Original Article

LIMNOLOGY FRESHWATER BIOLOGY

Exploratory study on the effects of Ibuprofen and Sucralose on Morphological Traits and Life Cycle of the Macroinvertebrate *Chironomus riparius* (Meigen, 1804)

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ABSTRACT. Pharmaceuticals and food additives increasingly contaminate freshwater ecosystems; ibuprofen and sucralose are prominent emerging contaminants frequently detected in aquatic environments worldwide today. This preliminary study investigates the effect of sub-lethal concentrations of the drug ibuprofen (IBU; 25 ng/L) and the artificial sweetener (AS) sucralose (SUCR; 500 ng/L and 5 g/L) on larval morphology (e.g. cephalic capsule size) and life cycle (from egg hatching to adult emergence) of *Chironomus riparius* (Diptera Chironomidae). The Kruskal-Wallis test was performed to evaluate differences in the five selected endpoints ($p < 0.05$). The two substances did not significantly affect the hatching time or the duration of the life cycle. Conversely, larvae exposed to 500 ng/L of SUCR had a longer and narrower head capsule, while IBU caused severe deformities of the mentum (e.g. tooth loss) in the larvae at III-IV instars. These findings address new insights on the toxicity of IBU and SUCR on benthic macroinvertebrates, increasing the awareness on the need for their monitoring and regulation.

Keywords: Chironomidae, emerging contaminants, drugs, food additives, larval morphology, mentum deformities

For citation: Sbarberi R., a, Bonciu A.C., a, Paoli F., Pozzi S., Piffer S., Lencioni V. Exploratory study on the effects of Ibuprofen and Sucralose on Morphological Traits and Life Cycle of the Macroinvertebrate *Chironomus riparius* (Meigen, 1804) // Limnology and Freshwater Biology. 2026. — No. 6. — P. 742–755. DOI: 10.31951/2658-3518-2026-A-6-742

1. Introduction

The presence of pharmaceutical and food additives in freshwater ecosystems has become a growing concern, with ibuprofen (IBU) and sucralose (SUCR) being two notable emerging contaminants (ECs), frequently detected in water bodies (Anim et al., 2020; Tkach et al., 2025).

IBU is a nonsteroidal anti-inflammatory drug (NSAID) derived from propionic acid (Mahfouz et al., 2024), used for the treatment of pain, fever and inflammation both in adults and children (Mazaleuskaya et al., 2015). It primarily works by inhibiting cyclooxygenase enzymes (COX-1 and COX-2), which play a key role in producing substances responsible for inflammation and pain (Upadhyay et al., 2021). In the last few decades, the prescriptions of NSAID as IBU has increased by 7% in Italy during the pandemic period, because these drugs were used to reduce the symptoms in people infected by the COVID-19 (Perrone et al., 2025). IBU enters the environment primarily through human excretion and improper disposal, such as flushing unused medication down the drain (Flórez-Restrepo et al., 2025).

On the other hand, SUCR is a synthetic, non-nutritive, zero-calorie artificial sweetener (AS), widely used in foods and beverages as a sugar substitute. The process of production entails the replacement of three hydroxyl groups in sucrose with chlorine atoms, a chemical modification that results in the development of an intense sweetness and remarkable stability at elevated temperatures and low pH levels. This renders it suitable for both culinary purposes and extended storage periods (AlDeeb et al., 2013).

Similarly to IBU, food additives production and use have recently risen. In fact, a study conducted in Australia reported that the use of SUCR increased by 45% and 29% in just 6 years (Li et al., 2021). This

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Received: July 09, 2026;
Accepted after revised: November 05, 2026;
Available online: November 20, 2026

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widely used AS is resistant to conventional wastewater treatment plants (WWTPs) and passes through municipal sewage systems unaltered (Alves et al., 2021).

Due to their ability to cross the WWTPs, these two ECs are frequently monitored in rivers as indicators of the pollution from urban wastewater (Stewart et al., 2025).

The study of (Marsik et al., 2017) investigated the presence of NSAIDs in the Czech Republic's area of the Elbe basin and revealed that IBU was the most present in all the rivers monitored, reaching a concentration of 3.210 μg/L. Similarly, a study conducted in Colombia (Cerón-Vivas and Mesa, 2024), suggests that IBU and Cephalexin are the most prevalent pharmaceutical pollutants in the Oro River Sub-basin with concentration of 3.156 μg/L and from 0.5 μg/L to 12.9 μg/L respectively. Even in the receiving water in Beijing (China), IBU was the most found drug with a mean concentration of 114.9 ng/L (Ma et al., 2019). Lencioni et al. (2018) detected a drug contamination even in high-altitude ecosystems, measuring concentrations of IBU from 327 to 619 ng/L in an Alpine stream, at 1799 m a.s.l. (Vermigliana, Tonale Pass, Trentino, NE Italy).

Moving to the SUCR, this AS was found in surface waters of the River Thames (UK) with concentrations ranging from < 0.01 to 5.9 μg/L (White et al., 2019). A recent study revealed the presence of higher levels in Kamphuan Stream, located in the southern region of Thailand where the level of SUCR reached the concentration of 9.070 μg/L (Lee et al., 2025). In the study conducted by (Marizzi del Olmo et al., 2025), the most prevalent contaminants (> 1μg/L) in the River Oniar (Spain) were caffeine, IBU and SUCR. The latter was detected with a concentration of 600 μg/L in the Vermigliana Stream mentioned above by (Lencioni et al., 2018), downstream of the Tonale Pass WWTP.

Prolonged exposure to mixture of ECs may damage macroinvertebrate communities, with cascade effects on the river food webs (Lencioni et al., 2018).

Lencioni et al. (2020) analysed the effects of a plethora of ECs, including IBU and SUCR, on eight species of insect larvae collected in an Alpine stream: *Baetis alpinus* Pictet, 1843, *Baetis rhodani* Pictet, 1843, *Diamesa cinerella* Meigen 1835, *Dictyogenus alpinus* Pictet, 1841, *Perlodes intricatus* Pictet, 1841, *Pseudodiamesa branickii* Nowicki, 1873, *Rhyacophila occidentalis* McLachlan, 1879, and *Rhyacophila tristis* Pictet, 1834. The study revealed that IBU presented the most acute toxicity, while SUCR was the least harmful when survival was the only endpoint considered. However, this AS was significantly genotoxic causing DNA damages in all tested species. These authors estimated a Lethal Concentration 10 (LC10, 24 h) ranging from 14 mg/L to a No Observed Effect Concentration (NOEC, 96 h) of 100 mg/L for IBU and LC10 96 h or NOEC 96 h of 43 g/L for SUCR. In a laboratory population of Chironomus riparius, IBU exhibited an LC10 (48 h) of 0.024 μg/L, along with oxidative damage and alterations in genes related to endocrine and immune functions (Muñiz-González, 2021).

Regarding the toxicity of SUCR and other sweeteners, most of studies have focused principally on fish and crustaceans, as (Goveas, 2025) review indicates. In zebrafish (*Danio rerio* Hamilton, 1822) embryos, environmentally relevant concentrations of SUCR caused several deformities and the activation of the antioxidant enzymes (Nguyen et al., 2024). Similar oxidative stress effects were observed in the Cladocera *Daphnia magna* Straus, 1820, together with alterations on swimming and feeding behaviours and acetylcholinesterase (AChE) activity (Eriksson Wiklund et al., 2014).

Recent reviews highlighted the limited monitoring and risk assessment of ECs in aquatic environments and their potential adverse ecological impacts that are not yet fully understood (Rocha et al., 2024). Specifically, there are few studies addressing the implications of IBU and SUCR on macroinvertebrates, despite the key role of these animals for river ecological dynamics (Lencioni et al., 2020; Rocha et al., 2024; Villa et al., 2018).

Within this context, we evaluated the potential adverse effects of two sub-lethal concentrations of SUCR (5 g/L; 500 ng/L) and one of IBU (25 ng/L) on a wild population of Chironomus riparius (Diptera: Chironomidae). Utilizing indigenous populations significantly enhances the ecological realism of the toxicological assessment. Specifically, we have set up an experiment to assess alterations on: (i) the morphological traits (MTs) of larvae, focusing on the size of the cephalic capsule, including the size of the antennae, and the body length of the different instars; (ii) the presence and the severity of mentum; and the (iii) life cycle span considering the different phases (e.g. the hatching time, time to emerge, etc.).

The selection of *C. riparius* as a test organism ensures that the findings of this research are highly comparable and reproducible within the global ecotoxicological framework. Given its status as an OECD-validated model species, the toxicological responses documented here can be directly integrated into environmental risk assessment (ERA) protocols and water quality monitoring strategies (Foucault et al., 2019). Additionally, by focusing on *C. riparius* Meigen, 1804, this study addresses a critical node in aquatic ecosystems. As primary consumers and foundational links in freshwater food webs, any impairment in these larvae poses a risk to higher trophic levels.

Finally, a key strength of this study lies in the assessment of larval mouthpart deformities (teratogenesis). These morphological aberrations serve as sensitive early-warning signals (Langer-Jaesrich et al., 2010; Planelló et al., 2015).

2. Materials and methods

2.1. Insect culture

Owing to the comprehensive characterisation of the ecological and physiological requirements of *C. riparius* Meigen, 1804, highly standardized laboratory culture protocols have been established (Foucault et al., 2019). A culture of *C. riparius* Meigen, 1804 was developed from larvae (a mix of all instars) collected in the wild, maintained at room temperature (20 ± 1 °C) and natural photoperiod.

Larvae (all instars) were collected from a small lowland stream, Rio Gola, in NE-Italy (Trentino, 187 m a.s.l., 46°02′19.95″ N, 11°07′16.66″ E) in March 2021. The Rio Gola stream flows through the village of Ravina, cutting the settlement in two as it runs from the wild Gola Valley towards the Adige River (the second longest Italian river). Since Ravina's domestic wastewater is entirely diverted to the centralized Trento Sud Treatment Plant, the Rio Gola is theoretically isolated from direct pharmaceutical and food-additive contamination.

Point measurements of water temperature (19.7 °C), percent oxygen saturation (101%), dissolved oxygen concentration (9.5 mg/L), pH (8.0) and conductivity (2.55 mS/cm) were recorded in the field with a multiparametric probe Hydrolab Quanta in the time period from 11.00 to 13.00 h. Granulometry was fine (< 5 cm) and bottom was covered by algal mats (green algae and cyanobacteria).

Larvae were collected with a 30 x 30 cm pond net (100 µm mesh size) in the sediment - larvae are burrowers, dwell in tubes and feed on detritus - and transported to the laboratory in plastic bags within 1 h after sampling. Rearing was conducted in an aerated 38 L aquarium with a 5 cm layer of natural substrate covering the bottom.

The substrate consisted of stream mud dried in an oven at 60 °C for 48 h, sieved on 250 µm mesh and sterilized by UV radiation with a UV/Whitelight transilluminator® TFP-C/WL (254 nm) for 30 min, according to Lencioni et al. (2016) and Bernabò et al. (2017).

The aquarium was filled with reconstituted water (RW) composed by 192 mg/L sodium bicarbonate (NaHCO3), 120 mg/L calcium sulphate (CaSO4), 120 mg/L magnesium sulphate (MgSO4) and 8 mg/L potassium chloride (KCl). Water temperature was maintained at 20 ± 1 °C by a refrigerator TECO TR15, with natural photoperiod. A digital logger (Onset Hobo TidbiT®) was placed in the water to record the temperature constantly. The aquarium was covered with a mosquito net with a mesh size of 250 µm to avoid the adults from escaping.

Larvae were fed with fish food flakes (Tetra-Min®) every 2 days, while the adults with a cotton ball soaked with a concentrated sugar solution. The experiment was performed within five weeks after sampling, when sufficient egg masses were available to begin.

2.2. Test solutions

Two experimental groups were designed to assess the effects of IBU (Fig. 1): a control group (Ic) and a test group exposed to a concentration of 25 ng/L (I1). A higher dose (50 ng/L) was tested but excluded from the experiment because no larvae hatched from the egg ropes. The concentration of 25 ng/L was chosen based on the IBU-LC_{10} 48h proposed by Muñiz-González (2021), well below the concentration detected in mountain streams (e.g. up to 619 ng/L in the Vermigliana stream downstream of a sewage plant, Trentino, Italy; Lencioni et al., 2020) and in northern Italian rivers (e.g., 121.2 ± 20 ng/L in the Lambro river; Zuccato et al., 2006). Additionally, I1 was lower than the Predicted Environmental Concentration (PEC = 4.96 µg/L) and the Predicted No Effect Concentration (PNEC = 9.06 µg /L) proposed to identify the risk of ibuprofen to aquatic organisms (Tollefsen et al., 2012).

SUCR was tested across three experimental groups (Fig. 1): a control group (Sc,) and two test groups, 500 ng/L (S1) and 5 g/L (S2). While S1 corresponds to the minimum concentration assessed by Wiklund et al. (2012) to assess the effects of this artifi-

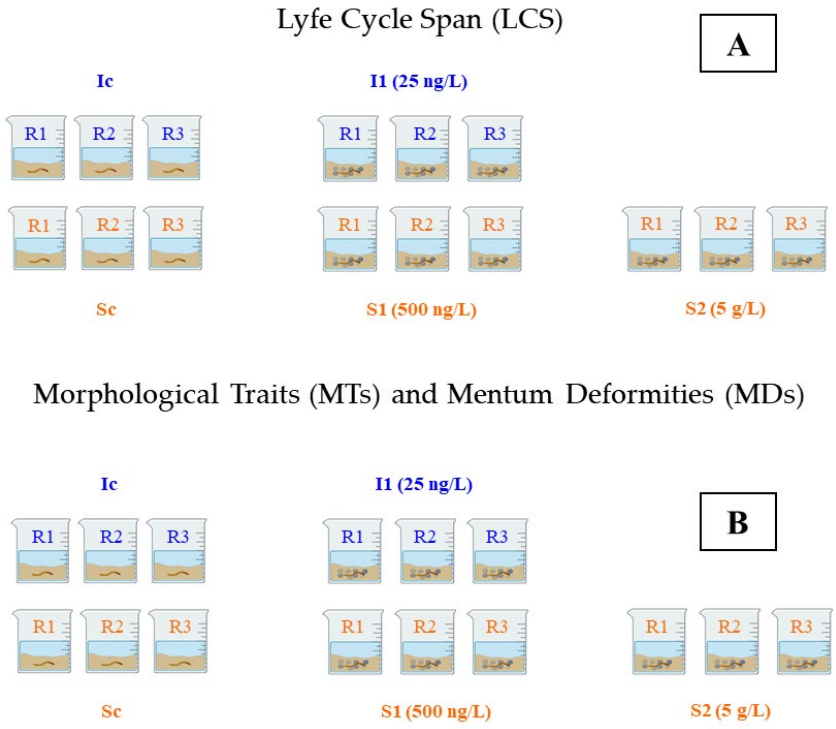


Fig. 1. Experimental design of the two independent experiments: (A) Life Cycle Span test, assessing the effects of ibuprofen and sucralose exposure on larval development and life cycle and (B) morphological assessment of *Chironomus riparius* larvae exposed to the tested substances.

cial sugar on freshwater crustaceans (*Gammarus* spp.), S2 represents a 1,000-fold increase over their highest experimental dose for acute toxicity, exploring the upper limits of potential impact on freshwater invertebrates. Additionally, both concentrations is below the environmental value recorded in mountain streams (up to 600 μg/L in the Vermigliana stream downstream of a sewage plant, Trentino, Italy; Lencioni et al., 2020), and several European rivers (e.g. 0.004–10 μg/L in receiving waters from sewage plants and 0.001-1.9 μg/L in surface waters; Jones et al., 2002). Additionally, S1 is lower than the Predicted Environmental Concentration (PEC = 10 μg/L) and the Predicted No Effect Concentration (PNEC = 0.93 mg/L) proposed to identify the risk of sucralose to aquatic organisms by Tollefsen et al. (2012).

For the IBU exposure, the I1 was obtained with serial dilutions. was prepared through serial dilutions. Due to the low water solubility of ibuprofen, 75% ethanol was used as a solvent. The initial stock solution (I-Stock1) was prepared by dissolving 50 mg of IBU (Sigma-Aldrich I4883) in 1 mL of 75% ethanol. Subsequently, 100 μL of I-Stock1 were diluted with 10 mL of reconstituted water (RW) to obtain a second stock solution (I-Stock2) with a concentration of 500 μg/mL. Finally, to reach the third stock (I-Stock3) at 5 μg/L, 10 μL of I-Stock2 was added to 10 mL of RW. Finally, 50 μL of I-Stock3 was diluted in 1 L of RW, yielding the test concentration (I1) of 25 ng/L. The control group (Ic) was prepared by adding 100 μL of 75% ethanol in 1 L of RW to ensure consistency in solvent exposure.

In contrast, SUCR is highly water-soluble; therefore, its control group (Sc) consisted exclusively of RW. The lower concentration (S1 = 500 ng/L) was prepared via serial dilutions: first, an initial stock (S-Stock1) was created by diluting 100 mL of S1 in RW. Then, 1 mL of S-Stock1 was diluted in 1 L of RW, yielding S-Stock2. Finally, 10 mL of S-Stock2 was mixed with 1 L of RW, achieving the target concentration of 500 ng/L. For the higher concentration (S2 = 5 g/L) 5 g of SUCR (Sigma-Aldrich 69293) were dissolved in 1 L of RW. The stability of IBU and of SUCR in such solution is plainly demonstrated in literature (Quinlan and Jenner, 1990; Grice and Goldsmith, 2000; Walker et al., 2011). Consequently, analytical verification of the concentrations during or at the end of the exposure period was deemed unnecessary.

Finally, the volume lost to evaporation was carefully replenished by adding droplets of ultra-pure deionised water in order to maintain the exposure concentrations constant throughout the duration of the entire experiment rather than only at the initial stage.

2.3. Life cycle span design

Fig. 1A represents the experimental design of the Life Cycle Span (LCS) test. For each group and for both the tested substances, 3 replicates (R1, R2, R3) were applied. The beakers filled with RW (200 mL) for the controls (Ic, Sc) and test solutions (200 mL) for the test groups (I1, S1, S2) were placed in a thermostatic chamber (Memmert HPP IPP plus) with temperature of 20 ± 1 °C and 16 h photoperiod with light intensity of 1000 lux (OECD, 2010; OECD, 2011). To maintain the correct level of dissolved oxygen, an aeration system was implemented into the test vessels.

After setting the experiments conditions, 18 egg masses necessary for the test were collected from the culture tank and placed one in each replicate. With the NIS-BR software (camera DS-Ri2 on the microscope Nikon Eclipse), the length of each rope and the size of random eggs per rope were measured. Finally, the number of eggs per rope, number of loops, and eggs per loop were counted and recorded. These data were crucial to obtain similar starting conditions among the tested concentration groups and the respective control.

Even the date of egg rope production and consequentially insertion into the replicate was recorded as starting point to count the number of days required for hatching and the time from hatching to the first adult.

2.4. Morphological traits and mentum deformities

The same experimental design and conditions for LCS were applied for the analysis of morphological traits (MTs), as reported in Fig. 1B. Measurements were taken every three days on three larvae, giving priority to deceased individuals. Both were conserved in 75% ethanol.

Measures were taken before press the larvae in the microscope slide in Canada Balsam (Wirth and Marston, 1968) in order to avoid deformation caused by the compression of the slide cover. The experiment was concluded once all larvae had either died or successfully pupated.

The MTs measures and larvae photos were taken with the NIS-BR software (camera DS-Ri2 on the microscope Nikon Eclipse). In detail, the first MT considered was the length of the body of the larvae. The other parameters measured referred to the structures present on the head and the cephalic capsule itself (Table 1, Fig. 2).

A total of 430 cephalic capsules were used for the MT measurements. The head parameters evaluated were: the length without labrum (L1, Table 1, Fig. 2), ventral length (VL, Table 1; Fig. 2) and width (W, Table 1, Fig. 2). Moving to the mouth parts, mentum (LMe, Table 1, Fig. 2), mandibles length (LM, Table 1, Fig. 2) and ventromental plates were considered (LVP, Table 1, Fig. 2). Furthermore, the size of the antennae was measured totally (LA, Table 1, Fig. 2).

Table 1. Summary of the abbreviations of the MTs measured for each larva.

Abbreviation	Morphological trait
L1	Cephalic capsule length without labrum
VL	Ventral length
W	Cephalic capsule width
LM	Mandible length
LMe	Mentum length
LVP	Ventromental plate length
LA	Antenna length

These parameters were evaluated as endpoints to assess the effects of the two test substances on the growth of the animal structures but were also used to obtain the larval instar based on (Richardi et al., 2013). The identification and separation of the instars was necessary to evaluate how the effects on the MTs changed in different growth phases.

The evaluation of the tested ECs effects on the same larvae was assessed also as the presence of mentum deformities (MDs) with the same microscope, camera, and software. The MDs (Fig. 1B) in the examined specimens were categorized into three distinct classes based on their severity and morphological features, according to (Goretti et al., 2019). Class 1 includes specimens that are morphologically normal or exhibit only mechanical damage, such as broken or worn teeth. Class 2 represents mild deformities, characterized by one or two rounded or filed teeth, the presence of a missing or additional tooth, a single bifid or serrated tooth, two fused teeth, or slight asymmetry. Class 3 refers to severe deformities, including very rounded or filed teeth, two or more missing or additional teeth, multiple bifid teeth, serrated dentition, three or more fused teeth, pronounced asymmetry, the presence of a Köhn gap, or a combination of these morphological anomalies.

2.5. Statistical analyses

Normality of data distribution was assessed for each variable set, which included developmental traits, egg traits, morphometric measures of the head capsule, larval body length and deformity scores of the teeth and mentum (scored on a scale from 1 to 3 according to Goretti et al., 2019), using the Shapiro–Wilk test from the stats package (R Core Team, 2024). Homogeneity of variances among groups was subsequently evaluated using Levene's test from the car package (Fox and Weisberg, 2018), ensuring that variance assumptions were consistent across the datasets.

As the normality assumptions were violated for all the variables, non-parametric methods were applied using the Kruskal–Wallis rank sum test from the stats package, with significant effects further examined through post hoc pairwise comparisons using Dunn's test from the FSA package (Ogle et al., 2023) and Holm-Bonferroni correction applied to account for multiple testing.

In addition, a dedicated script was specifically developed to determine the larval instars of *Chironomus riparius* (Bonciu, 2025). This script employs morphometric measurements based on the methodology of (Richardi et al., 2013) to assign instars in a standardized and objective manner. By applying these established criteria, the script ensures reproducibility and consistency across the dataset, providing a robust framework for linking larval developmental stages with the measured biological traits and allowing accurate comparisons among individuals and experimental groups.

Data manipulation, cleaning, and tidying were performed using the dplyr and tidyr packages (Wickham et al., 2023; Wickham et al., 2024), which facilitated structured and reproducible data handling.

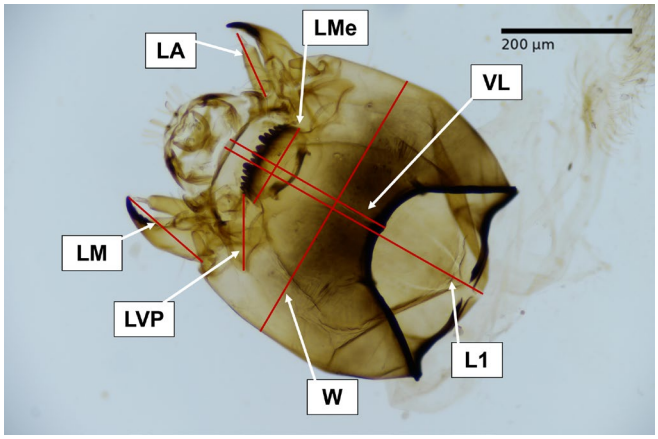


Fig. 2. Cephalic capsule of IV instar larvae of *Chironomus riparius*. The position of the MTs (L1; W; LVP; LM; LA; LMe; VL) are indicated with white arrows. The red lines highlight the dimension measured for each MPs.

All statistical analyses were performed in R (version 4.1.2) using the RStudio graphical interface (Posit Team, 2024), providing an integrated and reproducible platform for coding, visualization, and data analysis.

In the barplots, the y-axis range has been selected on the basis of the range of values in order to highlight any potential discrepancies.

3. Results

3.1. Egg ropes differences

To ensure the experimental validity of the treatment groups (SUCR and IBU), it was imperative to establish that baseline biological conditions were consistent with the control group. We assessed this initial homogeneity by analysing key reproductive parameters, including the number of egg masses (loops), eggs per mass, and the total length of the egg ropes. As illustrated in Fig. S-1, the lack of statistically significant differences across all evaluated metrics confirms that the egg masses used at the onset of the experiment were uniform. In particular, the number of loops per each rope of the SUCR control group (Sc; Fig. S-1A; Table S-1) presented a mean number (mean ± SE) of 27 ± 1.5 similar to the IBU control (IC; Fig. S-1 A: Table S-1) which presented values of 24.7 ± 1.9. S1, S2 and I1 (Fig. S-1A; Table S-1) presented the mean number (mean ± SE) of 27.7 ± 2.6; 25.7 ± 2; 24.7 ± 1.9, respectively. The number of eggs per each loop was similar in all the groups with a mean (mean ± SE) value around 13 in all the groups (Fig. S-1B; Table S-1). Consequently, even the number of eggs in each rope maintained no differences in the tested groups compared with the respect control. In detail, the rope in Ic had 330.7 ± 47.5 (mean ± SE) eggs while in I1 331.3 ± 46.3 eggs were counted (Fig. S-1 B; Table S-1). Same situation happened with the SUCR, where the values ranged from 342.3 ± 24.1 in Sc to 358 ± 29.3 in S1 (Fig. S-1 B; Table S-1). Additionally, from the morphological point of view, the conditions of the starting eggs were uniform with no significant differences. In fact, the dimensions of the ropes in term of length in the two controls ranged from 5.2 ± 0.7 mm in the Sc to 6.0 ± 0.5 mm in Ic group (mean ± SE; Fig. S-1D;

Table S-1). The longest rope measured 7.5 ± 2.2 mm in S2, followed by S1 (7.4 ± 2.1) and I1(5.6 ± 1 mm; mean ± SE; Fig. S-1D; Table S-1).

3.2. Life cycle span

Fig. S-2 illustrates the time in which the tested organisms complete their life cycle. In both the end-points evaluated, no significant difference occurred between the controls (Sc and Ic) and the organisms tested with the two ECs (Fig. S-2; Table S-2). The panel A (Fig. S-2), titled "Time to hatch", represents the period between the collection of the egg and the first larva hatched expressed as mean ± SE number of days. The I instar larvae of the Sc group hatched (Fig S-2A; Tab S-2) within 3 ± 0.58 days, similarly to S1 (3.33 ± 0.33) and S2 (3.67 ± 0.33). In the IBU test, larvae of Ic group needed 3.33 ± 0.33 days to hatch while I1 3.67 ± 0.88 days suggesting no effects on development of the eggs caused by IBU and SUCR. As demonstrated in Fig. S-2B, titled "Time from larva to adult", the two substances did not affect the time required for each larva to emerge. Indeed, the mean ± SE of days to emerge in Sc group was 28 ± 1.15 (Fig. S-2B; Table S-2),almost identical compare to the lowest concentration of SUCR (S1 = 28 ± 0.58; Fig. S-2B; Table S-2) and five days from the highest (S2 = 33 ± 1.73; Fig. S-2B; Table S-2). The number of days needed to emerge were almost the same even in IBU experiment (Ic = 34.33 ± 1.45 and I1 = 34.67 ± 0.33; Fig. S-2B; Table S-2).

3.3. Morphological traits

Results for the I and II instars were grouped as 'I-II' due to the absence of significant differences between them. In contrast, the III and IV instars were analysed separately, as they showed the expected significant divergence in size and developmental complexity typically seen in late-stage *C. riparius* larvae (Olafsson, 1992; Richardi et al., 2013).

3.3.1. Larval length

Relevant differences between groups ($p < 0.05$) appeared only in the IV instar (Fig. 3C, Table S-3). Although a significant difference was also detected in the I-II instar (Fig. 3A; Table S-3), this result was not biologically relevant because occurred between S1 and I1 groups and no effects were detected compared with their respective control groups. In the III (Fig. 3B; Tab S-3), on the other hand, the measures of the larval were remarkably similar across the groups. In Fig. 3C and Table S-3 relative to the IV instar, it is possible to notice that the mean size of the organisms tested with the highest dose of SUCR was significantly lower respect the control group. Indeed, the Sc (Table S-3) larvae presented a mean size (mean ± SE) equal to 11.32 ± 0.56 mm compared to 9.08 ± 0.62 mm of S2 (Table S-3). This group, however, was not significantly different ($p > 0.05$) compared to S1 group (Table S-2) which presented a mean (mean ± SE; Table S-3) equal to 11.27 ± 1.48 mm. The larvae of I1 were significantly shorter than the larvae in SC (Fig. 3; Table S-3) but had comparable size of the Ic. For this reason, this statistic was considered not strictly relevant.

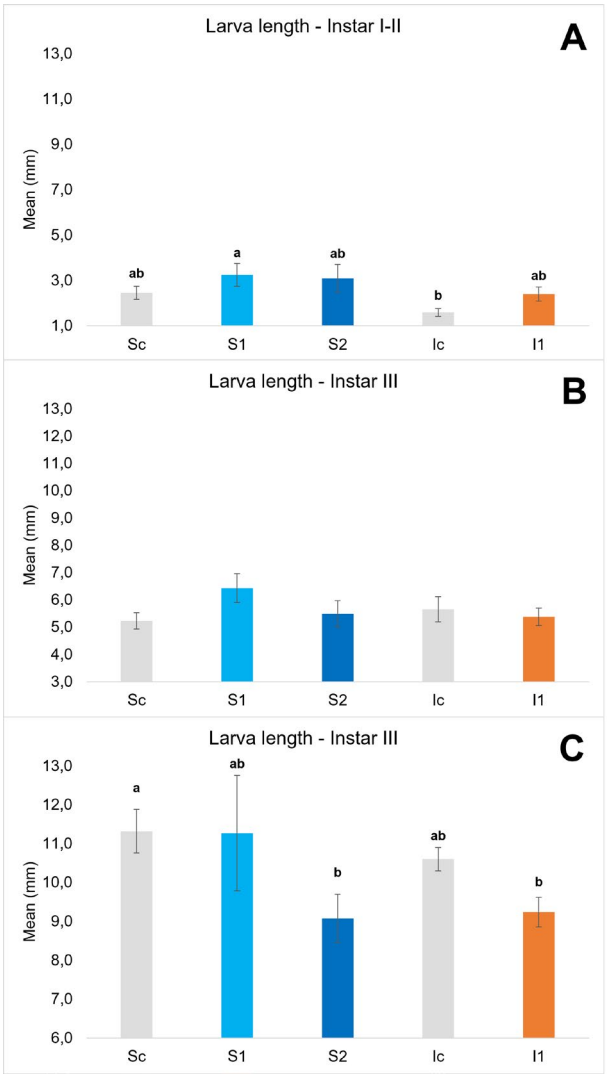


Fig. 3. MTs endpoint represented by the length (mm; median ± SE) of the larvae bodies divided in I-II (A), III (B) and IV (C) instar at the end of the test. Different letters above the boxes represents significative differences ($p < 0.05$) among groups according to Kruskal-Wallis and Holm-Bonferroni post hoc test.

3.3.2. Cephalic capsule

Morphometric parameters of the cephalic capsule (L1, VL, W, and LA) showed treatment-dependent differences across larval instars (Table 1). In the I-II instar, L1 differed significantly among treatments (Fig. 4A, 4B, 4C; Table S-4). In this instar, larvae exposed to the lowest SUCR concentration (S1) showed a significantly greater L1 than the solvent control (Sc) (212.22 ± 12.92 μm compared to 158.94 ± 10.04 μm). No significant differences were observed for S2 or I1 relative to the controls ($p > 0.05$).

In the III instar, L1 in larvae exposed to S1 (521.36 ± 24.74 μm) was significantly greater than in all other groups, including S2, Sc, Ic, and I1. In the IV instar, S1 maintained the highest L1 (711.95 ± 32.99 μm), which was significantly greater than I1 (591.33 ± 8.71 μm) but not different from Sc (636.16 ± 17.44 μm).

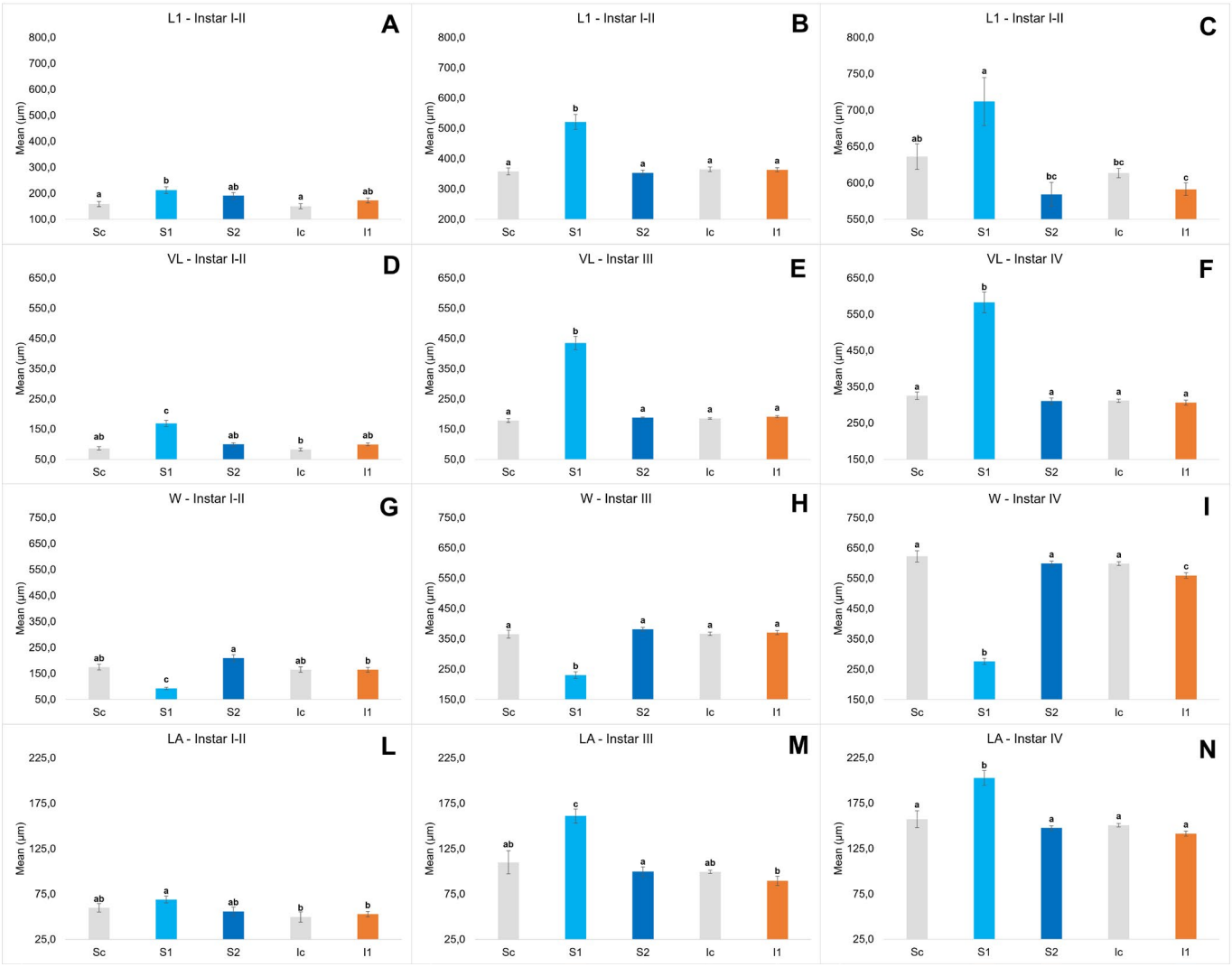


Fig. 4. MTs relate to the cephalic capsule dimensions (µm; median ± SE) expressed as length of the capsule without labrum (A, B, C), ventral length (D, E, F) and width (G, H ,I). Length of antennae (L, M, N) is also included in the cephalic capsule measures group. These graph are divided considering the instar of the larvae at the end of the test as follow: I-II (A, D, G, L), III (B, E, H, M) and IV (C, F, I, N) instar. Different letters above the boxes represents significative differences ($p < 0.05$) among groups according to Kruskal-Wallis and Holm-Bonferroni post hoc test.

Consistent with L1, VL was significantly increased in the S1 group across all instars (Fig. 4D, 4E, 4F; Table S-4). In S1 larvae, VL measured 168.92 ± 10.55 µm in the I–II instar, 435.18 ± 21.84 µm in the III instar, and 582.89 ± 28.24 µm in the IV instar. By contrast, VL in Sc increased from 86.17 ± 5.42 µm (I–II) to 325.52 ± 10.29 µm (IV). Larvae in S2 and I1 did not differ significantly from their respective controls (Sc and Ic) at any developmental stage.

W measurements revealed an inverse pattern. At all larval stages, S1 larvae exhibited the narrowest cephalic capsule compared with controls, higher SUCR concentration (S2), and IBU-treated groups (Fig. 4G, 4H, 4I; Table S-4). In the IV instar, I1 larvae also showed a significantly reduced W compared with the IBU control (Ic). Specifically, W ranged from 92.37 ± 4.78 µm in S1 to 209.42 ± 12.56 µm in S2 during the I–II instar. This trend persisted in older instars, with S1 consistently showing the lowest mean W values.

Overall, LA did not differ significantly among groups when considering each instar (Fig. 4L, 4M, 4N; Table S-5). However, specific pairwise differences were observed. In the I–II instar, antennae in S1 larvae (68.84 ± 3.72 µm) were significantly longer than in I1 (52.86 ± 2.95 µm) and Ic (49.86 ± 5.76 µm).

In the III instar, these differences became more pronounced: S1 antennae reached 161.27 ± 7.61 µm and were significantly longer than all other groups, including Sc and S2. Conversely, I1 larvae displayed significantly shorter antennae than S2. In the IV instar, antenna lengths converged across treatments, although S1 larvae still showed significantly higher mean values (202.89 ± 8.22 µm) compared with all other groups.

3.3.3. Mouthpart structure

Morphometric analyses of mouthparts included mentum length (LMe), mandible length (LM), and ventromental plate length (LVP; Table 1; Fig. 5).

Across all the instars, larvae exposed to the lowest SUCR concentration (S1) showed a significantly longer mentum than all other groups (Fig. 5A, 5B, 5C; Table S-6). In S1, LMe increased from 183.16 ± 12.95 µm in instars I–II to 449.50 ± 20.50 µm in instar III and reached 576.86 ± 25.69 µm in instar IV.

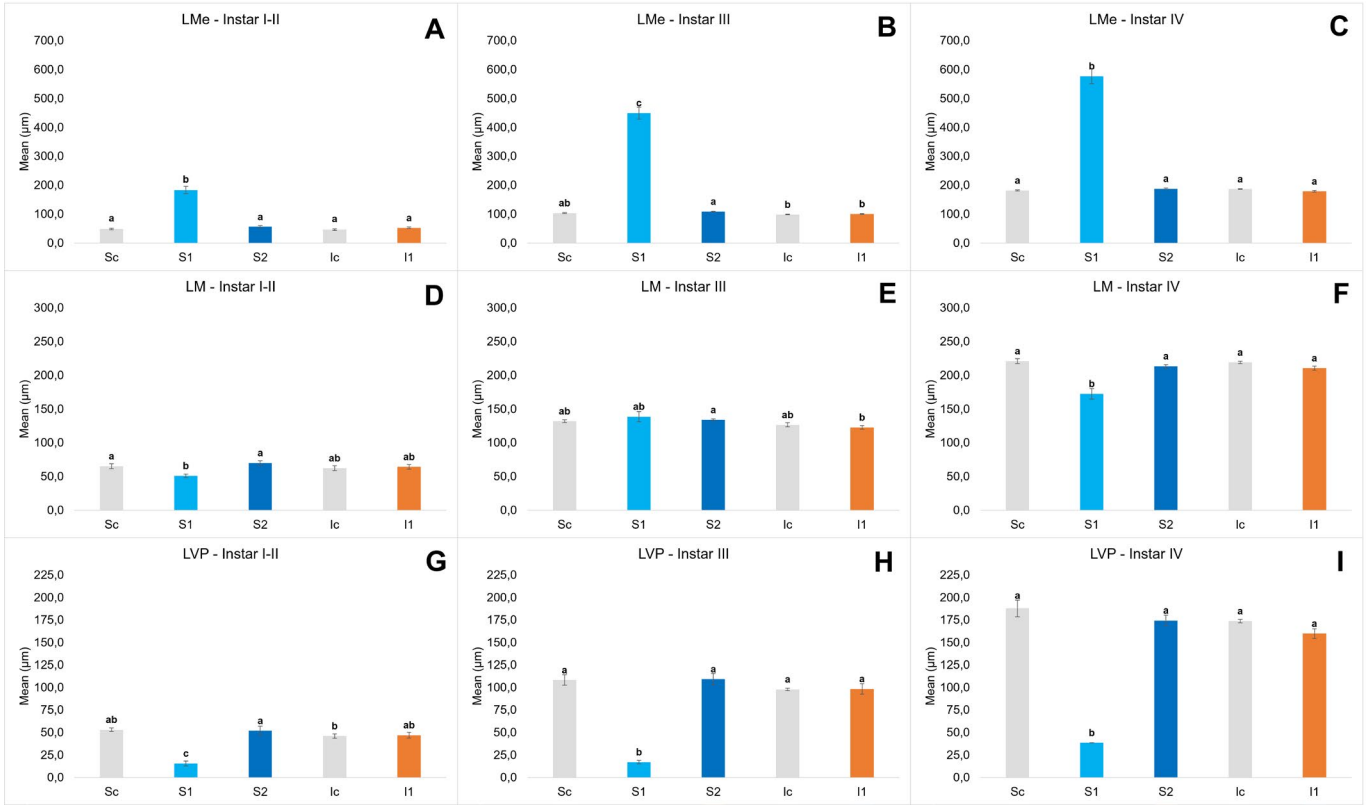


Fig. 5. MTs relate to the mouth part (µm; median ± SE) expressed as width of the mentum (A, B, C), length of the mandibles (D, E, F) and length of the ventromental plate (G, H, I). These graph are divided considering the instar of the larvae at the end of the test as follow: I-II (A, D, G), III (B, E, H) and IV (C, F, I) instar. Different letters above the boxes represents significative differences ($p < 0.05$) among groups according to Kruskal-Wallis and Holm-Bonferroni post hoc test.

In contrast, mentum growth in Sc, S2, Ic, and I1 was gradual and comparable among treatments, with values ranging from 46–57 µm in instars I–II to 179–188 µm in instar IV (Table S-6). In the III instar, I1 larvae showed a significantly narrower mentum than S1 and S2 but did not differ from the IBU control (Ic).

LM was significantly reduced in S1 larvae in the I–II and IV instars (Fig. 5D, 5F; Table S-6). In the I–II instar, LM value in S1 (51.03 ± 2.63 µm) was lower than in all other groups.

In the III instar, significant differences were detected only between I1 and S2, with I1 showing the lowest LM (122.81 ± 2.53 µm; Fig. 5E). In the IV instar, LM ranged from 172.52 ± 7.79 µm in S1 to 220.95 ± 3.48 µm in Sc, with S1 remaining significantly lower than the other treatments.

LVP was consistently reduced in S1 larvae at all developmental stages (Fig. 5G, 5H, 5I; Table S-6). In instars I–II and III, S1 exhibited significantly lower values than all other groups.

In instar IV, LVP in S1 remained significantly lower than Sc and S2 and did not differ statistically from I1, which was significantly reduced compared with Sc but not with its control (Ic). Overall, S1 larvae displayed the smallest ventromental plates throughout development.

3.4. Mentum deformities

Examples of the main deformities found divided per class of severity are reported in Fig. 6, which highlights how the severity of deformities increased moving from Class 1 (Fig. 6a, Fig. 6b and Fig. 6c) to Class 3 (Fig. 6g, Fig. 6h and Fig. 6i). Fig. 7 represents the distributions of the mentum deformities in the five tested groups considering all the larval instars and divided by the three classes of severity. The data are normalised posing the mean value of the Sc and Ic as 1 in order to obtain a single Ctrl for a better comparison between the two contaminants (Fig. 7). Observing these graphs (Fig. 7), is immediately notable that the highest number of deformed mentum was in the larvae exposed to IBU in all the larval stages. Moreover, the mentum in the I1 group were principally affected by the Class 3 type of deformities (Fig. 6g, Fig. 6h and Fig. 6i), therefore the most severe (Fig. 7). In detail, the Class 3 in I1 were 7.67 (23 % of the exposed larvae) times higher respect the Ctrl (Fig. 7, Table S-7). Considering the Class 1, Ctrl presented the highest values (1, Table S-7) respect S1 (0.97, Table S-7), S2 (0.84, Table S-7) and I1 (0.81, Tab S-7). Indeed, in the Class 1 were included also the larvae with a normal shape mentum, i.e. without any form of deformities as Goretti et al., 2019 suggested (Fig. 6 a). In fact, in larvae exposed to SUCR and IBU the number of deformed mentum increased progressively with the increasing of the deformation classes severity. Observing the orange bar of Fig. 7 and Table S-7, S1 had 1.36 more larvae than Ctrl affected by Class 2 deformities. Similarly, S2 and I1 presented 1.21 and 1.38 times more Class 2 deformities, respectively, compared to Ctrl. Following this trend, in the Fig. 7 is possible to notice that the Class 3 (grey bars) constantly increased from S1 (5 times more than Ctrl, Table S-7) to S2 (5.50 times higher than Ctrl, Table S-7) and I1 (7.67

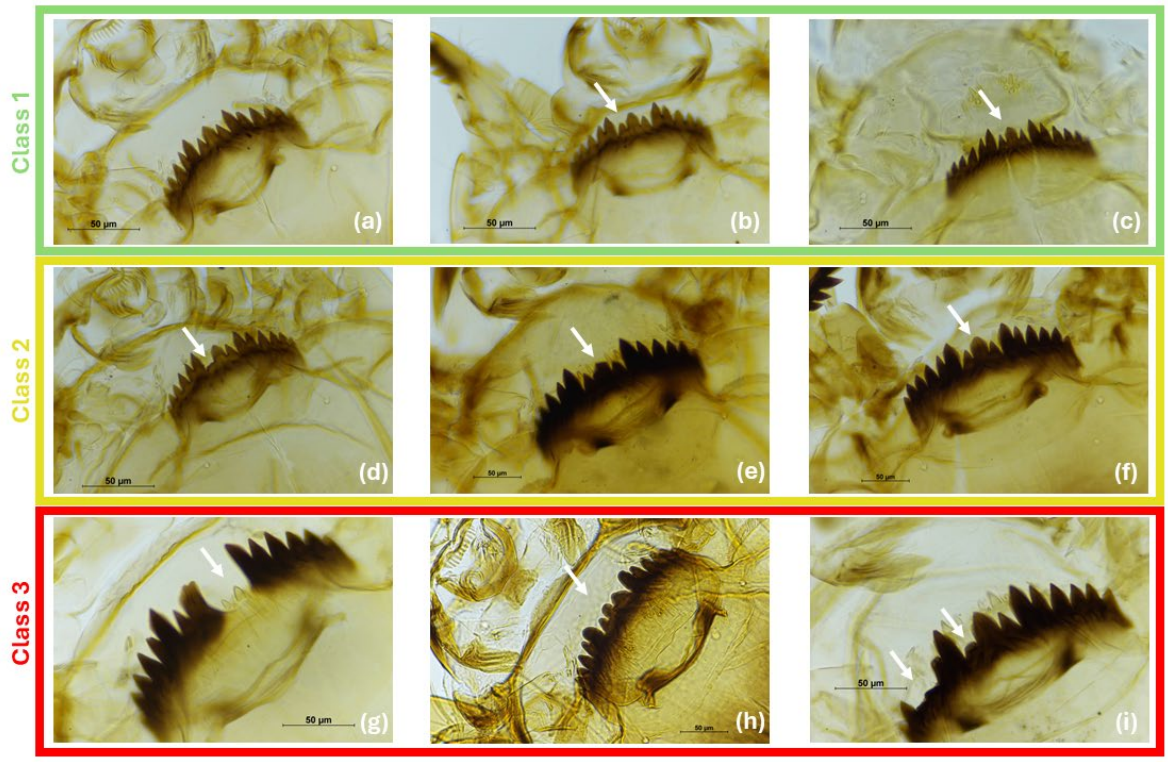


Fig. 6. *Chironomus riparius* images showing Class 1 deformities: (a) a normal-shaped mentum, (b-c) a normal-shaped mentum with mechanical damage and the various mentum deformity types of Class 2 and Class 3: (d) mentum with a missing tooth, (e) mentum with a chipped tooth, (f) mentum with a rounded tooth, (g) mentum with a Köhn gap (arrow), (h) mentum with rounded teeth (arrow), and (i) mentum with a combination of different deformities (arrows).

times higher than Ctrl, Table S-7). The deformities were also evaluated based on the larval instars (Fig. 8, Tab S-8). Also in this case, Ic and Sc were normalised with the median value equal to 1. Due to the absence of significant differences in I-II instar based on Kruskal-Wallis (Table S-8), the relative graph was not reported in Fig. 8. In contrast, a significant increase of Class 3 (Fig. 8) deformities was detected in instar III, with I1 (mean ± SE, 1.89 ± 0.14 deformities Class) exhibiting more severe deformities compared to the IBU control group (mean ± SE; Ic = 1.19 ± 0.10 deformities Class, Fig. 8A, Table S-8). Similarly, in instar IV, deformation was significantly unbalanced towards Class 3 in I1, with a mean ± SE Class of 2.07 ± 0.13, compared to Ic (1.50 ± 0.09 mean ± SE Class),Sc (1.32 ± 0.10 mean ± SE Class), S1 (1.46 ± 0.16 mean ± SE Class) and S2 (1.65 ± 0.13 mean ± SE Class, Fig. 8B, Table S-8).

4. Discussion

4.1. Sucralose effects

There is currently a lack of research concerning the sub-lethal impacts of SUCR on *Chironomus riparius*. Acute toxicity and genotoxicity of SUCR were tested in other chironomids (larvae of *Diamesa cinerella* gr.) by Lencioni et al., 2018; 2020) and, within dipterans, in *Musca domestica* Linnaeus, 1758 and *Drosophila melanogaster* Meigen, 1830 that are terrestrial species (Lee et al., 2021). Eriksson Wiklund et al. (2012) studied its acute toxicity and accumulation in aquatic species but not dipterans, i.e. *Daphnia magna, Nitocra spinipes*, and two gammarid species: *Gammarus oceanicus* and *Gammarus zaddachi*. Staying within the realm of freshwaters, Saucedo-Vence et al. (2017) assessed the SUCR effects in common carp (*Cyprinus carpio*).

Our results demonstrated that the timing of hatching and emergence in *C. riparius* was not significantly affected by SUCR at either tested concentration (500 ng/L and 5 g/L). In this species these developmental milestones are regulated by molecular mechanisms primarily involving the ecdysone hormone pathway and key genes, such as the ecdysone receptor and the ultraspiracle (Planelló et al., 2015). Although ecdysone levels are modulated through complex pathways often

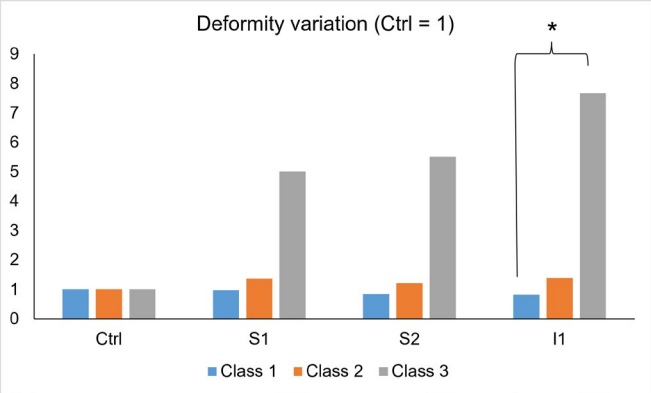


Fig. 7. Numbers of the deformities found in all the larvae observed divided per deformation type class and per tested substances group. The blue bars represent the Class 1 deformity types, the orange part is the Class 2 deformities, and the grey are related to the most severe deformities (Class 3). Results are normalised assuming the value of the Ctrl group as 1 for each deformation class.

linked to carbohydrate metabolism, direct evidence correlating ecdysone regulation with exposure to artificial sweeteners in insects remains elusive (Suzuki and Gu, 2025).

Regarding the (MTs), the effects of SUCR on larval body length were not dose-dependent, as increased larval length was observed exclusively at the lowest concentration and only during the early and intermediate developmental stages (I-II and III instars), while higher concentrations did not differ from controls and the effect disappeared in the final instar. Even in all the other MTs, the lowest concentration (500 ng/L) proved to be the most impactful. Notably, aberrations in the cephalic capsule and mouthparts were consistently observed across all larval instars. The occurrence of significant changes across multiple larval stages suggested an early and persistent onset of toxic effects during development, rather than an effect restricted to the last instar; this interpretation is consistent with evidence that mouthpart deformities arise from disturbances during larval molting (Meregalli et al., 2001) and with the high stability, water solubility, and low biodegradation of SUCR in aquatic systems (Tkach et al., 2025). This phenomenon may be interpreted based on recent findings in humans. Given the environmental persistence of SUCR and its capacity to induce oxidative stress and cellular damage even at trace levels, our results suggest the occurrence of non-monotonic dose-response (NMDR) patterns. In such scenarios, lower concentrations may elicit more pronounced adverse effects, whereas higher doses might trigger compensatory mechanisms or distinct homeostatic pathways (Chiang et al., 2025; Tkach et al., 2025). Furthermore, the biological response to xenobiotics is inherently complex, influenced by factors such as bioaccumulation kinetics, microbiome modulation, and the activation of specific metabolic pathways, which frequently exhibit a non-proportional correlation with the exposure dose (Singh et al., 2024; Tkach et al., 2025).

Previous studies have substantiated that SUCR exerted significant behavioural alterations in crustaceans and induces oxidative damage to lipids and proteins within the gills, musculature, brain, and liver of *Cyprinus carpio* Linnaeus, 1758 (Saucedo-Vence et al., 2017). Eriksson Wiklund et al. (2014) also found a significant positive correlation between SUC concentration (0.0001–5 mg/L) and neurological and oxidative alterations in *Daphnia magna*, which could induce sub-lethal effects.

These ethological disruptions represented critical early-warning indicators, as deviations from standard behavioural repertoires could trigger cascading effects at ecosystem level (Eriksson Wiklund et al., 2012).

In other Diptera Chironomidae species, i.e. *Diamesa zernyi* Edwards, 1933 and *Diamesa cinerella* Meigen, 1835 (Lencioni et al., 2018) and on other alpine macroinvertebrate species (Lencioni et al., 2020), SUCR, tested with higher concentrations respect the current study (i.e. 47 g/L), caused genotoxicity despite was the least effective tested contaminant in term of acute toxicity. In *C. riparius* Meigen, 1804, the detoxification response could vary with the concentration of the contaminant; for example, some enzymes

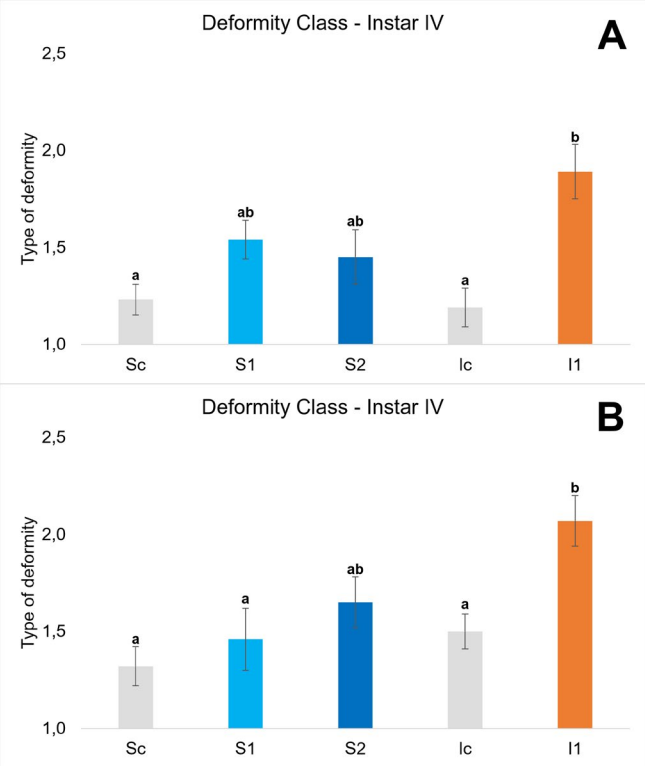


Fig. 8. Distributions of the deformation classes divided by the tested substances group, instar I-II (A), instar III (B) and instar IV (C). The letters above the boxes indicate significative differences after the Kruskal-Wallis and Holm-Bonferroni post hoc test.

were upregulated at higher concentrations, indicating an increased detoxification effort, while lower concentrations might not trigger significant gene expression changes or enzyme activity (Martínez-Guitarte, 2018; Martínez-Paz, 2018). Studies on other xenobiotics like caffeine (Rodrigues et al., 2025) and chlorantraniliprole (Rodrigues et al., 2015) showed that higher doses induced oxidative stress and alter enzyme activities related to detoxification and energy metabolism, suggesting that detoxification mechanisms were more actively engaged at elevated contaminant levels. This differential response might be due to the energetic cost of detoxification and the threshold at which the organism perceives chemical stress as harmful enough to activate these pathways. At low concentrations, the detoxification system might not be sufficiently stimulated or might rely on baseline metabolic processes, whereas at high concentrations, enhanced expression of detoxification genes and enzymes helps mitigate toxicity. Overall, *C. riparius* Meigen, 1804 seems to detox higher concentrations of non-nutritive sweeteners or similar xenobiotics by upregulating specific detoxification pathways, while lower concentrations may not elicit a strong detoxification response (Martínez-Guitarte, 2018; Martínez-Paz, 2018; Rodrigues et al., 2015).

Regarding the mouth part structures, the results in S1 group suggested an asymmetry among mentum, mandibles and ventral plates. In details mentum in larvae exposed to 500 ng/L of SUCR tended to increase in size while mandibles and ventromental plates tended to decrease. As reported in the research of (Servia et al., 2004), the presence of asymmetry in MTs could

be symptomatic of a stress in the organism. Moreover, each mouth structure could react differently against the same conditions of stress as reported in the study of (Arambourou et al., 2012). The same work suggested that gene regulations and molecular signalling may alter the mouth parts formation and growth (Blasco and Trombini, 2023).

5. Ibuprofen effects

The principal effects of the IBU on MTs appeared in the mature larvae, i.e. IV instar, as in the case of the mentum deformities. (Muñiz-González, 2021) emphasized that IBU exposure at concentrations < 100 μg/L had minimal effects in short-term (24 h) exposure, but leads to significant adverse effects on survival, development, and molecular responses with longer exposure durations (96 h) in *C. riparius*. In fact, the LC_{10} was higher (252.31 μg/L) at 24 h than at 48 h (LC_{10} = 0.024 μg/L). Moreover, the longest exposure (96 h) revealed higher values of Glutathione-S-transferase (GST), and the expression of genes linked with the stress response and immune system (Muñiz-González, 2021).

Blasco and Trombini (2023) reported that IBU and diclofenac in marine environments also reported that chronic exposure scenarios revealed more substantial risks to invertebrate species compared to acute or short-term exposures.

More evident effects in older larvae may be caused also by the persistence and the bioaccumulation of IBU in the organisms. This anti-inflammatory is often described as a pseudo-persistent contaminant in aquatic environments, because its low degradation maintain prolonged exposure conditions; therefore, larvae can remain in contact with the compound over time, which may favour the appearance of morphological alterations such as deformities (Walker et al., 2011). Previšić et al. (2021) highlighted that larvae of two caddisfly species (*Drusus croaticus* Marinković-Gospodnetić, 1971 and *Potamophylax* spp.) bioaccumulated pharmaceuticals, including IBU when exposed to 500 ng/L for 21 days. IBU was detected also in the adults of larvae reared in such conditions, demonstrating the bioaccumulation in the tissues even after the metamorphosis (Previšić et al., 2021). Furthermore, a temporal increase in diphenhydramine (an antihistamine drug) concentrations was observed in caddisfly larvae, rising from 13.6 ng/L to 19.2 ng/L and 29.9 ng/L over the course of the exposure (Previšić et al., 2021).

Another study conducted on the macroinvertebrate community of an Alpine stream highlighted as IBU is bioaccumulated by eight taxa with different diet and trophic habit, with concentrations ranging from 9.4 ng/g d.w., in larvae of *Isoperla* sp., to 543.3 ng/g d.w. in larvae of Limnephilidae (Lencioni et al., 2020). Renault et al. (2026) highlighted also that the bioaccumulation is drug and species-specific. Based on the literature (Lencioni et al., 2020; Previšić et al., 2021; Renault et al., 2026), IBU was accumulated in the tissue larvae during the growth, with the most evident effects on morphology (i.e. severe mentum deformities, Class 3) in the latest instars (III- IV instar). Based on recent studies on IBU effects at sub-organism level we might address these deformities to DNA damages, oxidative stress and interference with endocrine and developmental pathways (Lencioni et al., 2018; Hou et al., 2025; Muñiz-González, 2021; Sánchez-Aceves et al., 2021; Godoi et al., 2025; Hughes and Kaufman, 2000).

Lencioni et al. (2018) highlighted that IBU is particularly toxic and genotoxic respect to other ECs, such as other drugs (furosemide and trimethoprim), personal care products (triclocarban, tonalid and SUCR) and pesticides (boscalid, captan, chlorpyrifos, metolachlor, and terbuthylazine). Specifically, in *Diamesa cinerella* Meigen, 1835, IBU caused a significant DNA damage after 24 h and the permanence of the genotoxic activity after 96 h (Lencioni et al., 2018).

DNA damages together with histopathology, i.e. epithelial damages, were found also in another freshwater organism, the Asian clam *Corbicula fluminea* Müller, 1774 exposed to IBU (Hou et al., 2025).

Muñiz-González (2021) highlighted that IBU exposure at concentrations <=100 μg/L causes oxidative stress, endocrine disruption, and developmental alterations in *C. riparius*. Similarly, Godoi et al. (2025) highlighted effects on two hormones (17β-estradiol and testosterone) in the fish *Astyanax lacustris* Lütken, 1875 exposed to IBU <= 1000 ng/L. Additionally, they highlighted an increase in the activity of GST, and a reduction of superoxide dismutase (SOD) and catalase (CAT), symptoms of oxidative stress in the South American catfish *Rhamdia quelen* Quoy & Gaimard, 1824 exposed to concentrations <= 10 μg/L of IBU (Godoi et al., 2025).). Oxidative stress was found in *Danio rerio* tissues exposed to IBU (0.1 μg/L and 0.05 mg/L) and aluminium (11 μg/L and 6 mg/L) singularly and blended (Sánchez-Aceves et al., 2021). In the tissues was recorded the increasing of the production of reactive oxygen species (ROS) caused by IBU alone.

The effects on the mouth part structure could also link with endocrine systems. Hox genes such as Deformed (Dfd), proboscipedia (pb), and Sex combs reduced (Scr), in *Drosophila* species, played crucial roles in specifying mouthpart identity and morphology. The variations in their expression and regulatory interactions contributed to the diversity and potential deformities in mouthpart structures (Hughes and Kaufman, 2000).

Another molecular mechanism underlying mouthpart deformities may be linked to developmental disruptions during the moulting process. In *C. riparius* larvae, such deformities often result from physiological disturbances during hormonally regulated moulting stages, suggesting that endocrine disruption can actively induce these phenotypic aberrations (Meregalli et al., 2001).

Although only subtle effects were observed in overall MTs, the occurrence of numerous and severe deformities confirms the toxicity of IBU even at sub-lethal concentrations. Such malformations may impair the feeding efficiency of this benthic species, which plays a pivotal role in both freshwater and terrestrial food webs, alongside other key river macroinvertebrates (Renault et al., 2026; Middendorf et al., 2025).

6. Conclusion

Under chronic exposure conditions, both SUCR and IBU induced significant developmental alterations in *C. riparius*. These effects were particularly evident at the lowest SUCR concentration (S1), which triggered cephalic aberrations characterized by asymmetrical development and head elongation compared to the control group. In contrast, IBU exposure—at a concentration corresponding to the LC10 48h, induced mentum deformities, primarily in late-stage larvae. Our findings demonstrate that even at concentrations lower than the global average reported in surface freshwaters (S1 = 500 ng/L and I1 = 25 ng/L), these emerging contaminants elicit measurable biological responses. This underscores the necessity of continuous environmental monitoring. Furthermore, integrating multi-omic approaches with traditional biomarkers could further elucidate the specific biochemical and molecular pathways targeted by these substances.

Acknowledgements

This research was funded by the MUSE – Science Museum of Trento (Italy). Part of the data presented in this study was included in the Bachelor's thesis of S. Piffer, conducted as part of the BSc programme in Biotechnology at the University of Ferrara (Italy).

Conflict of interest

The authors declare no conflicts of interest.

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