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Toxicological assessment of benzalkonium chloride using planaria mobility: A comparison of manual and digital tracking methods

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ABSTRACT

The principle of the 3Rs—Reduction, Refinement, and Replacement—encourages minimizing animal use, improving experimental design, and developing alternative models for toxicology testing. Among such models, planaria (aquatic flatworms) have gained increasing attention in pharmacology, regenerative medicine, and toxicology because of their simple anatomy, high environmental sensitivity, exceptional regenerative ability, and ease of laboratory maintenance. In this study, we examined the effects of benzalkonium chloride (BAC)—a commonly used pharmaceutical excipient with antimicrobial and permeability-enhancing properties, as well as a known environmental toxicant—on the locomotor activity of *Schmidtea mediterranea* using both manual assessment and Lolitrack video-tracking software. Six concentrations of BAC (5–1000 µg/mL) and a negative control were tested. Both approaches showed an overall reduction in locomotor activity over time, though manual analysis indicated a transient stimulation at lower concentrations. The software-based method demonstrated greater reliability, precision, and objectivity, making it preferable for toxicity evaluation in planaria.

1. Introduction

First defined by William Russell and Rex Burch in their book *The Principles of Humane Experimental Technique* (Russell and Burch, 1959), the 3Rs provide guiding principles when using animals in scientific investigations or for product testing aiming to **reduce** the number of animals used in studies without compromising the quality of data obtained, to **refine** protocols to minimise pain and distress experienced by test subjects, and to **replace** the use of animals with non-animal methods and/or with organisms from earlier branches of the phylogenetic tree (Stokes, 2015).

One promising approach to reducing the use of mammals in animal experimentation is the replacement of vertebrates with simpler organisms, such as live invertebrates. Unlike vertebrates, most invertebrates—except for certain cephalopods—are not classified as protected animals in many countries, meaning their use in research typically does not require ethical approval or licences. Live invertebrates have a long and impactful history in physiology, biology, pharmacology, and toxicology, with several Nobel Prizes awarded for research involving these organisms. Among the most widely used models are *Drosophila melanogaster* (Tower, 2019) and *Caenorhabditis elegans* (Zhang et al., 2020).

Planaria are freshwater non-parasitic flatworms of the phylum *Platyhelminthes* that populate unpolluted streams and constitute a significant element of the aquatic ecosystem, as both predator and prey (Li, 2007; Alonso and Camargo, 2011). Despite their simplicity, they offer a useful alternative model in various fields of toxicology, pharmacology, and neuroscience, due to, amongst others, their sensitivity to toxins, their cephalisation and human-like neurotransmitters (e.g. serotonin, dopamine, glutamate) (Sarnat and Netsky, 1985), and their remarkable regenerative properties allowing full re-growth from fragments of the worm (Hagstrom et al., 2016; Rompolas et al., 2009). Toxicant testing can use various endpoints including mortality, regeneration, movement, behaviour, feeding, fertility (Alonso and Camargo, 2011) and epithelial integrity assessed via fluorescence microscopy (Shah et al., 2020). Furthermore, these flatworms' sensitivity to toxins and preference for well-aerated water make them a natural biological indicator of the cleanliness of water in their environment (Kustov et al., 2014).

Benzalkonium chloride (BAC) is a quaternary ammonium compound widely used for its disinfectant, surfactant, and broad-spectrum antimicrobial properties in domestic, clinical, industrial, pharmaceutical/medical, and industrial settings (Jia et al., 2024). A common active ingredient in disinfectants, BAC is used in personal hygiene and cosmetic

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products (e.g. soaps, shampoos, conditioners, and lotions) and in ophthalmic and nasally delivered solutions, drops, and sprays as an antimicrobial excipient (Merchel Piovesan Pereira and Tagkopoulos, 2019; Moiseev et al., 2019). In ocular drug delivery specifically, BAC also functions as a penetration enhancer, improving drug permeation across the corneal barrier (Moiseev et al., 2019). Such widespread use, with domestic and industrial disposal, has led to BAC discharge into the environment, mainly through wastewater effluent, despite its recognised toxicity to the aquatic ecosystem (Barber and Hartmann, 2021). Lavorgna et al. (2016) showed that BAC at concentrations lower than in the environment were toxic to the water flea models *Daphnia magna* and *Ceriodaphnia dubia*, with effects on mortality, movement, reproduction, and clear genotoxicity. Notably, the COVID-19 pandemic led to a rapid increase in use of BAC-based sanitisers and disinfectants worldwide, intensifying the environmental burden of this contaminant (Liao et al., 2023).

Common sublethal endpoints for planarian toxicity testing focus on behavioural changes exhibited during/following exposure to a toxicant. Planarian locomotion can be monitored in order to measure the effects of a chemical, with most studies reporting results obtained using a traditional gridline counting locomotor assay. First introduced by Raffa et al. (2001), the planarian locomotor velocity (pLMV) method aligned with techniques to quantify rodent behaviours (Hagstrom et al., 2016). It has since been used in toxicology assays of various central nervous system agents such as the anti-Parkinsonism drug benserazide (Carolei et al., 1975; Raffa et al., 2013b), cocaine (Raffa and Valdez, 2001), nicotine and opioid-antagonist naloxone (Raffa et al., 2013a) and psychiatric drugs such as carbamazepine and fluoxetine (Ofoegbu et al., 2019a, 2019b). Moreover, this model and assay were also used to study the effects of environmental toxicants such as the insecticide chlorantraniliprole (Rodrigues et al., 2016), heavy metals including copper and cadmium (Plusquin et al., 2012; Majid et al., 2022), and pollutants such as the organometallic compound tributyltin (Ofoegbu et al., 2016). In addition to its use as an endpoint in laboratory assays, locomotion also provides a measure for “ecological fitness”. Because locomotor behaviour directly underpins ecological functions such as foraging, hunting, and predator avoidance, robust assessment of movement in sentinel species like planaria is critical to understand sublethal ecological impacts of environmental contaminants (Wu and Li, 2018; Plaut, 2001; Fu et al., 2019).

The previously used gridline-based assay (Raffa et al., 2001), while easy to replicate and cost-effective, presents limitations that require consideration of alternative approaches. In addition to the longer time required to manually analyse all video recordings, the main limitation is the subjectivity of the method, since counting of the number of gridlines crossed is heavily reliant on individual interpretation, thus leading to variability and observer bias. Moreover, the use of gridline crossings as a proxy for distance moved may lead to oversimplification and misrepresentation of locomotor activity due to variations in movement trajectories. These limitations underscore the need for a more robust and standardised method to provide precise, objective, and reproducible measurements of locomotion. This study compares the effects of BAC on planarian locomotion using two approaches: the traditional manual pLMV method and use of the video tracking software Lolitrack, which has not previously been used in planarian research. The advantages and limitations of both methods are investigated. By evaluating and refining methods for locomotor assessment, this study aims to advance the reliability of behavioural endpoints in planarian toxicology, enabling a more accurate assessment of sublethal contaminant effects and supporting the broader adoption of invertebrate models in ecotoxicological research.

2. Materials and methods

2.1. Planarian husbandry

Asexual strains of *Schmidtea mediterranea* were kindly donated by Dr Jordi Solana from Oxford Brookes University (UK) to establish our culture. Worms were kept in 1X Montjuïc water (M-water, commonly referred to as artificial pond water or APW) prepared using 1.6 mM NaCl, 1.0 mM $\text{CaCl}_2 \cdot 6 \text{H}_2\text{O}$, 1.0 mM MgSO_4 , 0.1 mM MgCl_2 , 0.1 mM KCl, 1.2 mM NaHCO_3 in deionised water (pH adjusted to ~7.0) (Merryman et al., 2018). Planaria were housed in 30 litres capacity aquaria equipped with a sponge filter to provide aeration and aid in maintaining the water clean. Fish tank gravel was added to the bottom of each aquarium (after 3 washes in M-water) to better mimic a riverbed. Planaria were fed twice weekly with organic calf liver and water was changed after each feed. Additional water changes were carried out if the water quality was sub-optimal, i.e. if its odour was foul or if worms were found floating at the surface as these are both indicators of poor water cleanliness. Tanks were kept in the dark, with exposure to light occurring only during water changes, tank cleaning and experimentation. The aquaria were kept at room temperature within a range of 18–20°C. Planaria were removed from the breeding tanks and placed in a separate plastic container and starved for at least 5 days before testing, and each worm was only used once. Each 250 mL container contained approximately 30 planaria required for each round of experimentation. Worms were randomly chosen after ensuring they were healthy (did not exhibit signs of illness such as lesions, black spots or increased mucous secretions) and were fully regenerated.

2.2. Materials

Benzalkonium chloride ($\geq 95\%$ purity) was purchased from Sigma-Aldrich (Gillingham, UK). Single use plastic Petri dishes (7 cm diameter) were obtained from Thermo Fisher Scientific (Hemel Hempstead, UK). The Lolitrack v5 software was purchased from Loligo Systems (Viborg, Denmark), and an RS Pro light box was acquired from RS Components Ltd. (Corby, UK).

2.3. Traditional pLMV assay

This locomotor assay follows the protocol described by Raffa et al. (2001) with slight modifications to gridline spacing and duration of analysis. Planaria were placed individually into clear plastic observation Petri dishes placed over graph paper with major gridlines spaced 1 cm apart in a square pattern. Quantification of locomotor activity was done by counting the number of gridlines crossed and re-crossed by each worm per minute for a period of 5 min. Each planarian was allowed a period of acclimation of 30 s in the observational dish, which began as soon as the worm was placed in the experimental medium. A control solution of M-water and 5 concentrations of BAC in M-water (5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 50 µg/mL, 1000 µg/mL) were tested with five independent replicates each. Petri dishes were filled with 25 mL of test solution prior to each recording. The counting of gridline crossings was repeated thrice to minimise human error and the same values were obtained in all counts.

2.4. Lolitrack software method

Since gridlines interfered with worm-recognition by the software, planaria were placed individually into clear plastic observation Petri dishes located over 3 plain white A4 sheets of 80 gsm paper on a light box. Each worm was allowed the same acclimation period and the same control and concentrations of BAC were used as in the traditional assay. All videos were recorded in HD at 30 fps, suitable for digital processing by the software, for 5 mins 30 s using the camera of an iPhone 13 Pro and were analysed using the Lolitrack v5 video tracking and

behaviour analysis software to quantify the distance moved and percentage of activity of all replicates. All recordings were made in the same laboratory under standard laboratory lighting conditions. The activity threshold was set to 0.1 pixel/frame.

2.5. Statistical analysis

The detection of potential outliers was carried out using the modified z-score method. The modified z-score (M_i) for each data point (x_i) was calculated using the formula: $M_i = \frac{0.6745(x_i - \text{Median})}{\text{MAD}}$, where MAD is the Median Absolute Deviation. This approach resulted in 2 planaria being removed from the entire experimental population. Data points with a score higher than 3.5 or lower than -3.5 are considered outliers and are thus trimmed from the data set for a more robust statistical analysis (Iglewicz and Hoaglin, 1993). Statistical significance of the data was established on GraphPad Prism 8.0.2 by confirming normality of the data using the Shapiro-Wilk test before running one-way ANOVA tests with repeated measures. P values ≤ 0.05 were considered statistically significant.

3. Results

Both methods aim to investigate the toxicological effects of BAC by assessing the planaria locomotor velocity (pLMV) at different concentrations.

The traditional method quantifies the movement of each planarian in terms of gridline (re)crossings during their exposure to the tested solution. The data obtained was used to calculate mean and S.E.M. values of the cumulative numbers of gridline crossings and summarised as shown in the exemplary graph (Fig. 1). Exemplary graphs only display part of the data for clarity purposes – the full data sets are available to view in Supporting Information (Figure S1).

Previously, Pagán et al. (2009) proposed calculating the slope (gradient) of cumulative gridline crossings to analyse and compare planarian locomotion. In the present study, the gradient from the plots in Fig. 1, representing planarian velocity, was determined for each BAC concentration. Fig. 2 shows that when exposed to BAC at the lowest concentration tested (5 $\mu\text{g/mL}$), locomotion increased by approximately a third ($p = 0.0164$) when compared to M-water and then declines as concentration increases. This may reflect minor toxicity caused by BAC at the lowest concentration which could potentially promote planarian motility, presumably to escape the unpleasant environment. However, planaria locomotion then decreases with increasing BAC concentration, attributed to increased toxicity and its effects on the worms and their mechanism of locomotion and/or overall survivability. The significance

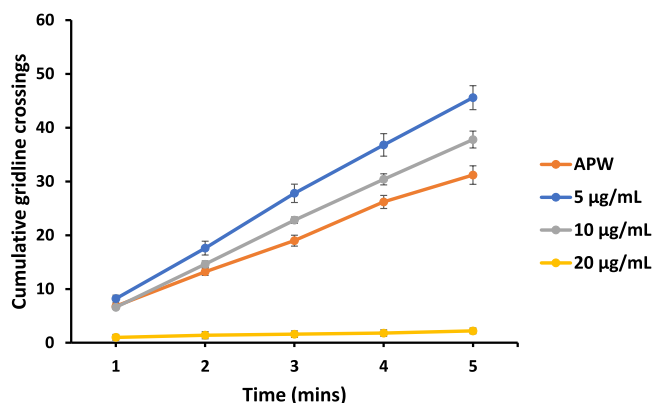


Fig. 1. Cumulative gridline crossings over 5 min for planaria exposed to increasing concentrations of BAC in M-water. Mean cumulative gridline crossings $\pm$ S.E.M for planaria in M-water (control) and test concentrations of 5, 10, and 20 $\mu\text{g/mL}$. $n = 5$ for each concentration. A representative video of planaria from the experiment is provided in Supporting information.

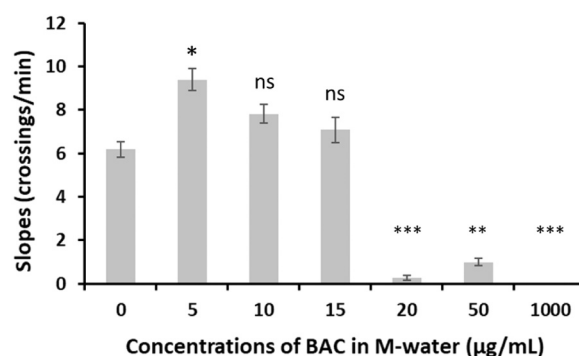


Fig. 2. Mean slope values (crossings/min) $\pm$ S.E.M. derived from cumulative gridline crossings of planaria exposed to M-water and increasing concentrations of BAC in M-water (in $\mu\text{g/mL}$). $n = 5$ per concentration. Significance of data: ns ($P > 0.05$), *** ($P \leq 0.001$), compared to the control.

of data is shown in comparison to the M-water negative control.

Digital tracking quantifies the movement of planaria as distance moved (cm) during solution exposure. The data obtained was analysed as above, with distance moved each minute recorded. Fig. 3 shows an example for some of the concentrations of BAC chloride tested (data expressed as mean $\pm$ SEM). The exemplary graph only displays part of the data for clarity purposes – the full data sets are available to view in Supporting Information (Figure S2). The slope values were plotted for comparison (Fig. 4), along with a graph depicting the percentage of activity (Fig. 5). The significance of the data was illustrated in comparison to the M-water negative control.

The data obtained through digital tracking illustrates a gradual decrease in pLMV with increasing BAC concentration. A solution of 0.1 % BAC in M-water (1000 $\mu\text{g/mL}$) was also tested as positive control. The overall activity against the concentration of BAC shows a similar decline in planarian activity time with increasing BAC concentrations.

To investigate the relationship between the manual and digital data, a Pearson correlation analysis was conducted and the 95 % confidence interval calculated and illustrated in Fig. 6. The scatter plot displays the relationship between the data obtained using both analysis methods. The trendline shows a positive correlation between the two variables distinguished by the moderate upward slope fitted to the data points. The Pearson correlation coefficient was calculated using Microsoft Excel ($r = 0.65$, 95 % CI [0.39, 0.81], $p < 0.001$) and further affirms a moderate positive correlation (Akoglu, 2018) despite the presence of outliers. Deviations from the line of best fit suggest some variability in the extent of movement detected by each method. Specifically, higher manual counts were occasionally associated with lower digital distance values, reflecting instances in which planaria displayed confined or

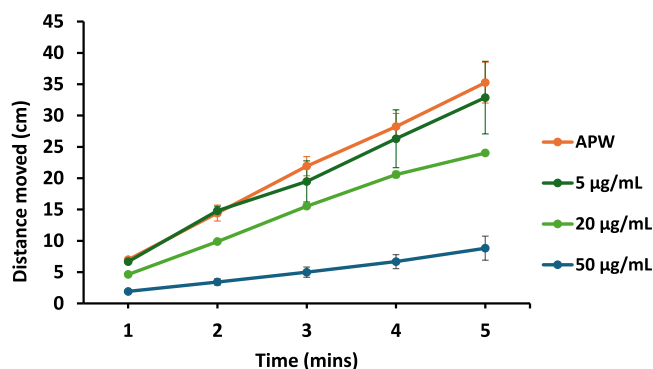


Fig. 3. Digitally tracked distance moved (in cm) $\pm$ S.E.M. by planaria exposed to M-water (control) and increasing concentrations of BAC in M-water (5, 20, and 50 $\mu\text{g/mL}$) over 5 mins. $n = 5$ for M-water, 20 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$. In 5 $\mu\text{g/mL}$, $n = 4$ for mins 1 and 2 and $n = 5$ for rest.

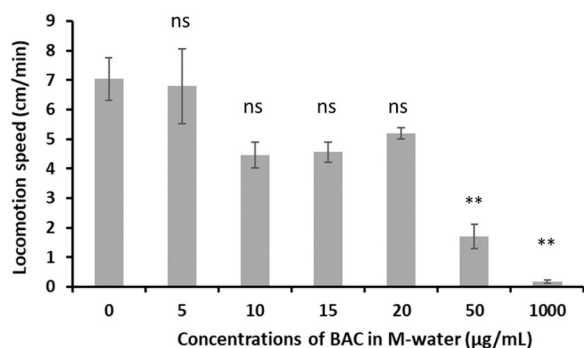


Fig. 4. Mean slope values in cm/min $\pm$ S.E.M of digitally tracked locomotion data of planaria exposed to M-water and increasing concentrations of BAC in M-water (in $\mu\text{g/mL}$). $n = 5$ per concentration. Significance of data: ns ($P > 0.05$), *** ($P \leq 0.001$), compared to the control.

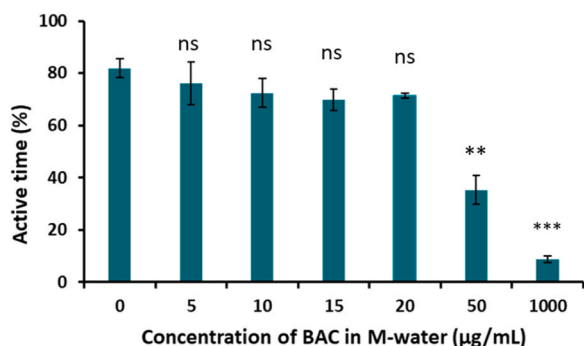


Fig. 5. Overall activity (in %) $\pm$ S.E.M. of planaria exposed to increasing concentrations of BAC in M-water (in $\mu\text{g/mL}$) over 5 mins. $n = 5$ for all concentrations tested. Significance of data: ns ($P > 0.05$), *** ($P \leq 0.001$), compared to the control.

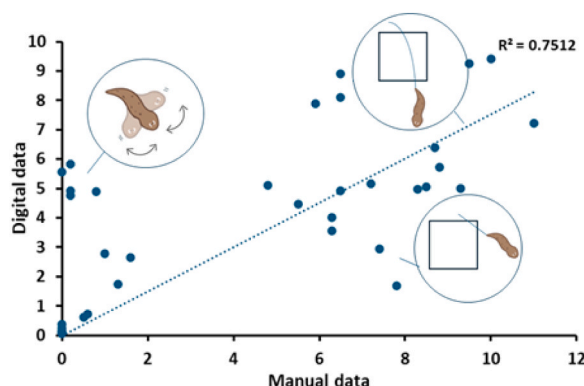


Fig. 6. Pearson's correlation scatter plot illustrating a moderate positive correlation between the manual slope data (x axis) and the digital slope data (y axis). The dotted line indicates the line of best fit ($R^2 = 0.7512$), showing a strong positive relationship between the two quantification methods. The diagrams illustrate representative behaviours of planaria observed during manual locomotion analysis following exposure to BAC: (top left) confined in-place movement tracked by digital method but not by manual method due to lack of gridline crossing; (bottom right) limited distance moved despite higher gridline crossing count in manual analysis due to the cutting of corners; (top right) direct, linear movement aligning between both methods.

oscillatory movements that were recorded as multiple gridline crossings but involved minimal actual displacement.

4. Discussion

A comparative analysis of the results obtained from both methods shows that the higher concentrations of BAC tested induced an overall reduction of locomotion despite the differences observed in trends. A previous study carried out on *Dugesia lugubris* (*Schmidtea lugubris*) planaria using skin irritants (including BAC) and the manual locomotor assay also showed a reduction of gridline crossings and aided in the identification of strong irritant vs. non-irritant compounds without providing the precision required to discriminate between different levels of irritation (Shah et al., 2020).

The manual method enables quantification of movement through a cost-effective and simple approach; however, it exhibits significant variability of results depending on the experimental set up and subjective interpretation in gridline-counting protocols. The number of gridline crossings may vary with the initial position of the worm at the onset of recording, where worms may be positioned at the centre or the edge of a grid square. This variability is especially noticeable at higher concentrations tested where locomotion is impaired; under these conditions, planaria are less likely to cross gridlines when placed centrally compared to positions nearer to the grid boundaries. Moreover, additional variability could occur if data analysis is performed by different individuals and if standardised gridline counting guidelines are not set, underscoring the importance of clear, consistent instructions and triplicate analysis to mitigate potential observer bias. In addition to such variability, it is important to note that the use of gridlines as a unit of quantification can be flawed in that two gridline crossings can represent different distances depending on the planaria's trajectory. For instance, a worm traversing a grid square diagonally versus one merely skirting its corner, will both register as crossing a single gridline, despite covering different distances. Ambiguities also emerge when evaluating specific behaviours, such as whether a worm cutting a grid right at its corner has crossed one or two gridlines. Similarly, when a worm exhibits restricted locomotion with repetitive movements along a gridline (e.g. head bobbing without swimming), questions arise regarding whether each instance of head crossing should be tallied as a gridline crossing or considered a non-locomotive behaviour effect. Fig. 6 is annotated to illustrate data points characteristic to such behaviours.

Similarly, a study conducted by Rodrigues et al. (2016) investigating the behaviour of *Dugesia subtentaculata* planaria following exposure to the insecticide chlorantraniliprole, highlighted the uncertainty and variability associated with observer-induced bias when using the traditional pLMV method. They also found that small and unpredictable movements performed by the worms under stressful conditions were underestimated during analysis in the pLMV assay, whereas the usage of a video tracking and analysis software allowed for higher sensitivity and precision in locomotion detection and analysis.

The issues encountered in the manual data analysis are mitigated when employing the digital method, where pLMV is automatically quantified as distance moved (in cm) upon applying the appropriate filter settings and selecting the target object. Although some minimal movement artifacts may be quantified as locomotion despite the planarian remaining largely stationary (i.e. neither gliding nor swimming), these recorded movements were very minor in our experiments and did not significantly alter the locomotor effects observed. Similar phenomena have also been reported by Rodrigues et al. (2016), who noted that small erratic movements may reflect toxicants' effects on muscle contraction. Moreover, the software provides a modifiable activity threshold setting to set the pixel change per frame necessary to qualify as movement, enhancing the precision of activity tracking. Beyond quantification, the digitalisation of this assay allows additional behavioural analyses, such as chemotaxis assays, bend quantification, or detailed mapping of planarian trajectory and movement. It also enables

quantification of the proportion of time spent in active versus inactive states based on activity threshold settings, as shown in Fig. 5, providing complementary insight into chronic effects of chemicals and overall activity patterns in response to toxicant exposure.

Analysis of the locomotor data following BAC exposure can lead to different apparent trends in behaviour, depending on the data analysis method used. Thus, despite both methods displaying an overall decrease in locomotion with increasing concentrations of BAC, the manual pLMV method showed an initial increase in gridline crossings and higher velocities at low to moderate concentrations compared to the negative control. An analysis of such data alone could be interpreted as a stimulatory or irritant-induced hyperactivity response, a phenomenon which could be observed at sublethal toxicant concentrations where organisms exhibit increased locomotion or abnormal behavioural patterns as a stress response before inhibitory effects dominate at higher exposures (Buttarelli et al., 2008). Although such occurrence is most commonly associated with neurotoxic compounds, other substances such as irritants might also lead to increased agitation or escape-like behaviour (Arenas et al., 2017), consistent with nocifensive responses described in planarians (Reho et al., 2022). However, as explained previously, this pattern may also be a reflection of the methodological artifacts inherent to the gridline-counting approach of the pLMV assay. In contrast, the video tracking software does not reflect this initial increase in locomotion. Instead, it shows a more consistent concentration-dependent decrease in planaria velocity. This divergence in trends underscores the need for cautious interpretation of manually derived data and supports the use of automated tracking tools for more precise and objective measurement of locomotion in planaria assays.

At the mechanistic level, the concentration-dependent inhibition of locomotion observed with BAC exposure is consistent with its established toxicological mechanism of action as a cationic surfactant. In fact, BAC primarily causes membrane disruption, compromising the integrity of lipid bilayers and increasing cellular permeability (Wessels and Ingmer, 2013), which can impair osmoregulatory and neuromuscular function in aquatic invertebrates (Alberts et al., 2002). This disruption likely interferes with the planaria's epithelial cilia and smooth muscle coordination that are responsible for the worm's gliding and swimming behaviour. Furthermore, BAC also alters ion channel function and disturbs neurotransmission, particularly through inhibition of acetylcholinesterase and modulation of membrane-bound ion gradients. These effects can in turn impair the electrical excitability of muscle and neural cells (Herron et al., 2021), thus likely leading to the reduction in motility documented in this study. The initial/early membrane irritation and ionic imbalance may be associated with the transient hyperactivity or erratic movement occasionally observed at lower BAC concentrations due to the initial excitatory or irritant response before overt neuromuscular inhibition occurs following the progressive disruption of neural and membrane integrity.

5. Conclusion

Both data analysis methods employed demonstrated the toxicity effects of BAC on the planaria model, with higher concentrations of the pollutant hindering locomotion and eventually leading to death. Overall, the software method appears to offer improved consistency and precision relative to manual gridline crossing counting, whilst allowing faster data analysis and the potential for batch processing, opening the possibility of high-throughput screening. By enabling objective and scalable quantification of sublethal behavioural endpoints, this method supports and strengthens the use of planarians as a bridge model between invertebrate and vertebrate toxicological systems and provides a valuable framework for assessing neurotoxic and irritant effects of emerging contaminants.

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CRediT authorship contribution statement

Manel M. Habel: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Khutoryanskiy Vitaliy V:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Adrian C. Williams:** Writing – review & editing, Supervision, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vitaliy V. Khutoryanskiy reports financial support was provided by The Royal Society. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104850.

Data availability

Data will be made available on request.

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