7 min of returning the fish to anesthetic-free water.

Acknowledgments

To the laboratory of medicinal plants and phytochemistry of Embrapa Amazônia Ocidental, for providing peppermint essential oil; to the nucleus of studies in aquaculture pathology of Universidade Federal de Santa Catarina (UFSC), for the infrastructure used for the histological analyses; and to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for a productivity research fellowship granted to the sixth author.

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